NMR Spectroscopy

DOI: 10.1002/anie.200905941

2D TR-NOESY Experiments Interrogate and Rank Ligand–Receptor Interactions in Living Human Cancer Cells**

Silvia Mari, Chiara Invernizzi, Andrea Spitaleri, Luca Alberici, Michela Ghitti, Claudio Bordignon, Catia Traversari, Gian-Paolo Rizzardi, and Giovanna Musco**

Recent advances in cancer therapy include the design of molecules that interfer with tumor angiogenesis and moieties that recognize specific receptors expressed onto the tumor endothelium and/or onto tumor cells, thus allowing the ligand-directed targeted delivery of various drugs and particles to tumors. In this context, integrin αvβ3 and the membrane-spanning surface protein aminopeptidase N (CD13) play a pivotal role in tumor growth and metastatic spread, as they are important membrane-bound receptors highly expressed during angiogenesis.[1]

A crucial contribution to the efficacy of targeting approaches is the characterization of receptor–ligand interactions in their natural membrane environment. However, this is an inherently difficult goal to achieve. Usually reductionist approaches are adopted, where binding experiments are mostly performed on recombinant purified proteins. Nevertheless, these studies are often hampered by the limited availability of the target receptor, whose active form is often structurally undefined, as is the case for the CD13 isoform relevant in angiogenesis.[2] In addition, because of the intricate network of macromolecules simultaneously exerting different biological activities at the membrane level, binding assays using purified proteins often fail to reflect the true nature of the cellular environment. Therefore, the binding affinity and binding specificity of soluble forms may differ from the properties of the native receptor.[3]

Hence, drug-discovery studies may benefit from binding assays performed under physiological conditions, which might require accessory proteins contributing to ligand–receptor interactions. Herein, solution nuclear magnetic resonance (NMR) spectroscopy, because of its noninvasive nature, is increasingly and successfully utilized.[4–8] Ligand-observed NMR techniques,[9] including saturation-transfer difference (STD)[10] and transferred nuclear Overhauser effect (TR-NOE),[6] rely on the rapid and efficient transfer of spectroscopic characteristics between the free and the bound state of a ligand (mM–μM affinity range). As these techniques require only small amounts of purified receptor (pM–μM concentrations), they are routinely used to probe receptor–ligand interactions.[6,11] However, these techniques have some limitations and, until now, their in-cell application has not been fully exploited. STD has been successfully applied to monitor the binding of ligands to receptors localized on the surface of human cells; one-dimensional (1D) experiments were performed that allow rapid spectra acquisition.[3,12,13] Nevertheless, the applicability of STD in general may be hampered by the inability to discriminate between ligands with similar structures and chemical shifts; this is relevant in the screening of various ligands for the same receptor in competition experiments. Furthermore, STD and TR-NOE have been used only to study the interaction of ligands with a defined receptor, the prevalent receptor expressed on the cell surface.[3,12,14] Finally, although it has been employed on purified receptors,[15] organelles,[16] and platelets,[14] TR-NOE spectroscopy has not been performed with nucleated living human cells.

Here we successfully applied TR-NOE spectroscopy directly on patient-derived intact cancer cells to prove the selective binding of ligands to both molecularly undefined and structurally characterized receptors, such as the CD13 isoform expressed on angiogenic endothelial cells and αvβ3, respectively. In addition, we show that this method can be applied to screen various ligands for the same receptor by performing competition experiments, and thus an affinity ranking can be defined among different ligands in a physiological context.

We investigated the binding of various cyclopeptides containing the RGD and NGR motifs (see Figure 1 in the Supporting Information) onto two human cancer cell lines, including a non-small cell lung carcinoma (MR300) and a melanoma (MSR3) cell line, which display different phenotypes for CD13 and αvβ3. Both cell lines express αvβ3 but differ in the expression of CD13: MR300 cells express CD13 ($\alpha v\beta 3^+CD13^+$ cells), whereas MSR3 cells are negative ($\alpha v\beta 3^+CD13^-$ cells), (see Figure 2a,b in the Supporting Information). Published structural and biochemical data show that RGD and isoDGR motifs can efficiently bind (low μM affinity) to αvβ3,[17] unlike the NGR peptide, which

[*] Dr. S. Mari, Dr. A. Spitaleri, Dr. M. Ghitti, Dr. G. Musco
Dulbecco Telethon Institute
Laboratorio di RMN biomolecolare
Centro di Genomica, Bioinformatica e Biostatistica
S. Raffaele Scientific Institute
via Olgettina 58, 20132 Milano (Italy)
Fax: (+39) 02-2643-4153
E-mail: giovanna.musco@hsr.it
C. Invernizzi, L. Alberici, Prof. C. Bordignon, Dr. C. Traversari, Dr. G.-P. Rizzardi
MolMed SpA
via Olgettina 58, 20132 Milano (Italy)
Fax: (+39) 02-2127-7322
E-mail: paolo.rizzardi@molmed.com

[**] This work was supported in part by a research grant from the Italian Ministry of Research (FIRB). We thank Dr. Angelo Corti for critical reading of the manuscript and Renato Longhi for peptide synthesis.

Supporting information for this article, including the experimental details, is available on the WWW under http://dx.doi.org/10.1002/anie.200905941.

interacts with CD13 on tumor vessels,[2] thus indicating that NGR and RGD/isoDGR are separate moieties binding to different receptors.[17,18] We performed two-dimensional (2D) NOESY experiments at a field strength of 600 MHz using 5×10^6 cells in 200 μL deuterated phosphate buffer (PBS, 5 mM $MgCl_2$) at 37 °C for 2 h; cell suspension homogeneity and >75% cell viability were achieved throughout each experiment (see the Supporting Information). We first ran control experiments with cells alone (see Figure 2 d,e in the Supporting Information) and free ligands (0.5 mM) (Figure 1 a and Figure 1 a–e in the Supporting Information). Spectra of intact cells show background signals of metabolites along with line broadening arising from mobile lipids,[19] while spectra of free ligands show small positive NOEs, as expected for molecules of this size that have a fast tumbling rate; in contrast, slowly tumbling macromolecules generate large negative NOEs.[20] Next, NOESY spectra were acquired on each cyclopeptide, including CRGDC, CisoDGRC, CDGRC, and CARGC, in the presence of $\alpha v\beta 3^+CD13^-$ and $\alpha v\beta 3^+CD13^+$ cells (Figure 1 b,c,e–g). It is worth noting that, despite the high background generated by cellular components, NOE cross-peaks deriving from correlations of the arginine δ/γ protons can be identified unequivocally for each peptide (Figure 1). When CRGDC and CisoDGRC were studied under these experimental conditions, we observed the generation of negative NOE cross-peaks indicating that the average tumbling rate of these cyclopeptides is significantly slower than that in their free state (Figure 1 b,c,e). On one hand, this effect might be explained simply by the increased viscosity of the sample solution; on the other hand, it might suggest that a true TR-NOE is occurring, indicating that a molar fraction of the ligand molecules interacts with one or more receptors localized on the cell surface. To exclude the possibility that the change in NOE cross-peaks is related to the increased viscosity of the medium, we challenged CDGRC and CARGC, which are unable to bind CD13 and integrin αvβ3,[17] in $\alpha v\beta 3^+CD13^-$ (Figure 1 f) and $\alpha v\beta 3^+CD13^+$ cells (Figure 1 g). Herein, we observed the generation of positive NOE cross-peaks, indicating that these cyclopeptides are free in solution and do not interact with any surface receptor. Most importantly, CNGRC, a CD13 ligand, showed negative TR-NOE cross-peaks in the presence of $\alpha v\beta 3^+CD13^+$ cells (Figure 1 h), whereas no TR-NOE effects with $\alpha v\beta 3^+CD13^-$ cells were observed (Figure 1 i). Altogether, these results indicate that in experiments using living cells the flipped sign of the NOE cross-peaks is determined by a true ligand–receptor interaction, and that the decrease in the peptide tumbling rate does not occur nonspecifically because of medium viscosity. Thus, we conclude that TR-NOE in living tumor cells is a feasible approach.

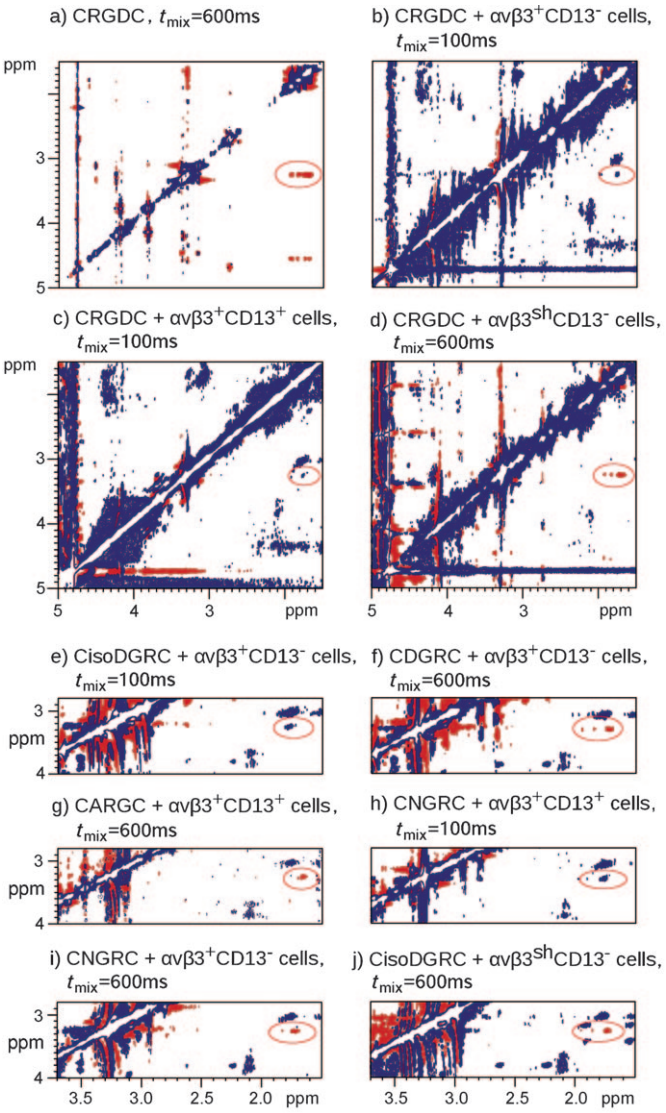

Figure 1. Ex vivo TR-NOE spectra showing protein–ligand interactions. The spectrum of the free ligand shows positive NOEs (opposite sign with respect to the diagonal). During ligand–receptor binding, the ligand transiently adopts the tumbling time of the receptor and can transfer the negative NOE (same sign with respect to the diagonal) of the protein complex to the population of the free molecule. a)–j) Ligand, cell line, and mixing time (t_{mix}) are indicated in each panel; spectra were acquired as described in the Supporting Information; the red circles indicate the cross-peaks deriving from correlations of the arginine δ/γ and δ/β protons; a) 2D NOESY spectrum of free CRGDC, and TR-NOESY spectrum of CRGDC in the presence of b) $\alpha v\beta 3^+CD13^+$ cells, c) $\alpha v\beta 3^+CD13^-$ cells, and d) β3-silenced ($\alpha v\beta 3^{sh}CD13^-$) cells; e)–j) selected regions of TR-NOESY spectra recorded for CisoDGRC, CDGRC, CARGC, CNGRC in the presence of different tumor cell lines.

We next tested whether TR-NOE can be applied to prove the specificity of the ligand–receptor interaction in living cells and to rank the affinity of varying ligands (Figure 2 a) for the same receptor. We took advantage of the use of living cells and transduced $\alpha v\beta 3^+CD13^-$ with a lentiviral vector coding for a β3-specific shRNA to achieve efficient silencing (>70%) of αvβ3 integrin in these $\alpha v\beta 3^{sh}CD13^-$ cells (see Figure 2 c,f in the Supporting Information). In the presence of $\alpha v\beta 3^{sh}CD13^-$ cells, CRGDC and CisoDGRC generated spectra with positive NOE effects, thus indicating that the peptides are free in solution (Figure 1 d,j; Figure 3 in the Supporting Information). Importantly, these results not only prove that CRGDC and CisoDGRC specifically target αvβ3, but also indicate that the application of TR-NOE in living cells can benefit from the possibility of mutating and altering

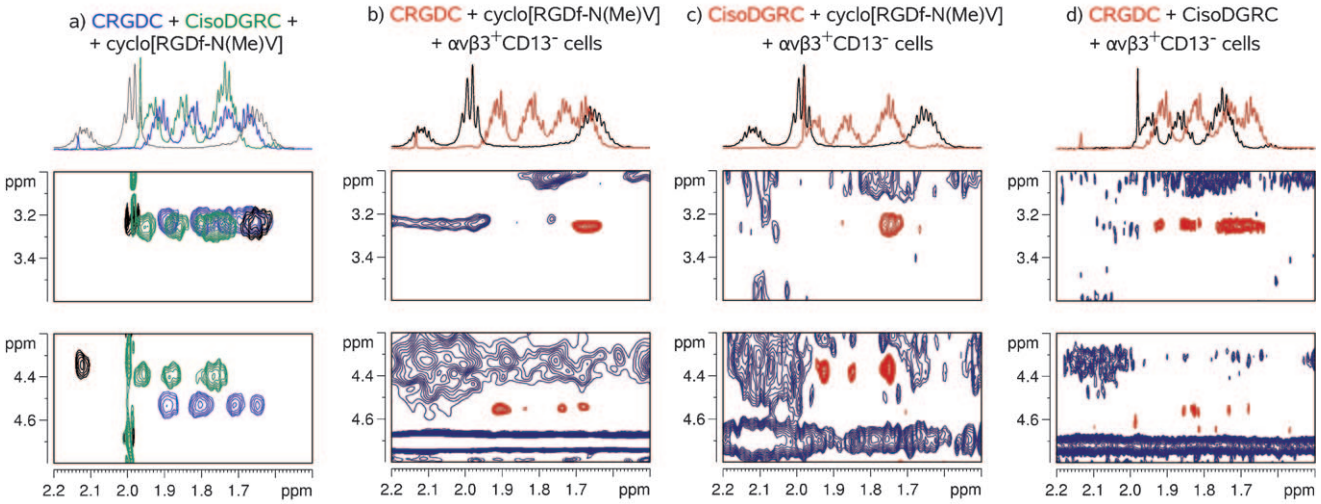

Figure 2. Ex vivo TR-NOE competition experiments and ligand-affinity ranking. Spectra were acquired as described in the Supporting Information. a) Superposition of 1D ^{1}H NMR spectra of free CRGDC (blue), CisoDGRC (green), and cyclo[RGDf-N(Me)V] (black) (upper panel), and superposition of NOESY spectra of ligand δ and α selected regions (middle and lower panels, respectively). b) CRGDC and c) CisoDGRC in the presence of $\alpha v\beta 3^+CD13^-$cells and equal amounts of cyclo[RGDf-N(Me)V] generate positive NOE cross-peaks, thus proving that cyclo[RGDf-N(Me)V] displaces the two ligands from $\alpha v\beta 3$ and has the highest relative affinity. d) A stoichiometric mixture of CisoDGRC and CRGDC is added to $\alpha v\beta 3^+CD13^-$ cells; CRGDC is free in solution generating positive NOE cross-peaks, thus indicating that CisoDGRC is able to displace CRGDC from the receptor.

the expression of a specific receptor. In addition, we performed competition experiments with CRGDC and CisoDGRC using equal amounts of cilengitide, the RGD-peptidomimetic cyclo[RGDf-N(Me)V], a well-described nM-affinity ligand for $\alpha v\beta 3$.[21] In these experiments, unlike in the absence of cilengitide (Figure 1b,c,e), CRGDC and CisoDGRC generated positive NOE cross-peaks, indicating that cilengitide displaces these two $\alpha v\beta 3$ ligands from the receptor and has the highest affinity (Figure 2b,c). To compare their relative binding strength to that of $\alpha v\beta 3$, equal amounts of CRGDC and CisoDGRC were challenged in the same TR-NOE competition experiment. We found that in $\alpha v\beta 3^+CD13^-$ cells CisoDGRC displaces CRGDC and shows a higher affinity for $\alpha v\beta 3$ (Figure 2d). Therefore, we conclude that it is feasible to rank various ligand–receptor affinities by performing TR-NOE experiments in living cells.

Overall, we have demonstrated that in living human tumor cell lines TR-NOESY methods can be successfully applied to monitor or screen ligand–receptor interactions and, in competition experiments, to obtain ligand-affinity ranking directly in a physiological context. In addition, by combining NMR methods with cell-manipulation techniques (e.g. shRNA-mediated receptor silencing) the specificity of ligand–receptor interactions can be investigated. Our approach makes it possible to verify the targeting of a receptor in a physiological amount in its cellular context without prior knowledge of its structure and posttranslational modifications, and the possibility that other proteins/components affect the binding and/or the receptor function. It is worth noting that ex vivo 2D TR-NOESY experiments will conceivably benefit from advances in NMR hardware along with fast NMR methods, including NMR covariance,[22] which can be used to reduce measurement time, a critical variable in experiments on living systems. In conclusion, these approaches may provide new platforms for the study of ex vivo protein–drug interactions.

Received: October 22, 2009
Published online: January 7, 2010

Keywords: drug design · integrins · NMR spectroscopy · protein–ligand interactions

[1] D. Neri, R. Bicknell, *Nat. Rev. Cancer* **2005**, *5*, 436.
[2] A. Corti, F. Curnis, W. Arap, R. Pasqualini, *Blood* **2008**, *112*, 2628.
[3] B. Claasen, M. Axmann, R. Meinecke, B. Meyer, *J. Am. Chem. Soc.* **2005**, *127*, 916.
[4] M. Pellecchia, I. Bertini, D. Cowburn, C. Dalvit, E. Giralt, W. Jahnke, T. L. James, S. W. Homans, H. Kessler, C. Luchinat, B. Meyer, H. Oschkinat, J. Peng, H. Schwalbe, G. Siegal, *Nat. Rev. Drug Discovery* **2008**, *7*, 738.
[5] J. W. Peng, J. Moore, N. Abdul-Manan, *Prog. Nucl. Magn. Reson. Spectrosc.* **2004**, *44*, 225.
[6] C. B. Post, *Curr. Opin. Struct. Biol.* **2003**, *13*, 581.
[7] B. J. Stockman, C. Dalvit, *Prog. Nucl. Magn. Reson. Spectrosc.* **2002**, *41*, 187.
[8] H. Widmer, W. Jahnke, *Cell. Mol. Life Sci.* **2004**, *61*, 580.
[9] T. Carlomagno, *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 245.
[10] M. Mayer, B. Meyer, *Angew. Chem.* **1999**, *111*, 1902; *Angew. Chem. Int. Ed.* **1999**, *38*, 1784.
[11] B. Meyer, T. Peters, *Angew. Chem.* **2003**, *115*, 890; *Angew. Chem. Int. Ed.* **2003**, *42*, 864.
[12] S. Mari, D. Serrano-Gomez, F. J. Canada, A. L. Corbi, J. Jimenez-Barbero, *Angew. Chem.* **2005**, *117*, 300–302; *Angew. Chem. Int. Ed.* **2005**, *44*, 296.

[13] F. M. Assadi-Porter, M. Tonelli, E. Maillet, K. Hallenga, O. Benard, M. Max, J. L. Markley, *J. Am. Chem. Soc.* **2008**, *130*, 7212.
[14] D. Potenza, L. Belvisi, *Org. Biomol. Chem.* **2008**, *6*, 258.
[15] L. Zhang, R. H. Mattern, T. I. Malaney, M. D. Pierschbacher, M. Goodman, *J. Am. Chem. Soc.* **2002**, *124*, 2862.
[16] L. Verdier, J. Gharbi-Benarous, G. Bertho, P. Mauvais, J. P. Girault, *Biochemistry* **2002**, *41*, 4218.
[17] 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.
[18] G. P. Rizzardi, C. Bordignon, *Blood* **2009**, *113*, 5366.
[19] J. D. de Certaines, L. Le Moyec, F. Seguin, P. A. Eliat, J. M. Constans, *Curr. Org. Chem.* **2007**, *11*, 529.
[20] D. Neuhaus , M. P. Williamson, *The NOE in structural and conformational analysis*, Wiley-VCH, New York, **2000**.
[21] D. Heckmann, H. Kessler, *Methods Enzymol.* **2007**, *426*, 463.
[22] R. Brueschweiler, *J. Chem. Phys.* **2004**, *121*, 409.

