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Efficient siRNA-peptide conjugation for specific targeted delivery to tumor cells

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Albert Gandioso,[a] Anna Massaguer,[b] Núria Villegas,[cd] Cándida Salvans,[c] Dani Sánchez,[c] Isabelle Brun-Heath,[c] Vicente Marchán,*[a] Modesto Orozco[cde] and Montserrat Terrazas*[c]

Despite the broad applicability of the Huisgen cycloaddition reaction, the click functionalization of RNAs with peptides remains still a challenge. Here we describe a straightforward method for the click functionalization of siRNAs with peptides of different size and complexity. Among them, a promising peptide carrier for the selective siRNA delivery into HER2+ breast cancer cell lines.

RNA interference (RNAi)[1] is a potent gene regulatory process triggered by 21-nt RNA duplexes (siRNAs)[2] that has attracted much attention both for biotechnological and biomedical points of view. Unfortunately, the poor ability of siRNAs to cross cell membranes dramatically limits their practical use.[1b] To overcome this limitation, much effort has been dedicated to develop general delivery vehicles[3] involving positively charged peptides,[3c] lipids,[3de] dendrimers and nanocarriers[3f] as well as receptor-mediated delivery approaches.[3g] This research has produced a great variety of modified siRNAs[3] that have contributed to progress in this area. For example, in a recent work,[3h] functionalization of siRNAs at the 3'-termini of both strands with the cell permeable peptide KALLAL has allowed to improve the combination of siRNA and cationic vectors, increasing the half-life of siRNAs and their release from the endosome. On the other hand, receptor mediated delivery approaches are particularly interesting since they involve linking the siRNA to a targeting ligand (e.g. folate or peptide) that binds a membrane receptor expressed in a specific cell line. Unfortunately, despite the potential of siRNA-peptide conjugates, their preparation can be troublesome. For example, solid-phase step-wise synthesis can present problems of functional group incompatibility.[3a] An alternative consists in synthesizing separately RNA and peptide fragments functionalized with potentially reactive groups, and then to chemoselectively react (post-synthetically) both fragments in solution.[3a] Among various oligonucleotide linking strategies developed to date, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, "click")[4] is one of the most selective and versatile.[5] This approach has produced a large number of DNAs functionalized with fluorescent probes,[6ab] lipids[6c] and other carriers.[6d] Although fewer in number, several DNA-, 2'-*O*-Me-RNA- and locked nucleic acid-peptide conjugates have also been synthesized.[7,3c] However, due to the inherent lability of RNA (with free 2'-OH) limited research has been devoted to RNA click conjugation.[8] Most of the examples have been focused on the conjugation of small molecules[3e,8a-c] and fluorescent labels.[8de] To the best of our knowledge, there are only two examples of click post-conjugation of RNA to small peptides,[8fg] both of them implying long reaction times, large excesses of the peptides and several synthetic steps for the incorporation of the azide to the RNA. Most of the methods that have been used to functionalize RNA with peptides have been restricted to RNAs modified with thiol[9a] or amino[9b,3a] groups, which are reacted with maleimide or N-hydroxysuccinimide (NHS) esters. However, although the NHS approach is more efficient than the maleimide-thiol reaction, it

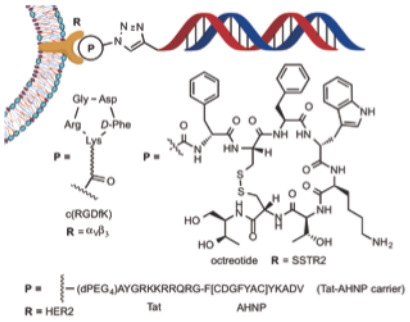

Fig. 1 siRNA-peptide conjugates used in this study.

[a] Department of Inorganic and Organic Chemistry, Section of Organic Chemistry, IBUB, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain.
[b] Department of Biology, University of Girona, Campus Montilivi, 17071 Girona, Spain.
[c] Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Joint IRB-BSC Program in Computational Biology, Baldiri Reixac 10-12, 08028 Barcelona, Spain.
E-mail: montserrat.terrazas@irbbarcelona.org
[d] The Join IRB-BSC Program in Computational Biology. Institute for Research in Biomedicine (IRB Barcelona).
[e] Department of Biochemistry and Biomedicine, University of Barcelona, 08028 Barcelona, Spain.

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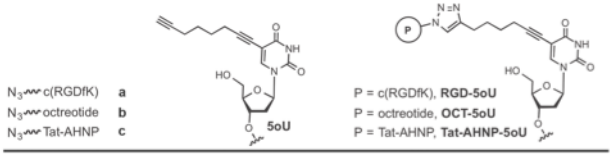
cannot be performed in the presence of free amino groups and requires slightly alkaline pH. Therefore, expanding the use of click chemistry to the conjugation of peptides to RNA would represent a great improvement in the synthesis and applications of RNA-peptide conjugates.

Here we present an efficient method for the Huisgen-based click functionalization of siRNAs with peptides targeting relevant receptors frequently overexpressed in major tumors: integrins, somatostatin and HER2 receptors (Fig. 1).

It is well-known that some integrins (specially, the $\alpha_v\beta_3$ subfamily) recognize the tripeptide motif RGD with high affinity and selectivity,[10] offering the possibility of using RGD-containing peptides to deliver drugs into specific cancer cell types overexpressing them. Another candidate for tumor-drug delivery is octreotide,[11a] a potent FDA-approved synthetic cyclooctapeptide agonist of the endocrine hormone somatostatin that displays high affinity for the somatostatin subtype-2 receptor (SSTR2), which is very prevalent in tumor cells.[11b] On the other hand, several attempts to overcome the limitations associated with antibody-based anti-HER2 therapies[12a] have led to the development of several small antigen-mimetic peptides of great therapeutic potential.[12bc] Among them, a peptide carrier based on a cyclic anti-HER2 peptide (AHNP) linked to a Tat cell-penetrating peptide (Tat-AHNP, Fig. 1) has been reported to enter HER2+ breast cancer cells with high efficiency.[12b] Unfortunately, there are no examples in the literature of the conjugation of vehicles based on this peptide to siRNAs. With the aim of targeting siRNAs directly to specific tumor cells overexpressing these receptors, we have explored the click functionalization of siRNAs with the cyclic RGD-containing cyclopentapeptide c(RGDfK), octreotide and the promising Tat-AHNP peptide carrier.

Because siRNAs modified at the ends of the sense strand are better tolerated by the RNAi machinery, we studied the CuAAC click reaction between a 5'-alkynyl-modified sense siRNA strand targeting the endogenous gene HER2 (**1**; Fig. 2) and azido-bearing peptides **a-c**. To achieve the RNA 5'-end conjugation, a deoxyuridine phosphoramidite with an octadiyne handle at C5 (5oU) was used during RNA synthesis.



siRNA	RNA	Sequence	T_m (°C)
	1	5'-**(5oU)**AAGCCUCACAGAGAUCUUGtt	
I	2	5'-**(RGD-5oU)**AAGCCUCACAGAGAUCUUGtt	75.8
	5	ttUUCGGAGUGUCUCUAGAAC-5'	
II	3	5'-**(OCT-5oU)**AAGCCUCACAGAGAUCUUGtt	76.2
	5	ttUUCGGAGUGUCUCUAGAAC-5'	
III	4	5'-**(Tat-AHNP-5oU)**AAGCCUCACAGAGAUCUUGtt	76.4
	5	ttUUCGGAGUGUCUCUAGAAC-5'	
IV	6	5'-AAGCCUCACAGAGAUCUUGtt	76.8
	5	ttUUCGGAGUGUCUCUAGAAC-5'	

Fig. 2 Sequences of 5'-alkynyl-sense RNA and guide RNA strands, azido peptides and siRNA-peptide click conjugates targeting the endogenous gene HER2, and T_m data.

Following previous approaches,[8e] we selected $CuSO_4$ and sodium ascorbate to carry out RNA-peptide conjugations, which were followed by HPLC and PAGE (Fig. 3 and Fig. S2-S4, ESI). We first studied the reaction between RNA **1** (0.15 µmol RNA; 1mM) and a two-fold excess of the RGD-containing azido peptide **a** (Fig. S1) in the presence of $CuSO_4$ (15 mM) and sodium ascorbate (15 mM) in a Tris·HCl-ACN (8:2 mixture). HPLC analysis of the reaction after 30 min revealed the formation of a new peak corresponding to the desired conjugate (**2**; as determined by MS analysis of the isolated peak), although conversion was far from being complete (Fig. S2, ESI). When larger excess of $CuSO_4$ and sodium ascorbate (45 equivalents; added in three separate additions) were used, conversion was quantitative within 90 min and conjugate **2** was obtained as a single product (as determined by HPLC and PAGE analyses of the reaction; Fig. S2-S4, ESI) in 40% isolated yield. By using these conditions, the larger and more complex octreotide and Tat-AHNP-azido peptides **b** and **c** (containing cysteine bridges and several trifunctional amino acids, including Lys; Fig. S1) were subsequently clicked very efficiently to the RNA **1**, to give conjugates **3** and **4** as single products (Fig. 3, Fig. S3-S4, ESI) (45% and 35% isolated yield, respectively). It is worth noting that complete conversion to the final product was observed in the three cases under the optimized conditions, and the three RNA-peptide conjugates were isolated with excellent purity using reversed-phase semi-preparative HPLC and characterized by MALDI-TOF MS (Table S1). Importantly, analysis of the purified conjugates by ICP-MS (ESI) revealed that copper contamination from CuAAC click reaction was negligible [10-25 ppb of copper for a 750 nM siRNA-peptide dose, see below, which is considerably below the average content of copper in human serum (1-2 ppm)].

Incorporation of c(RGDfK), octreotide or Tat-AHNP to the 5'-end of the siRNA did not cause negative effects on the stability of the double-helix (Fig. 2 and Fig. S5). Indeed, thermal denaturation studies with siRNA duplexes formed by 5'-peptidyl modified sequences **2-4** paired with the unmodified guide sequence **5** (**siRNAs I-III**) revealed melting temperature values very similar to that of the corresponding unmodified **siRNA IV** (formed by unmodified RNAs **5** and **6**).

To investigate the ability of siRNA-peptide conjugates **I-III** to selectively enter into cancer cells and induce RNAi-mediated inhibition of gene expression, we first focused on studying the level of expression of $\alpha_v\beta_3$ integrin, SSTR2 and HER2 receptors in a panel of tumor cell lines. The receptor's expression level on the cell surface was characterized by flow cytometry using

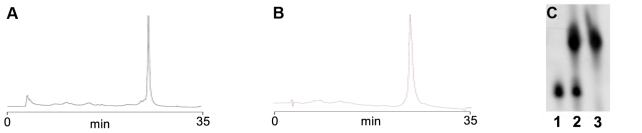


Fig. 3 (A, B) Reversed-phase HPLC analysis of sense-siRNA conjugate **3**. Panel A: reaction crude; panel B: fully purified conjugate. (C) Denaturing PAGE of the reaction of formation of conjugate **3** (lane 1: 5'-alkynyl RNA **1**, lane 2: mixture of RNA **1** and the reaction crude; lane 3: reaction crude).

specific antibodies (Fig. S6). We found that SK-BR-3 and BT-474 breast cancer cells expressed high levels of HER2 (mean fluorescence intensity of 1166.6 and 1302.2, respectively), which is in good agreement with data from the literature.[12b] Interestingly, the HER2+ SK-BR-3 cell line also expressed SSTR2 (mean fluorescence intensity of 52.1), which could be exploited to facilitate the internalization of siRNA-octreotide conjugate. However, we detected very low levels of $\alpha_V\beta_3$ integrin expression in this cell line (mean fluorescence intensity of 11.8). In contrast, SK-MEL-28 melanoma cells express high levels of $\alpha_V\beta_3$ integrin (mean fluorescence intensity of 278.9) and acceptable levels of HER2 (mean fluorescence intensity of 36.9). As expected, flow cytometry studies with FITC-labelled peptides confirmed that octreotide and Tat-AHNP were able to enter the SK-BR-3 cell line (Fig. S7). Satisfactory results were also obtained with FITC-c(RGDfK) peptide in SK-MEL-28 cells.[13] Identical results were obtained after exposing the peptides to siRNA annealing conditions (Fig. S7), suggesting that the active peptide structure responsible for interaction with the receptor remains unaffected after the annealing process required in inhibition studies (see below).

The overall data suggest that the breast cancer cell line SK-BR-3 is a good model to evaluate the biological activity of Tat-AHNP and octreotide-containing siRNA conjugates targeting the HER2 mRNA, while the SK-MEL-28 cell line can be used to test the c(RGDfK)-siRNA conjugate. Due to the significant relevance of the Tat-AHNP peptide carrier, we also used the HER2+ breast cancer cell line BT-474 to validate this system. The non-malignant HEK-293 cell line was used as negative control for Tat-AHNP-containing siRNA conjugates, as the level of HER2 expression is almost negligible in this cell line (Fig. S8, ESI).

We next studied the ability of siRNA-peptide conjugates to silence an endogenous gene (HER2) into the selected cell lines. With this aim, we hybridized the c(RGDfK), octreotide- and Tat-AHNP-sense strands (**2**, **3** and **4**, respectively) to the complementary unmodified guide strand **5** (Fig. 2) to give siRNA-peptide conjugates **I-III**, respectively. To investigate if the presence of c(RGDfK), octreotide and Tat-AHNP peptides at the 5'-end of the sense strand was tolerated by the RNAi machinery, we carried out a first series of RNAi studies in SK-BR-3 and SK-MEL-28 cells in the presence of the commercial transfecting agent Lipofectamine 2000. As a positive control we used the unmodified siRNA analogue **IV**. The effect of the siRNAs on HER2 protein levels was assessed by western blot 48 h after transfection. Dose-response experiments revealed that all siRNA-peptide conjugates (**I-III**) are well tolerated by the RNAi machinery, displaying a dose-dependent profile comparable to that of the unmodified siRNA (**IV**) (Fig. 4, A-C).

Encouraged by these observations, we next examined the ability of the siRNA-peptide conjugates to impart the desired RNAi effect in the selected cell lines in the absence of transfecting agent. Initial control experiments with unmodified siRNA **IV** in SK-BR-3 and SK-MEL-28 cell lines showed that the HER2 expression was not affected (Fig. 4, D and E). In contrast, we observed a significant HER2 knockdown in the presence of the octreotide-siRNA conjugate (**II**) in the SK-BR-3 cell lines (45% gene knockdown at 750 nM concentration; Fig. 4F). Encouraging results were also obtained for the Tat-AHNP- and cRGDfk-siRNA conjugates (**III** and **I**) in SK-BR-3 and SK-MEL-28 cells, respectively (28% gene knockdown for siRNA **III** at 1μM concentration and 27% gene knockdown for siRNA **I** at 2μM concentration; Fig. 4, G and H), proving uptake and activation of the RNAi machinery. Given the great potential of the Tat-AHNP carrier in the selective treatment of HER2+ cancer, we next proceeded to investigate the inhibitory activity of Tat-AHNP-siRNA conjugate **III** in another relevant HER2+ cancer cell line: BT-474. Very interestingly, inhibitory activity was also observed in this cell line in the absence of transfecting agent (30% gene knockdown at 750 nM siRNA **III** concentration; Fig. 4 I). To further confirm that the inhibitory activity of the Tat-AHNP bearing conjugate was a consequence of selective cell uptake, we next treated a non-malignant cell line expressing very low levels of HER2 (HEK-293) with a Tat-AHNP-bearing siRNA targeting the *Renilla* mRNA (Fig 5, A-C). To carry out these experiments, we conjugated the Tat-AHNP carrier to the 5'-end of the sense strand of a siRNA targeting the *Renilla* luciferase gene (RNA **7**; Fig. 5A). After hybridization with the unmodified complementary guide RNA strand (**8**), the resulting Tat-AHNP siRNA conjugate (**V**) was used to study the *Renilla* gene knockdown in HEK-293 cells previously transfected with *Renilla* and Firefly luciferase plasmids. A first series of experiments performed in the presence of transfecting agent (Fig. 5B) confirmed that Tat-AHNP *Renilla*-siRNA conjugate was able to activate the RNAi machinery in this cell line, with activities comparable to that of the corresponding unmodified siRNA analogue (**VI**) (96% inhibition of *Renilla* expression in both cases, at 90 nM siRNA concentration). In contrast, the levels of *Renilla* luciferase in this cell line were not affected after treatment with both siRNAs in the absence of transfecting agent (Figure 5C). These results confirm that the Tat-AHNP carrier displays the internalization properties required to deliver siRNAs into HER2 overexpressing cancer cell lines in a selective manner.

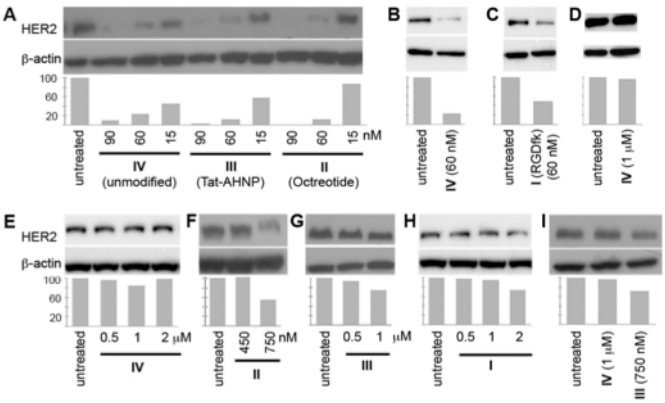


Fig. 4 Western blot analysis of cells treated with siRNA analogues in the presence (A-C) and in the absence of transfecting agent (D-I). Panels A, D, F and G: SK-BR-3 cells; Panels B, C, E and H: SK-MEL-28 cells; panel I: BT-474 cells. β-actin was used as loading control. Untreated cells: cells treated with cell medium alone.

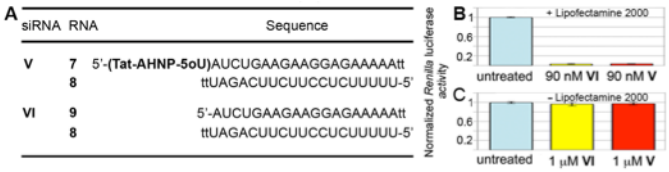


Fig. 5 (A) Sequences of 5'-Tat-AHNP-siRNA conjugate **V** and unmodified siRNA **VI** targeting the *Renilla* luciferase gene. (B, C) RNAi assays with siRNAs **V** and **VI** in the presence (B) or in the absence (C) of transfecting agent Lipofectamine 2000 in HEK-293 cells previously transfected with *Renilla* and Firefly luciferase plasmids. Untreated cells: cells treated with plasmids alone.

Importantly, WB analysis of cells treated with peptide vehicles alone confirmed that the inhibitory effect (Fig. 4) was exclusively induced by the siRNA-peptide conjugates, as peptide treatment did not cause any decrease in the HER2 protein level (Fig. S9). Furthermore, cell viability assays ruled out any toxicity from the peptides themselves (Fig. S10).

In summary, we have presented here a mild and efficient methodology for conjugating peptides to RNA via click chemistry in excellent yield and purity. This approach enables the efficient construction of siRNAs modified with receptor-binding peptides of different size and complexity at the 5'-end of the sense siRNA strand. By using this approach we have also been able to induce selectively gene silencing in HER2+ cancer cell lines with novel siRNA-peptide conjugates, making particularly appealing the use of Tat-AHNP targeting peptide. Although 750 nM-1 μM doses are needed, our approach offers the ability to selectively deliver the siRNA inside tumor cells overexpressing specific receptors, which would minimize undesired effects in healthy cells.

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Notes and references

1 (a) A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806; (b) G. F. Deleavey and M. J. Damha, *Chem. Biol.*, 2012, **19**, 937.
2 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494.
3 (a) A. Aviñó, S. Grijalvo, S. Pérez-Rentero, A. Garibotti, M. Terrazas and R. Eritja, *Meth. Mol. Biol.,* 2011, **751**, 223; (b) Y. Shing, P. Murat and E. Defrancq, *Chem. Soc. Rev.*, 2010, **39**, 2054; (c) P. Boisguérin, S. Deshayes, M. J. Gait, L. O'Donovan, C. Godfrey, C. A. Betts, M. J. A. Wood and B. Lebleu, *Adv. Drug. Deliv. Rev.*, 2015, **87**, 52; (d) D. V. Morrisey, J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer, Ll. B. Jeffs, A. Judge, I. MacLachlan and B. Polisky, *Nat. Biotechnol.*, 2005, **23**, 1002; (e) T. Yamada, C. G. Peng, S. Matsuda, H. Addepalli, K. N. Jayaprakash, Md. R. Alam, K. Mills, M. A. Maier, K. Charisse, M. Sekine, M. Manoharan and K. G. Rajeev, *J. Org. Chem.*, 2011**, 76**, 1198; (f) A. Kwok, G. A. Eggimann, M. Heitz, J. -L. Reymond, F. Hollfelder and T. Darbre, *ChemBioChem*, 2016, **17**, 2223.; (g) W. Tai and X. Gao, *Adv. Drug Deliv. Rev.*, 2016, http://dx.doi.org/10.1016/j.addr.2016.08.004; (h) X. Fang, Y. Zhang, X. Liu, H. He, Y. Ma, J. Sun, Y. Huang, X. Wang, Y. Wu, L. Zhang and Z. Yang, *Bioconjugate Chem.*, 2016, **27**, 1131.
4 (a) H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.,* 2001, **40**, 2004; (b) C. W. Torn∅e, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057.
5 J. E. Hein and V. V. Fokin, *Chem. Soc. Rev.*, 2010, **39**, 1302.
6 (a) F. Seela and S. A. Ingale, *J. Org. Chem.*, 2010, **75**, 284; (b) P. M. E. Gramlich, S. Warncke, J. Gierlich and T. Carell, *Angew. Chem., Int. Ed.*, 2008, **47**, 3442; (c) G. Godeau, C. Staedel and P. Barthélémy, *J. Med. Chem.*, 2008, **51**, 4374; (d) H. Lönnberg, *Curr. Org. Synth.*, 2009, **6**, 400.
7 (a) M. Jezowska, D. Honcharenko, A. Ghidini, R. Strömberg and M. Honcharenko, *Bioconjugate Chem*., 2016, **27**, 2620; (b) I. K. Astakhova, L. H. Hansen, B. Vester and J. Wengel, *Org. Biomol. Chem.*, 2013, **11**, 4240.
8 (a) K. Brunner, J. Harder, T. Halbach, J. Willibald, F. Spada, F. Gnerlich, K. Sparrer, A. Beil, L. Möckl, C. Bräuchle, K. -K. Conzelmann and T. Carell, *Angew. Chem., Int. Ed.*, 2015, **54**, 1946; (b) J. Willibald, J. Harder, K. Sparrer, K. -K. Conzelmann and T. Carell, *J. Am. Chem. Soc.*, 2012, **134**, 12330; (c) W. Wang, K. Chen, D. Qu, W. Chi, W. Xiong, Y. Huang, J. Wen, S. Feng and B. Zhang, *Tetrahedron Lett.*, 2012, **53**, 6747; (d) E. Paredes and S. R. Das, *ChemBioChem*, 2011, **12**, 125; (e) T. Santner, M. Hartl, K. Bister and R. Micura, *Bioconjugate Chem.*, 2014, **25**, 188; (f) M. Fonvielle, D. Mellal, D. Patin, M. Lecerf, D. Blanot, A. Bouhss, M. Santarem, D. Mengin-Lecreulx, M. Sollogoub, M. Arthur and M. Ethève-Quelquejeu, *Chem. Eur. J.*, 2013, **19**, 1357; (g) M. Fonvielle, I. de la Sierra-Gallay, A. H. El-Sagheer, M. Lecerf, D. Patin, D. Mellal, C. Mayer, D. Blanot, N. Gale, T. Brown, H. van Tilbeurgh, M. Ethève-Quelquejeu and M. Arthur, *Angew. Chem., Int. Ed.*, 2013, **52**, 7278.
9 (a) P. Z. Qin and A. M. Pyle, *Methods*, 1999, **18**, 60; (b) S. Solomatin and D. Herschlag, *Methods Enzymol.*, Vol. **469**, Elsevier, San Diego, 2009, p. 47.
10 H. M. Sheldrake and L. H. Patterson, *J. Med. Chem.*, 2014, **57**, 6301.
11 (a) W. Bauer, U. Briner, W. Dopfener, R. Haller, R. Huguenin, P. Marbach, T. J. Petcher and J. Pless, *Life Sci*., 1982, **31**, 1133; (b) A. Janecka, M. Zubrzycka and T. Janecki, *J. Pept. Res*., 2001, **58**, 91.
12 (a) R. Y. Tsang and R. S. Finn, *Br. J. Cancer*, 2012, **106**, 6; (b) M. Tan, K. -H. Lan, J. Yao, C. -H. Lu, M. Sun, C. L. Neal, J. Lu and D. Yu, *Cancer Res.*, 2006, **66**, 3764; (c) V. R. Fantin, M. J. Berardi, H. Babbe, M. V. Michelman, C. M. Manning and P. Ledder, *Cancer Res.*, 2005, **65**, 6891.
13 A. Massaguer, A. González-Cantó, E. Escribano, S. Barrabés, E. Artigas, V. Moreno and V. Marchán, *Dalton Trans.*, 2015, **44**, 202.

