Drug Selectivity

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# Increasing αvβ3 Selectivity of the Anti-Angiogenic Drug Cilengitide by N-Methylation**

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The drug Cilengitide, *c*(RGDf(*N*Me)V), is a cyclic RGD pentapeptide (R = arginine, D = aspartic acid, G = glycine) currently in clinical phase III for the treatment of brain tumors and in phase II for other cancer types.[1] The antitumoral properties of this peptide are based on its antagonistic activity for pro-angiogenic integrins, such as αvβ3, αvβ5, or α5β1. However, the specific roles of these integrin subtypes in angiogenesis and cancer are not yet clear and fully understood. In this work, we present di-N-methylated analogues of the stem peptide *c*(RGDfV) which retain an αvβ3-binding activity in the nanomolar range but have lost most of the activity for integrins αvβ5 and/or α5β1. Highly active and selective peptides for αvβ3 are important tools to study the specific role of this integrin in angiogenesis and cancer.

Integrins are heterodimeric receptors that govern cell–cell and cell–extracellular matrix (ECM) interactions, and play crucial roles in a plethora of cellular functions.[2] The fact that many integrins are involved in pathological processes, such as tumor angiogenesis, has stimulated their study as therapeutic targets.[3] A number of integrin receptors recognize and bind the tripeptide sequence RGD, which is a prominent cell-adhesion motif present in ECM proteins.[4] Mimicking this tripeptide sequence with RGD-peptides or peptidomimetics is hence a promising approach to target integrins involved in angiogenesis and to develop anti-cancer agents.[1,3b,5]

It is known that αvβ3 and αvβ5 are involved in two different angiogenic pathways.[6] Whereas angiogenesis induced by basic fibroblast growth factor (bFGF) or tumor necrosis factor-α depends on αvβ3, angiogenesis triggered by vascular endothelial growth factor (VEGF) or transforming growth factor-α is αvβ5-dependent. These two integrins are also described to be important mediators in the regulation of hypoxia in glioblastomas.[7] However, mice lacking either αv or β3 and β5 integrins showed extensive angiogenesis.[8] These intriguing results were a matter of debate and challenged our understanding about the role of these two integrins in angiogenesis.[9] The integrin α5β1 is also highly expressed in angiogenic vasculature by several angiogenic stimuli, such as bFGF but not by VEGF.[10] Since αvβ3, αvβ5 and α5β1 have partially overlapping ligand affinities,[4b] it is plausible that α5β1 might substitute the pro-angiogenic activity of the other integrins. Paradoxically, another recent study showed that low concentrations of Cilengitide stimulates VEGF-mediated angiogenesis.[11] Although the doses used in this study are far lower than therapeutic concentrations[12] and hence such a "pro-angiogenic" effect is not likely to be observed in the clinical studies, it becomes evident that a better understanding of anti-angiogenic agents is necessary.[13]

It has been shown by us and others that N-methylation can increase the selectivity towards specific receptor subtypes.[14] These biological effects are often caused by the induction of conformational constraints in the peptide backbone, which lead to preferred single conformers essential for biological activity.[14a,d,h,15] Thus, we envisioned that further N-methylation of Cilengitide could result in enhanced selectivity profiles. For this reason we designed a library containing all the di-N-methylated analogues of *c*(RGDfV) (Figure 1).

Note that the synthesis of *N*Me peptides (especially if they are cyclic) is not without challenges that need to be carefully considered.[14a,16] In the first place, although many N-methyl amino acids are commercially available, most of them are still expensive. Therefore, we synthesized, in solution, the *N*Me residues of Gly, Val, and D-Phe by reduction of the corresponding oxazolidinone using Freidinger conditions.[17] Alternatively, Arg and Asp were methylated on resin using the Miller and Scanlan method,[18] later optimized by Biron et al.,[19] which is compatible with acid-sensitive side-chain

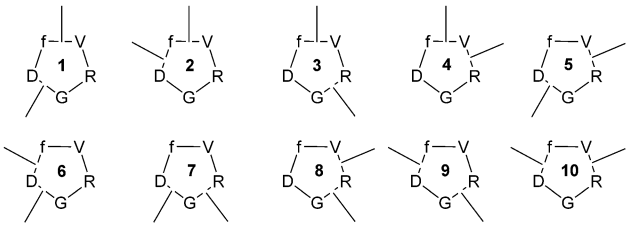


***Figure 1.*** Schematic representation of our library of di-N-methylated analogues of *c*(RGDfV).

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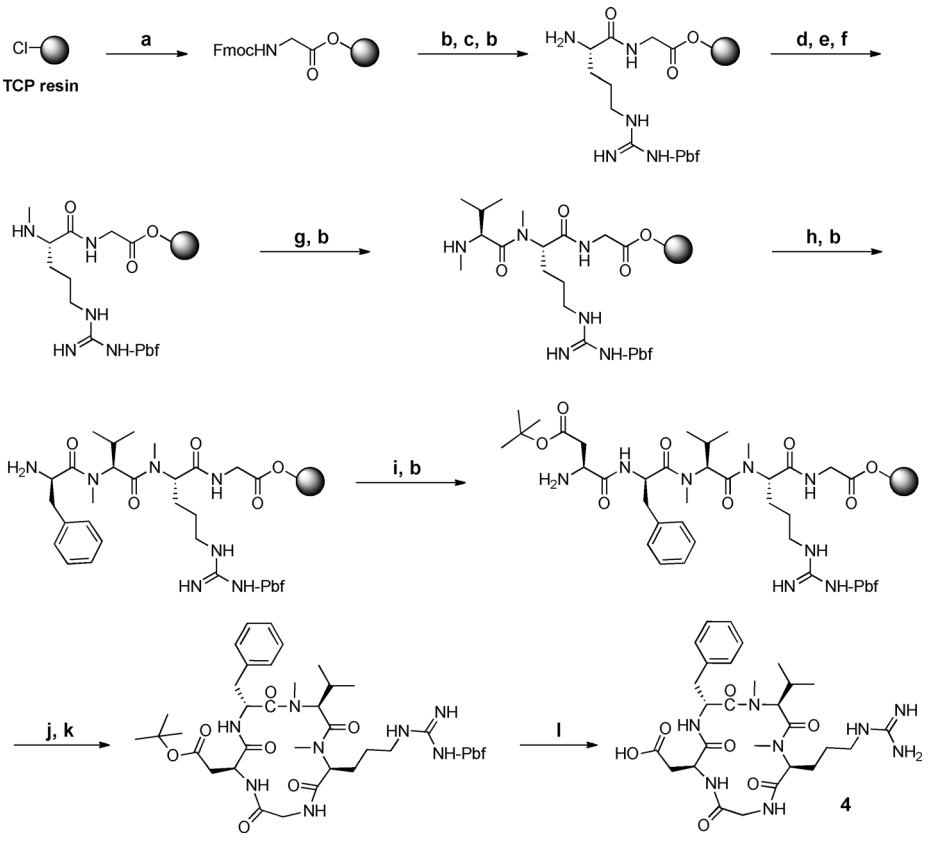
protecting groups. Moreover, other limitations were encountered. For instance, the presence of *N*MeGly at the C-terminus resulted in diketopiperazine formation. Also, to improve coupling efficiency, powerful coupling reagents, such as HATU, were required. Another critical point was the cyclization step: to favor cyclization *N*Me amino acids were avoided at the N-terminus and whenever possible Gly was fixed at the C-terminus to prevent racemization. Finally, both reaction time and TFA concentration were optimized to avoid peptide fragmentation during side chain deprotection. The synthesis of peptide **4**, which summarizes all these considerations, is shown in Scheme 1 (see the Supporting Information for a detailed description of the synthesis of all the analogues).

The impact of the extra N-methylation on Cilengitide in terms of integrin binding activity and selectivity was evaluated using a solid-phase binding assay for the pro-angiogenic integrins αvβ3, αvβ5, and α5β1 as well as for the platelet receptor αIIbβ3 (Table 1). The analogues in which Val was non-methylated (**5**–**10**) showed a dramatic decrease in αvβ3-binding activity, regardless of the position of the N-methylated residues. This effect was particularly observed for peptides **9** and **10**, in which the antagonistic activity for the vitronectin receptor was totally lost. In contrast, when Val was N-methylated, the resulting analogues (**1**–**4**) displayed low nanomolar activity for the αvβ3 integrin receptor. These results indicate that *N*MeVal is a crucial residue to retain the activity for this receptor, probably by inducing a preferred bioactive αvβ3-binding conformation.[5a,20] Analogues **9** and **10**, which are totally inactive, are both N-methylated at D-Phe. It could be hypothesized that this biological effect was due to the loss of a hydrogen-bond donor at this position;[21] however, peptide **2**, which also has *N*Me-D-Phe unit, exhibits a remarkable nanomolar antagonistic activity. In this regard, the effect of *N*Me at an Arg residue is also interesting. In a previous study, a peptide with a single N-methylation of this residue showed an $IC_{50}$ of 5.5 nM.[5a] Herein, the presence of *N*MeArg is found in peptides with activities ranging from superpotent (1.9 nM, **4**), moderate (142 nM, **5**), low (> 1000 nM, **8**) and very low (> 10000 nM, **10**). These data clearly indicate that the biological activity of these peptides more strongly depends on their overall conformation rather than on the local effects of a single N-methylation.[22] Noteworthy, most members of the library are inactive for the integrins αvβ5 and α5β1. If we focus on peptides **1** to **4** (highly active for αvβ3) only **4** shows nanomolar activity for these receptors, with selectivity ratios very similar to Cilengitide. In contrast, in peptides **1** and **2** the activity for αvβ5 is strongly or fully suppressed, with selectivity ratios much higher than those found for Cilengitide (> 500-fold for **1** and > 250-fold for **2**). Compound **3** does not show an improved selectivity towards αvβ5 but towards α5β1. For all these compounds the selectivity of Cilengitide against αIIbβ3 was either maintained or improved. A strong reduction in binding activity for αvβ5 and α5β1 was also observed for analogues **5**–**10**. However, these peptides are of lower biological interest due to their low (or absent) affinity for αvβ3.




***Scheme 1.*** Solid-phase synthesis of analogue **4**. a) Fmoc-Gly-OH, DIEA, DCM; b) piperidine-NMP (1:4); c) Fmoc-Arg(Pbf)-OH, TBTU, HOBt, DIEA, NMP; d) NBS-Cl, collidine, NMP; e) $Ph_3P$, MeOH, DIAD, THF; f) HS-$CH_2$-$CH_2$-OH, DBU, NMP; g) Fmoc-*N*MeVal-OH, HATU, HOAt, DIEA, NMP; h) Fmoc-D-Phe-OH, HATU, HOAt, DIEA, NMP; i) Fmoc-Asp(O*t*Bu)-OH, TBTU, HOBt, DIEA, NMP; j) HFIP-DCM (2:8); k) DPPA, $NaHCO_3$, DMF; l) TFA-DCM-$H_2O$-TIS (60:35:2.5:2.5). Fmoc = 9-fluorenylmethoxycarbonyl, DIEA = ethyldiisopropylamine, DCM = dichloromethane, NMP = *N*-methylpyrrolidine, TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate, NBS-Cl = nitrobenzylsulfonylchloride, DIAD = diisopropylazodicarboxylate, DBU = 1,5-diazabicyclo[5.4.0]undec-5-en, HATU = *O*-(7-azabenzotriazol-1-yl)-tetramethyluronium hexafluorophosphate, HFIP = hexafluoroisopropylalcohol, DPPA = diphenylphosphorylazide, TFA = trifluoro acetic acid, TIS = Triisopropylsilane.

To rationalize these findings and to explain the selectivities obtained in a better way, three peptides were chosen for structural studies: peptide **1**, a selective ligand that shows nanomolar activity for αvβ3 and low activity for αvβ5; peptide **4**, which is active for both receptors in the nanomolar range and therefore not selective; and peptide **10**, totally inactive for

***Table 1:*** The ten di-N-methylated analogues of Cilengitide and their binding activity ($IC_{50}$ in nM) towards αvβ3, αvβ5, α5β1, and αIIbβ3.[a]

| Peptide | | αvβ3 | αvβ5 | α5β1 | αIIbβ3 | αvβ5/αvβ3 | α5β1/αvβ3 |
|---|---|---|---|---|---|---|---|
| Cil | c(-R-G-D-f-***V***-) | 0.65(±0.07) | 11.7(±1.5) | 13.2(±0.6) | 815(±60) | 18 | 20 |
| **1** | c(-R-G-***D***-f-***V***-) | 5.9(±2.5) | >3000 | 270(±95) | >1000 | >500 | 46 |
| **2** | c(-R-G-D-***f***-***V***-) | 36.2(±8.1) | >10000 | >2000 | >10000 | >250 | 55 |
| **3** | c(-R-***G***-D-f-***V***-) | 13.2(±1.8) | 313(±122) | >1000 | >2000 | 24 | >75 |
| **4** | c(-***R***-G-D-f-***V***-) | 1.9(±0.3) | 40.9(±3.2) | 39.5(±1.3) | >1000 | 22 | 21 |
| **5** | c(-***R***-G-***D***-f-V-) | 142(±33) | >10000 | >2000 | >10000 | >70 | >14 |
| **6** | c(-R-G-***D***-***f***-V-) | 173(±12) | >10000 | >5000 | >10000 | >58 | >29 |
| **7** | c(-R-***G***-***D***-f-V-) | 965(±96) | >10000 | >1000 | >10000 | – | – |
| **8** | c(-***R***-***G***-D-f-V-) | >1000 | >10000 | >10000 | >2000 | – | – |
| **9** | c(-R-***G***-D-***f***-V-) | >10000 | >10000 | >10000 | >2000 | – | – |
| **10** | c(-***R***-G-D-***f***-V-) | >10000 | >10000 | >10000 | >10000 | – | – |

[a] Residues in bold and italics are N-methylated.

all integrins. Based on NMR spectroscopic assignments (Supporting Information, Table S2), ROEs, homo- and heteronuclear scalar coupling constants, $H^N$ temperature gradients, and on distance geometry calculations, distinct preferred structures could be derived for the three peptide backbones (Figure 2). The structures of **1**, **4**, and **10** possess pronounced differences that are described in detail in the Supporting Information along with additional information about their dynamics. Further, molecular-docking studies of these peptides were attained into the αvβ3 X-ray structure[20] as well as in the newly constructed αvβ5 homology models. Docking of **1** on αvβ3 showed that this peptide is able to interact with this receptor similarly to Cilengitide (Figure 3a). Nevertheless, note that the substitution of the Asp residue in Cilengitide with *N*MeAsp in **1** does affect to a certain extent the binding mode to αvβ3. In particular, this modification causes the loss of a hydrogen bond with (β3)-D216 CO but more importantly an evident relocation of the lower part of the peptide occurs (see Figure 3b). This result explains why **1** has approximately tenfold lower affinity to αvβ3 than Cilengitide (5.9 nM and 0.65 nM, respectively). For docking to the αvβ5 receptor, 100 αvβ5 homology models, differing in the specificity determining loop (SDL) conformation, were generated. Prior to docking calculations, all 100 models were tested for their capability to host the unselective Cilengitide and only those able to bind were further considered for docking of **1**, **4**, and **10**. Predictably, in these models Cilengitide assumed a binding pose similar to the experimentally determined bound state in αvβ3.[20] Interestingly, analysis of the multiple docking simulations performed on the αvβ5 selected models demonstrated that in the case of **1**, a well-defined binding mode could not be easily identified. Therefore, in this case the ligand Asp N-methylation causes a pronounced effect on the binding to αvβ5. In an attempt to rationalize such a behavior, the predicted **1**/αvβ3 complex was superimposed to the modeled αvβ5 receptor structure. As represented in Figure 3c, it is clear that the (β3)-A252/(β5)-D279 mutation results in a remarkable restriction of the available space. Therefore, the methyl group of the *N*MeAsp would be hardly adapted in the same binding fashion as in the **1**/αvβ3 complex. This, in turn, seems to strongly affect the RGD binding to αvβ5.

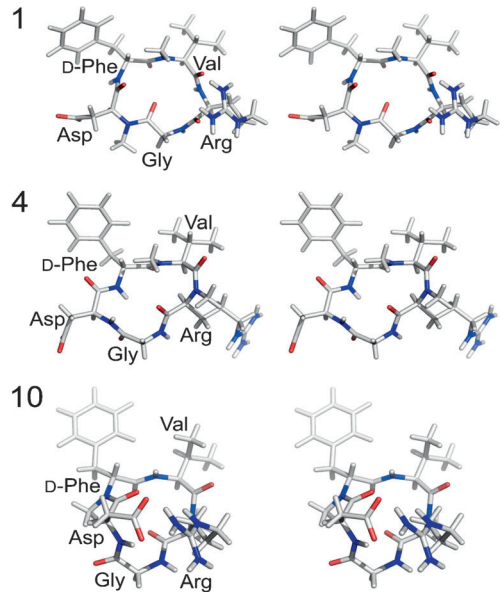



***Figure 2.*** Stereoviews of **1**, **4**, and **10**, as determined from NMR-based distance geometry calculation and subsequent minimization (see the Supporting Information for details).

Docking studies were also helpful in suggesting why the N-methylation of Arg residue (**4**) is ineffective in producing the αvβ3/αvβ5 selectivity (Table 1). In fact, such a modification, while inducing a different peptide conformation with respect to Cilengitide, does not influence the binding of **4** which is still assured by the conserved RGD sequence (Supporting information, Figure S1). Conversely, docking of **10** revealed that this peptide is unable to efficiently bind to the metal-ion-dependent adhesion site (MIDAS) and the αv subunit β propeller at the same time. Indeed, a comparison between the NMR solution structure of **10** and the X-ray bound conformation of Cilengitide showed that the double methylation of Arg and D-Phe residues (**10**) induces marked differences in distance between the Arg and Asp Cα atoms (5.0 Å and 6.4 Å for **10** and Cilengitide, respectively) as well as in the orientation of the Cα–Cβ bond vectors of the same residues (Supporting Information, Figure S2). Both features are well known to be critical for integrin binding and selectivity.[1a,23] Hence, unlike in **1** and **4**, the double N-methylation in **10** seems to induce a non-productive peptide conformation that prevents binding to αvβ3 and αvβ5 integrins.

In conclusion, we have demonstrated that double N-methylation of the peptide backbone allows fine tuning of the peptide's biological activity by inducing preferred bioactive conformations. Certain members of our library of di-N-methylated *c*(RGDfV) retained nanomolar affinity for αvβ3

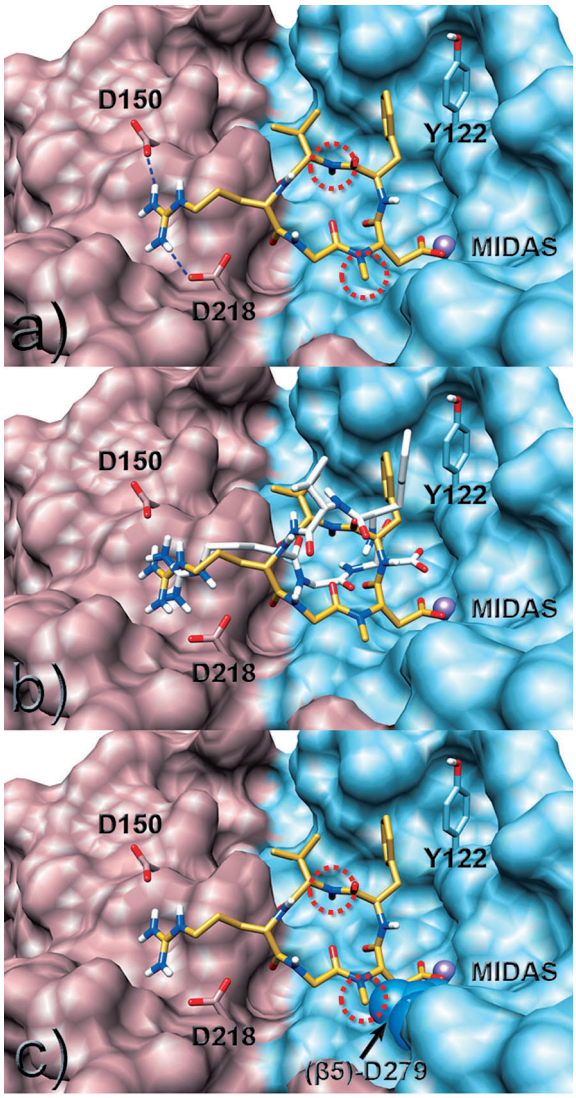



***Figure 3.*** a) Structure of **1** (yellow) docked in the αvβ3 integrin binding pocket. The αv and β3 subunits are represented by the pink and cyan surfaces, respectively. In both subunits the amino acid side chains important for the ligand binding are represented as sticks. The metal ion in the MIDAS region is represented by a magenta sphere. For comparison, the X-ray structure of b) Cilengitide (white sticks) as well as c) the (β5)-D279 residue (blue spheres) are shown. Red circles highlight N-methyl groups in (a) and (c).

but were totally inactive for the integrin subtypes αvβ5 and α5β1, thus improving the selectivity of Cilengitide. Compounds displaying such selectivity profiles represent new promising tools to study the role of closely related integrins in essential biological processes.

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