Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

A dimeric bicyclic RGD ligand displays enhanced integrin binding affinity and strong biological effects on U-373 MG glioblastoma cells

Giovanni Sacco,[a] Alberto Dal Corso,*[a] Daniela Arosio,[b] Laura Belvisi,[a,b] Mayra Paolillo,[c] Luca Pignataro[a] and Cesare Gennari*[a,b]

A C_2-symmetric bicyclic peptide bearing two RGD motifs was developed as dimeric ligand, and displayed enhanced inhibition of ECM protein binding to purified integrin receptors as compared to monomeric RGD analogues. Moreover, the dimeric bicyclic ligand induced cell detachment and inhibited FAK phosphorylation in U-373 MG glioblastoma cells.

Introduction

Among modern strategies for targeted cancer therapy, the use of monoclonal antibodies (mAbs) relies on their unique ability to bind biological targets with a high selectivity. The presence of 7 mAbs among the 10 best-sold drugs in 2018 speaks for the enormous impact of mAbs on modern oncology and other pharmaceutical needs.[1] While high-affinity mAbs can be routinely generated against virtually any protein antigen,[2] there are unfortunately important drawbacks related to their large size which leads to long half-lives in blood, slow extravasation, hindered tissue penetration and potential immunogenicity.[3] For these reasons, there is a considerable interest in the development of high-affinity small molecule ligands, which may show important benefits such as better pharmacokinetic profiles, oral availability, cell permeability and synthetic accessibility.[4] One way to strengthen ligand-receptor interactions is to generate multimeric ligands, where multiple copies of a binding unit are displayed on a multivalent scaffold. According to this design, the cooperativity effect of multiple interactions may stabilize the ligand-receptor complex by a combination of thermodynamic (i.e. reduction of entropy loss) and kinetic factors (i.e. increased rates of binding events).[5] In general, cooperativity may either result from the binding of each individual unit (x and y, Figure 1) to a different copy of target receptor (i.e. the so-called "cluster effect", Figure 1A) or from the alternation of different units on the same epitope (i.e. "rebinding" effect, Figure 1B).[6] Multimeric RGD ligands have been developed to effectively bind integrin $\alpha_v\beta_3$,[7] a transmembrane receptor over-expressed in many cancer cells,[8] and have shown promising results in several pharmacological and biochemical studies.[7,9] However, the physico-chemical properties of branched multivalent scaffolds (e.g. size, charge, solubility, etc.) may influence the targeting performances *in vivo*,[10] and sometimes resulted in lower tumour : organ uptake ratios in mice compared to the monovalent RGD analogues.[11]

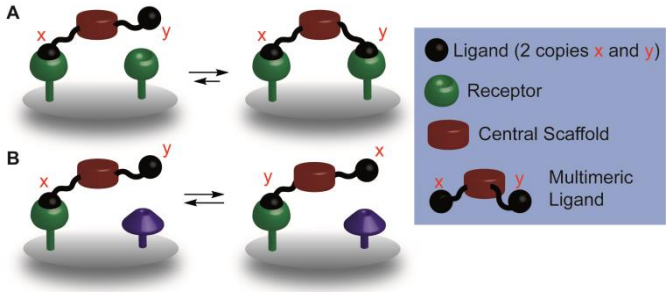

Figure 1. Two modes of multimeric ligand binding: A) The "cluster effect" results in recruitment of different receptor copies on the cell surface to allow for multiple receptor-ligand interactions; B) The "rebinding effect" allows ligands to efficiently bind a single receptor, based on increased local effective ligand concentration.[6]

Results and discussion

Design

As an alternative to the traditional "branched" structures, while maintaining the advantages of multivalent interactions, we report here the design of a "condensed" dimeric bicyclic RGD ligand (**1**, Figure 2). In compound **1**, the RGD sequence is displayed on both rings of the bicyclic structure, which is endowed with a disulfide bridge.[12] The resulting ligand is C_2-symmetric, and the C_2 axis crosses the disulfide bond. Ideally, compound **1**, possessing two identical pharmacophores on the same molecule, should exhibit increased binding affinity for the cognate receptor, relying on the above-mentioned "rebinding" effect (Figure 1B). To investigate this possibility, a bicyclic peptide (compound **2**, Figure 2) featuring both a RGD and a low-binding Arg-β-Ala-Asp (RβAD) motif was designed as monomeric analogue of **1**. A third bicyclic peptide bearing two RβAD motifs (compound **3**, Figure 2) was designed as negative control. Finally, to evaluate the impact of the bicyclic structure on the integrin binding affinity, we designed a monocyclic RGD analogue endowed with a disulfide bridge (compound **4**, Figure 2).

[a] *Università degli Studi di Milano, Dipartimento di Chimica, Via C. Golgi, 19 I-20133, Milan (Italy). E-mail: alberto.dalcorso@unimi.it; cesare.gennari@unimi.it*
[b] *CNR, Istituto di Scienze e Tecnologie Molecolari (ISTM), Via C. Golgi, 19 I-20133, Milan (Italy).*
[c] *Università degli Studi di Pavia, Dipartimento di Scienze del Farmaco, Viale Taramelli 6, 27100 Pavia (Italy)*
Electronic Supplementary Information (ESI) available: synthetic procedures, HPLC and MS data, procedures for biochemical assays.

Synthesis

Bicyclic peptides **1-3** were prepared by standard solid-phase peptide synthesis protocol using 2-chlorotrityl resin. The linear octapeptides, featuring acid-labile protecting groups at Asp, Arg and Cys side chains, were treated with HATU/HOAt coupling reagents under high dilution conditions (1.4 mM), affording the macrocyclic peptides. Final compounds **1**-**3** were obtained upon side-chain deprotection and disulfide bond formation using iodine. Monocyclic compound **4** was synthesized in a similar manner, using a Rink amide 4-methylbenzhydrylamine resin. Peptide cleavage from the resin afforded a linear deprotected intermediate which was oxidized with iodine affording the disulfide bridge in monocyclic compound **4**. All compounds were characterized by high resolution mass spectrometry (HRMS) and the purity was assessed by analytical HPLC. The experimental details for the preparation of compounds **1-4** are reported in the Electronic Supplementary Information (ESI).

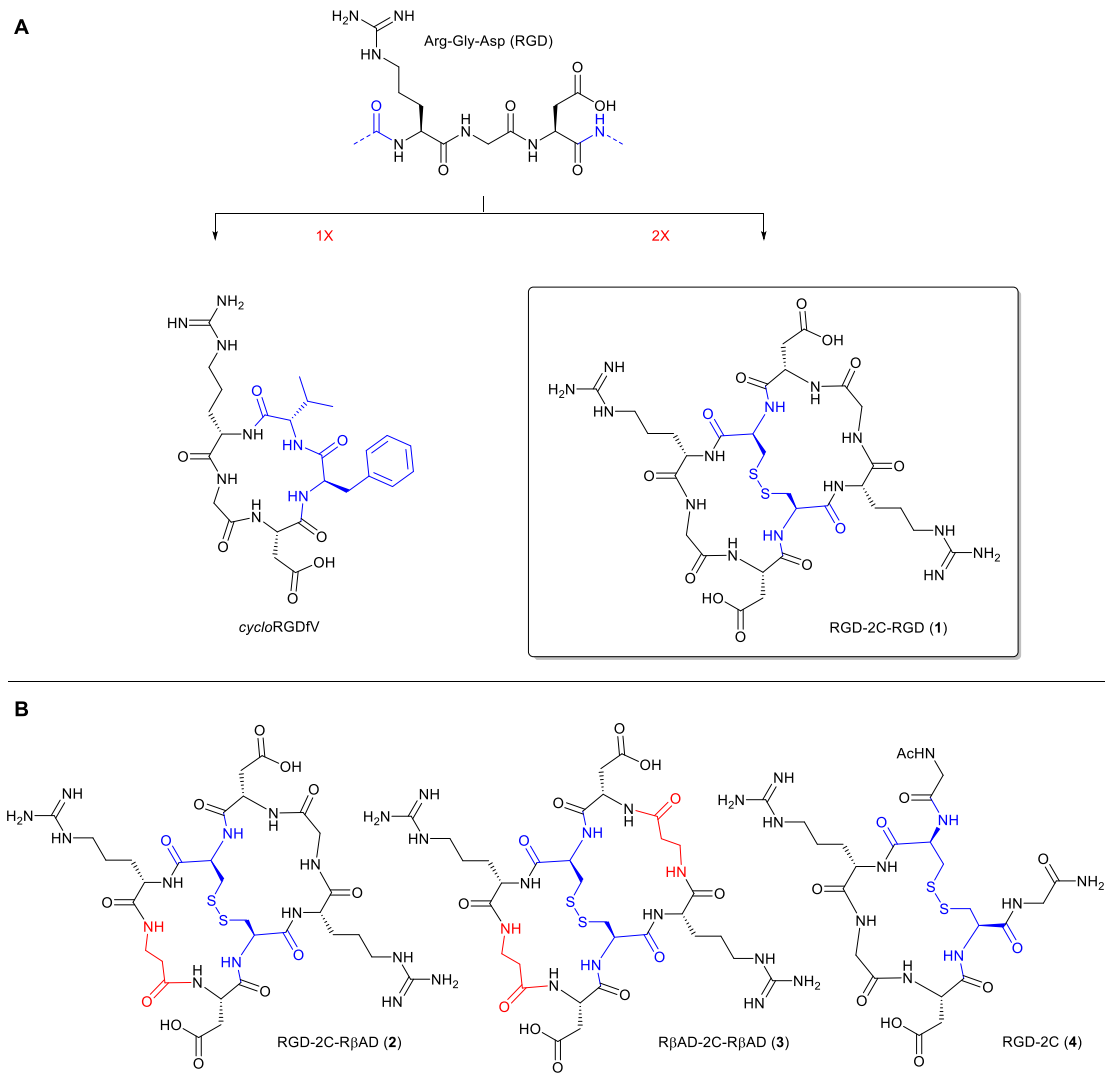

Figure 2. A) Molecular structures of a well-known monocyclic integrin ligand *cyclo*RGDfV, and the C_2-symmetric dimeric bicyclic peptide RGD-2C-RGD (compound **1**); B) Monomeric bicyclic peptide RGD-2C-RβAD (compound **2**), negative control bicyclic peptide RβAD-2C-RβAD (compound **3**), and monocyclic peptide RGD-2C (compound **4**).

Biological Tests

The newly prepared compounds **1-4** were examined *in vitro* for their ability to inhibit the binding of extracellular matrix (ECM) proteins (i.e. biotinylated vitronectin and fibronectin) to the corresponding purified $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors, using a previously reported protocol.[13] IC_{50} values are reported in Table 1.

Table 1. Inhibition of biotinylated ECM protein binding to the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors.

	IC_{50}[a] [nM]	
Compound	$\alpha_v\beta_3$	$\alpha_5\beta_1$
*cyclo*RGDfV	1.60 ± 0.90	105 ± 5
RGD-2C-RGD (**1**)	1.02 ± 0.68	263 ± 115
RGD-2C-RβAD (**2**)	5.97 ± 3.77	1000 ± 27
RβAD-2C-RβAD (**3**)	811 ± 59	>100000
RGD-2C (**4**)	6.39 ± 0.37	728 ± 142

[a] IC_{50} values were determined as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding to integrin $\alpha_v\beta_3$ or biotinylated fibronectin binding to integrin $\alpha_5\beta_1$, as estimated by GraphPad Prism software. All values are the arithmetic mean ± the standard deviation (SD) of triplicate determinations.

In particular, dimeric bicyclic RGD ligand **1** inhibited vitronectin binding to the purified $\alpha_v\beta_3$ integrin receptor at low nanomolar concentrations, slightly better than the reference peptide *cyclo*RGDfV.[14] Compound **1** proved significantly more active towards $\alpha_v\beta_3$ integrin than the monomeric bicyclic peptide **2**. As expected, negative control **3** showed low integrin binding affinity. In addition, the similar IC_{50} values shown by the monomeric bicyclic peptide **2** and the monocyclic peptide **4** indicate that the bicyclic structure is not a strict requirement for optimal binding profiles. The trend observed with $\alpha_v\beta_3$ was substantially confirmed in binding assays to purified integrin $\alpha_5\beta_1$. Reference peptide *cyclo*RGDfV proved to be the best $\alpha_5\beta_1$ integrin binder in the series and compound **1** inhibited fibronectin binding at lower concentrations than monomeric bicyclic peptide **2**, monocyclic peptide **4** and, by far, negative control compound **3**. Also in this case, monocyclic compound **4** and monomeric bicycle **2** proved similarly active. In summary, these data show that the dimeric bicyclic peptide **1** displays an enhanced integrin binding affinity as result of the dual presentation of the RGD pharmacophore. The effect of multivalency on binding of peptide **1** to the cognate receptors was estimated by the calculation of the relative potency Rp and the Rp/n values.[7d,15] For both receptors, Rp/n values >1 (i.e. 2.93 for $\alpha_v\beta_3$ and 1.90 for $\alpha_5\beta_1$) account for the multivalent effect of ligand **1**, and indicate that the enhancements in binding are truly synergistic and not only statistical. Since integrins are not multivalent receptors and considering the small size of the condensed bicyclic structure as compared to traditional branched multimeric ligands,[7] it is conceivable that in this case the multivalent effect is exclusively due to the "rebinding effect" (Figure 1B) rather than the "cluster effect" (Figure 1A).

Encouraged by these results, we subjected bicyclic compounds **1-3** to a panel of biological assays on U-373 MG, a human glioblastoma cell line showing high levels of integrin expression.[16] In particular, we focused our attention on the ability of peptides **1**-**3** to induce cell detachment. U-373 MG cells were treated with increasing concentrations (5, 10, 20, and 50 μM) of bicyclic compounds **1**-**3** for 48 hours. Dimeric bicyclic peptide **1** induced cell detachment,[17] which is particularly pronounced at 50 μM, while the monomeric analogue **2** was much less effective and comparable to the negative control **3** (Figure 3A). This dramatic effect caused by compound **1** can possibly be ascribed to interferences with integrin signalling and downstream phosphorylation of specific kinases (e.g. Focal Adhesion Kinase, FAK).[18] FAK phosphorylation was assessed by western blot analysis on U-373 MG cells incubated with compounds **1**-**3** for 48 hours. An observable decrease of FAK phosphorylation was detected only when cells were treated with dimeric bicyclic peptide **1** (Figure 3B,C).

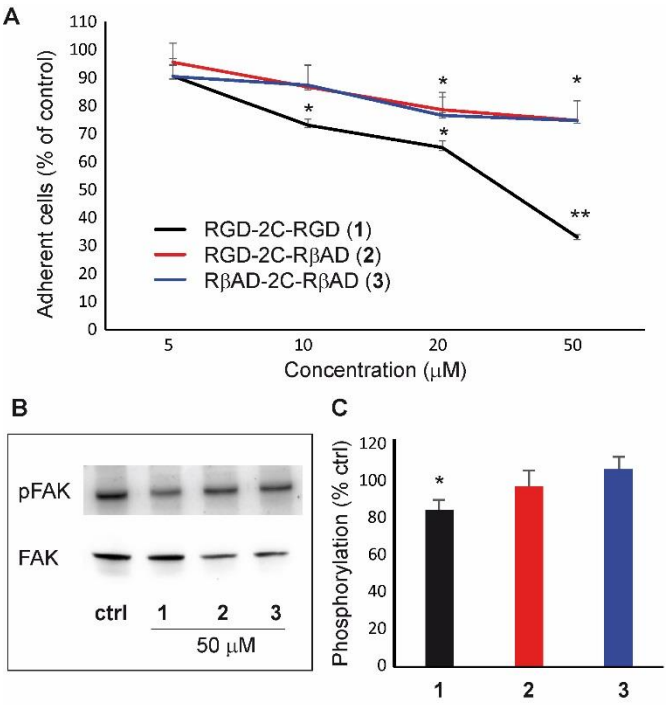

Figure 3. A) Induction of cell detachment by compounds **1-3**. Adherent U-373 MG cells were incubated with increasing compound concentrations for 48 h. Amounts of adherent cells in the wells were estimated by the MTS cell viability assay. Percentage of adherent cell was calculated by normalizing the values with no treatment control. Experiments were performed three times in quadruplicate. Lines represent mean ± standard deviation, $^{**}p < 0.05$, $^{*}p < 0.5$. B) Inhibition of FAK phosphorylation by compounds **1-3**. Cells were treated with a 50 μM solution of compounds **1-3** for 48 h; 30 μg of protein extracts for each sample were analysed by western blot. C) Densitometric analysis of western blot data, $^{*}p < 0.5$.

The effect of compound **1** on FAK phosphorylation was less pronounced than on the induction of cell detachment and this is possibly due to FAK involvement in other signal transduction pathways not directly connected to $\alpha_v\beta_3$ integrin signalling.

Conclusions

We reported here a dimeric integrin ligand featuring one RGD motif at each ring of a bicyclic structure and a disulfide bridge (compound **1**). The biological effects of the dual pharmacophore presentation in peptide **1** were evaluated in comparison to bicyclic peptides featuring either one (compound **2**) or two (compound **3**) copies of the poor integrin-binding motif RβAD, and a monocyclic RGD peptide (compound **4**). Among these compounds, dimeric peptide **1** exhibited a superior activity in competitive binding assays to the purified integrin receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$. This enhanced binding resulted in a potent interference of **1** with integrin signalling in U-373 MG glioblastoma cells, inducing a marked cell detachment and decrease of FAK phosphorylation. The inhibition of cell adhesion mediated by RGD integrin antagonists has raised interest in these compounds as potential antimetastatic agents.[19] The effects displayed by compound **1** account for the high local concentration of RGD pharmacophore in the surrounding of the receptor, which likely results in a "rebinding" mode that extends the ligand residence time in the integrin binding pocket. In addition to the multivalency effect, it is also possible that the second RGD-motif improves the integrin affinity of compound **1** via additional enthalpic interactions to the receptor binding site. In particular, while the first RGD motif is involved in the electrostatic clamp, interacting with charged regions of the integrin binding site,[20] the second RGD loop may contribute with stabilizing enthalpic interactions to proximal integrin residues, as reported recently by Timmerman and coworkers with monomeric RGD bicyclic peptides.[21] The field of bicyclic peptides has been dramatically expanding in recent years,[22] with important advances provided by the seminal work of Winter and Heinis on peptide phage display technologies,[23] as well as by the contribution of Pei and coworkers to the development of peptide ligands for intracellular proteins.[24] The data presented herein may further increase the interest around bicyclic peptides, opening to their use as high affinity dimeric ligands of clinically-relevant protein antigens.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Università degli Studi di Milano for a PhD fellowship (to G.S.). This work was supported by Ministero dell'Università e della Ricerca (PRIN 2015 project 20157WW5EH).

Notes and references

1 L. Urquhart, *Nat. Rev. Drug Disc.* 2019, **18**, 245.

2 G. Winter, A. D. Griffiths, R. E. Hawkins, H. R. Hoogenboom, *Annu. Rev. Immunol.* 1994, **12**, 433.

3 P. Chames, M. Van Regenmortel, E. Weiss, D. Baty, *Br. J. Pharmacol.* 2009, **157**, 220.

4 S. Cazzamalli, A. Dal Corso, F. Widmayer, D. Neri, *J. Am. Chem. Soc.* 2018, **140**, 1617.

5 a) M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem. Int. Ed.* 1998, **37**, 2754; b) C. Fasting, C. A. Schalley, M. Weber, O. Seitz, S. Hecht, B. Koksch, J. Dernedde, C. Graf, E.-W. Knapp, R. Haag, *Angew. Chem. Int. Ed.* 2012, **51**, 10472 c) M. Weber, A. Bujotzek, R. Haag, *J. Chem. Phys.* 2012, **137**, 054111

6 H. L. Handl, J. Vagner, H. Han, E. Mash, V. J. Hruby, R. J. Gillies, *Expert Opin. Ther. Targets* 2004, **8**, 565.

7 a) F. Thoreau, L. Vanwonterghem, M. Henry, J.-L. Coll, D. Boturyn, *Org. Biomol. Chem.* 2018, **16**, 4101; b) M. Degardin, D. Thakar, M. Claron, R. P. Richter, L. Coche-Guérente, D. Boturyn, *J. Mater. Chem. B*, 2017, **5**, 4745; c) L. Sandrin, D. Thakar, C. Goyer, P. Labbé, D. Boturyn, L. Coche-Guérente, *J. Mater. Chem. B*, 2015, **3**, 5577; d) A. R. M. Dias, A. Pina, A. Dal Corso, D. Arosio, L. Belvisi, L. Pignataro, M. Caruso, C. Gennari, *Chem. Eur. J.* 2017, **23**, 14410.

8 J. S. Desgrosellier and D. A. Cheresh, *Nat. Rev. Cancer*, 2010, **10**, 9.

9 Cyclic RGD peptides/peptidomimetics have shown widespread applications as angiogenesis inhibitors as well as for the targeted delivery of cytotoxic or imaging agents. For recent reviews, see: a) A. Dal Corso, L. Pignataro, L. Belvisi, C. Gennari, *Curr. Top. Med. Chem.* 2016, **16**, 314; b) D. Arosio, C. Casagrande, *Adv. Drug. Deliv. Rev.* 2016, **97**, 111; c) R. De Marco, A. Tolomelli, E. Juaristi, L. Gentilucci, *Med. Res. Rev.* 2016, **36**, 389.

10 N. Krall, F. Pretto, D. Neri, *Chem. Sci.* 2014, **5**, 3640.

11 a) M. Janssen, W. J. G. Oyen, L. F.A.G. Massuger, C. Frielink, I. Dijkgraaf, D. S. Edwards, M. Radjopadhye, F. H. M. Corstens, O. C. Boerman, *Cancer Biother. Radiopharm.* 2002, **7**, 641; b) Z. H. Jin, T. Furukawa, M. Degardin, A. Sugyo, A. B. Tsuji, T. Yamasaki, K. Kawamura, Y. Fujibayashi, M. R. Zhang, D. Boturyn, P. Dumy, T. Saga, *Mol. Cancer Ther.* 2016, **15**, 2076; c) Y. Yang, S. Ji, S. Liu, *Bioconjugate Chem.* 2014, **17**, 1720.

12 For previous examples of cyclic integrin ligands endowed with disulfide bridges, see: a) D. J. Burkhart, B. T. Kalet, M. P. Coleman, G. C. Post, T. H. Koch, *Mol. Cancer Ther.* 2004, **3**, 1593; b) E. Koivunen, B. Wang, E. Ruoslahti, *Biotechnology* 1995, **13**, 265; c) A. Spitaleri, S. Mari, F. Curnis, C. Traversari, R. Longhi, C. Bordignon, A. Corti, G. P. Rizzardi, G. Musco, *J. Biol. Chem.* 2008, **283**, 19757.

13 a) S. L. Goodman, G. Hölzemann, G. A. Sulyok, H. Kessler, *J. Med. Chem.* 2002, **45**, 1045; b) b) M. Marchini, M. Mingozzi, R. Colombo, I. Guzzetti, L. Belvisi, F. Vasile, D. Potenza, U. Piarulli, D. Arosio, C. Gennari, *Chem. Eur. J.* 2012, **18**, 6195; c) I. Guzzetti, M. Civera, F. Vasile, D. Arosio, C. Tringali, U. Piarulli, C. Gennari, L. Pignataro, L. Belvisi, D. Potenza, *ChemistryOpen* 2017, **6**, 128.

14 M. Aumailley, M. Gurrath, G. Müller, J. Calvete, R. Timpl, H. Kessler, *FEBS Lett.* 1991, **291**, 50.

15 The relative potency Rp is obtained by dividing the measured IC_{50} values of the monomeric compound **2** by the IC_{50} of dimeric compound **1**. Rp/n values were calculated by dividing Rp of the dimeric ligand by the valency (n) of the ligand itself (i.e. 2). A true multivalent effect is observed if Rp/n is higher than 1, see: D. Alvarez-Dorta, D. T. King, T. Legigan, D. Ide, I. Adachi, D. Deniaud, J. Désiré, A. Kato, D. Vocadlo, S. G. Gouin, Y. Blériot, *Chem. Eur. J.* 2017, **23**, 9022.

16 a) M. Paolillo, M. C. Galiazzo, A. Daga, E. Ciusani, M. Serra, L. Colombo, S. Schinelli, *Int. J. Oncol.* 2018, **53**, 2683; b) Integrin expression was assessed by quantitative RT-PCR (qRT-PCR), see the Electronic Supplementary Information (ESI).

17 During the cell detachment experiments, observation by optical microscopy did not reveal any apparent toxic effect on detached cells.

18 FAK is a cytoplasmic tyrosine kinase that promotes cell motility, survival, and proliferation, see: a) F. J. Sulzmaier, C. Jean, D. D. Schlaepfer, *Nat. Rev. Cancer* 2014, **14**, 598; b) D. S. Harburger, D. A. Calderwood, *J. Cell Sci.* 2009, **122**, 1472.

19 M. Paolillo, M. Serra, S. Schinelli, *Pharmacol Res.* 2016, **113**, 55.

20 J. P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* 2002, **296**, 151.

21 a) D. Bernhagen, V. Jungbluth, N. Gisbert Quilis, J. Dostalek, P. B. White, K. Jalink, P. Timmerman, *ACS Comb. Sci.* 2019, **21**, 598; b) D. Bernhagen, V. Jungbluth, N. G. Quilis, J. Dostalek, P. B. White, K. Jalink, P. Timmerman, *ACS Comb. Sci.* 2019, **21**, 198.

22 C. A. Rhodes, D. Pei, *Chem. Eur. J.* 2017, **23**, 12690.

23 a) K. Deyle, X. D. Kong, C. Heinis, *Acc. Chem. Res.* 2017, **50**, 1866; b) C. Heinis, G. Winter, *Curr. Opin. Chem. Biol.* 2015, **26**, 89; c) C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.* 2009, **5**, 502.

24 a) W. Lian, B. Jiang, Z. Qian, D. Pei, *J. Am. Chem. Soc.* 2014, **136**, 9830; b) B. Jiang, D. Pei, *J. Med. Chem.* 2015, **58**, 6306; c) T. B. Trinh, P. Upadhyaya, Z. Qian, D. Pei, *ACS Comb. Sci.* 2016, **18**, 75.