Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 5590–5594
Bioorganic & Medicinal Chemistry Letters

On-resin cyclization of peptide ligands of the Vascular Endothelial Growth Factor Receptor 1 by copper(I)-catalyzed 1,3-dipolar azide–alkyne cycloaddition

Victor Goncalves,[a,b] Benoit Gautier,[a,b] Anne Regazzetti,[c] Pascale Coric,[d,e] Serge Bouaziz,[d,e] Christiane Garbay,[a,b] Michel Vidal[a,b,*] and Nicolas Inguimbert[a,b,*]

[a]*Université Paris Descartes, UFR biomédicale, Laboratoire de Pharmacochimie Moléculaire et Cellulaire, 45 rue des saints Pères, F-75006 Paris, France*
[b]*INSERM U648,45 rue des saints Pères, F-75006 Paris, France*
[c]*Université Paris Descartes, UFR des Sciences Pharmaceutiques et Biologiques, Laboratoire de Chimie Analytique, 4 avenue de l'observatoire, F-75006 Paris, France*
[d]*Université Paris Descartes, UFR des Sciences Pharmaceutiques et Biologiques, Laboratoire de pharmacologie chimique et génétique, 4 avenue de l'observatoire, F-75006 Paris, France*
[e]*INSERM U640, 4 avenue de l'observatoire, F-75006 Paris, France*

Received 15 June 2007; revised 26 July 2007; accepted 28 July 2007
Available online 22 August 2007

Abstract—Cyclic peptides were obtained, on-resin, by the copper (I) catalysed 1,3-dipolar cycloaddition of azides and alkynes. The reaction led exclusively to the formation of the expected cyclomonomeric products which acted as ligands of the Vascular Endothelial Growth Factor receptor 1.

In the few years since its discovery, copper (I)-promoted [3+2] Huisgen cycloaddition of azides with terminal alkynes has proved to be one of the most efficient 'click reactions' with widespread applications in organic chemistry and drug discovery.[1] It has been notably used with interest in the field of peptide chemistry to produce 1,2,3-triazoles as peptide bond isosteres[2–4] and to synthesize cyclic peptides. Up to now, two strategies have emerged to perform cyclization of such peptides. The first one was the solution phase method which led to monomeric,[3] dimeric[2] or mixtures of monomeric and dimeric cyclic peptides depending on the nature of amino acids introduced[5] and substrate concentrations.[6] The second strategy was the on-resin cyclization of peptides, as performed by Punna et al.[7] Unexpectedly, this method led to the formation of cyclodimeric products despite the pseudo-dilution effect of the resin.

Herein, we describe the application of copper(I)-catalyzed 1,3-dipolar azide–alkyne cycloaddition to the solid-phase synthesis of cyclic peptides targeting the Vascular Endothelial Growth Factor Receptor 1 (VEGFR1). Interestingly, this method specifically allowed the formation of monomeric peptides.

An increasing number of reports tend to indicate that VEGFR1 is strongly implicated in pathological angiogenesis, a biomarker of cancer, and that its inhibition may constitute an attractive strategy for stopping tumour growth and metastasis.[8–10] VEGFR1 is stimulated by the binding of its pro-angiogenic ligands, the Vascular Endothelial Growth Factor (VEGF) and the Placenta Growth Factor (PlGF). An interesting approach for inhibiting the receptor activation consists in developing peptides, or small molecules, that act as ligands of the receptor extra-cellular domain and therefore are able to displace VEGF/PlGF binding to VEGFR1.[11] Based on the structural[12,13] and mutagenesis[14,15] data available, several amino acids of the VEGF, essentials for VEGF-VEGFR1 interaction were identified (Fig. 1). Considering the spatial proximity existing between some of these

Keywords: Click chemistry; Huisgen reaction; VEGF receptors; Antagonist; Resin supported synthesis; Cyclic peptide.
* Corresponding authors. Tel.: +33 01 42 86 21 26; fax: +33 01 42 86 40 82; e-mail: nicolas.inguimbert@univ-paris5.fr

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.07.087

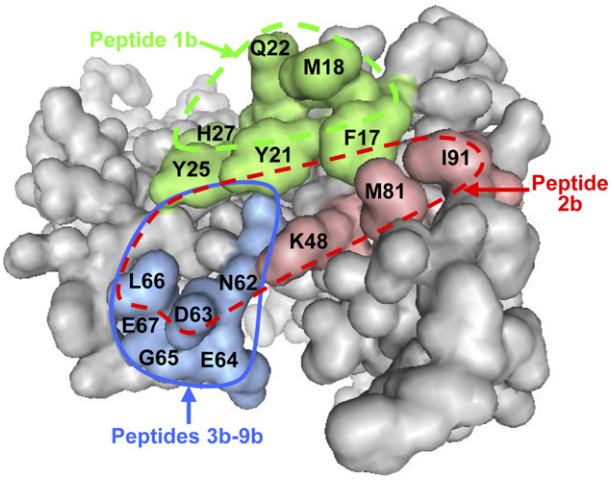


Figure 1. Surface rendering of $VEGF_{165}$ based on the crystal structure of VEGF–$VEGFR1_{d2}$ complex.[12] Residues involved in VEGF–VEGFR1 interaction are colored and labeled. The encircled areas were mimicked by the different synthesized peptides.

residues at the surface of the VEGF, we designed small VEGF mimics as peptides containing these amino acids.

These peptides were cyclised in order to obtain the desired circular arrangements of amino acids. Following this approach, three families of peptides were synthesized (Table 1).

The cyclic peptide **1b** mimics the VEGF residues H27, Q22, M18, F17, Y21, and Y25 (Fig. 1, green line).

The cyclopeptide **2b** mimics L66, D63, N62, K48, M81, I91, F17, W21 and Y25 amino acids (Fig. 1, red line).

A series of peptides (**3b**, **4b**, **5b**, **6b**, **7b**, **8b**, **9b**) imitates the area covered by the VEGF residues Y21, Y25, D63, E64, G65, L66 and E67 (Fig. 1, blue line).

For each cyclic peptide, the corresponding linear product was prepared.

First, the linear peptide **1a** (N_3-GHQMFYY*Pra*-NH_2) was synthesized by standard N^{α}-Fmoc chemistry on Rink amide MBHA resin[16] (substitution: 0.62 mmol/g) in 0.25 mmol scale. Couplings were carried out with in situ-activating reagents (HBTU, HOBt in the presence of DIPEA) to generate HOBt esters.

The N-terminal α-azido glycine was synthesized according to the method developed by Lundquist et al.[17] and the alkyne moiety was introduced as a L-propargylglycine introduced in the C-terminal position. This amino acid, commercially available, presents the same stereochemistry as natural amino acids and permits the side-chain cyclization of the peptide. After the elongation completed, a third of the resin was removed, cleaved and deprotected with triisopropylsilane as scavenger, leading to the linear azido-peptide **1a**. The remaining peptidyl-resin was cyclized by exposure to 0.5 equivalents of copper (I) iodide, in presence of sodium ascorbate and 2,6-lutidine in NMP/DCM (Scheme 1, method A). The reaction progress was monitored every 24 h by analyzing a sample of the resin by IR-spectroscopy (disappearance of the 2100 cm^{-1} band characteristic of the azide) and by the modified Kaiser test described by Punna et al.[18]

After 48 h at room temperature, the conversion of the azide and terminal alkyne in 1,2,3-triazole was complete. The β-(1*H*-[1,2,3]triazol-4-yl)alanine amino acid generated from the propargylglycine is abbreviated as βtA in the following cyclopeptides. The peptide was then cleaved from the resin, deprotected and purified by HPLC to a purity of 95%. After these treatments, the cyclomonomeric peptide **1b** (cyclo[GHQMFYYβtA]-NH_2) was exclusively isolated. The linear and cyclic peptides **2**–**6** were synthesized according to the same

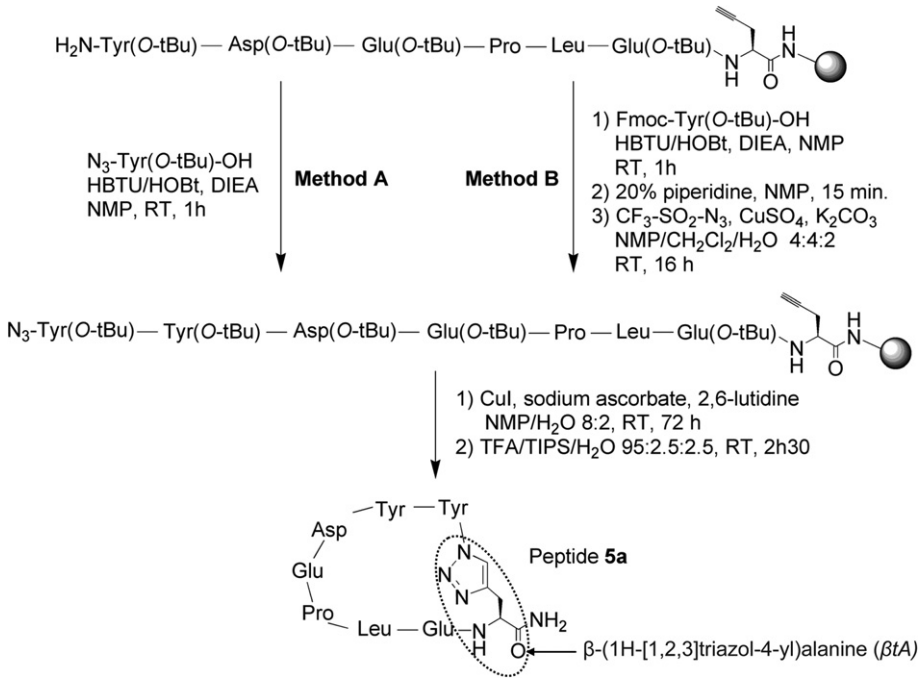
Table 1.

Compound	Peptide sequence[a]	Activity at 100 μM (%)[b]	IC50 (μM)
1a	N_3-GHQMFYYPra-NH_2	67	19.4 ± 2.2
1b	Cyclo[GHQMFYYβtA]-NH_2	35	121 ± 29
2a	N_3-GLDNKMIFWYPra-NH_2	95	22.9 ± 4.2
2b	Cyclo[GLDNKMIFWYβtA]-NH_2	83	31.1 ± 1.2
3a	N_3-GhFDEGLEPra-NH_2	0	ND
3b	Cyclo[GhFDEGLEβtA]-NH_2	10	ND
4a	N_3-GhFDEPLEPra-NH_2	0	ND
4b	Cyclo[GhFDEPLEβtA]-NH_2	29	ND
5a	N_3-YYDEPLEPra-NH_2	0	ND
5b	Cyclo[YYDEPLEPra]-NH_2	0	ND
6a	N_3-FYDEPLEPra-NH_2	55	96 ± 14
6b	Cyclo[FYDEPLEβtA]-NH_2	61	93 ± 18
7a	N_3-QYDEPLEPra-NH_2	0	ND
7b	Cyclo[QYDEPLEβtA]-NH_2	0	ND
8a	N_3-EYDEPLEPra-NH_2	6	ND
8b	Cyclo[EYDEPLEβtA]-NH_2	7	ND
9a	N_3-KYDEPLEPra-NH_2	27	151 ± 29
9b	Cyclo[KYDEPLEβtA]-NH_2	28	162 ± 24
SP5.2	NGYEIEWYSWVTHGMY-NH_2	99	28 ± 7

ND, not determined; In this set of experiments, recombinant human $VEGF_{165}$ displayed an IC_{50} of 387 ± 60 pM.

[a] hF, homophenylalanine; Pra, propargylglycine; βtA, β-(1*H*-[1,2,3]triazol-4-yl)alanine.

[b] The inhibitory activity corresponds to the percentage of biotinylated $VEGF_{165}$ displaced by 100 μM of peptide on VEGFR1.



Scheme 1. Synthesis of peptide **5b**. Method A: Azido-acid coupling followed by Cu(I)-promoted on-resin cyclization. Method B: Direct solid-phase conversion of the N-terminal amine into azide by diazo-tranfer followed by the cyclization of the peptide.

protocol. For all of the studied peptides, the comparison of the IR spectra in KBr tablets allowed clearly to distinguish the linear peptides, which exhibited a strong absorption at 2100 cm^{-1}, from the cyclic peptide in which such band was absent. Furthermore all the peptides were purified by HPLC to a purity of at least 95% and their structure confirmed by ESI-mass and NMR spectroscopy. Comparison of the 1H NMR spectra of the linear and cyclic forms for the compounds **1**–**9** allowed the characterization of the triazole system. Indeed all the cyclic peptides spectra show a particular resonance around 8 ppm corresponding to the HA proton of the triazole. Furthermore the α-proton Hα of the amino acid engaged in the triazole ring resonates at 5.5 ppm and is low field shiefted by around 1.5 ppm when compared to the same proton in the linear form. The assignment of these signals was accomplished after 2D NMR experiments performed on compounds **8a** and **8b**. The observed NOE interaction between the triazole hydrogen HA and the proton Hα of the peptide sequence attested for the ring closure. Moreover, many inter-residue NOE were observed between residues 1 and 8, in particular between proton Hb of residue 8 and protons Hβ* and Hγ* of residue 1 (See: supporting information).

The first peptide produced to mimic the VEGF residues Y21, Y25, D63, E64, G65, L66 and E67 was the peptide **3b** (cyclo[GhFDEGLE βtA]-NH_2. The homophenyl-alanine (hF) was introduced in the sequence in order to allow its aromatic ring to mimic the position occupied by the tyrosine 25 aromatic ring. Compared to the previous peptides, **1b** and **2b**, cyclization of **3b** proved to be sluggish (96 h) and gave raise to unidentified by-products observed by HPLC analysis of the crude reaction mixture. Thus, we attempted to replace the glycine contained in this peptide by a proline (peptide **4b**) to facilitate the cyclization which, indeed, appeared easier with a shortening of the reaction time (72 h) and a crude product with higher purity. Therefore, we chose to conserve the proline in the following peptides mimicking this sequence of amino acids.

Subsequently, we proceeded to a structure–activity study by evaluating the influence of the nature of the N-terminal residue on both cyclization efficiency and VEGFR1 binding affinity of the peptides. First, the peptides **5b** (cyclo[YYDEPLE βtA]-NH_2) and **6b** (cyclo[FYDEPLE βtA]-NH_2) were synthesized. For these experiments, $N_3{}^{\alpha}$-Tyr(*O*-tBu)-OH and $N_3{}^{\alpha}$-Phe-OH were prepared in respectively 90% and 77% yields following the Lundquist method. In the peptide **5b** the tyrosine are expected to mimic the Y21 and Y25, present in the VEGF while, in **6b**, the first tyrosine was replaced by a phenylalanine. These two peptides were obtained, respectively, in 8.4% and 7.0% yields and only compound **6b** behaved as a VEGFR antagonist. Furthermore, the cyclization did not lead to the expected increase of activity when compared to the linear compound **6a**.

Alternatively, we used a more direct method for synthesizing the peptide **5b**, i.e. the direct, on-resin, conversion of the N-terminal amine into azide by diazo-transfer,[19] followed by the Cu(I)-promoted cyclization of the peptide (Scheme 1, method B). After the solid-phase elongation of the peptide, the N-terminal Fmoc protecting group was cleaved by 20% piperidine in NMP. Then, an excess of trifluoromethanesulfonic azide in DCM was added to the resin and the suspension was stirred overnight at room temperature, leading to the complete conversion of the amine in the corresponding azide. Finally, the cyclization was realized following the method described previously and the peptide was deprotected

and cleaved by acidic treatment. This direct method proved to be as efficient as the previous one leading to the formation of the crude peptide with the same yield and level of purity.

The second method was applied to the synthesis of the linear peptides **7a**–**9a** and of their corresponding cyclic peptides. Overall, the cyclic peptides were obtained after RP-HPLC purification with 4–9% yields while linear peptides were recovered with 10–30% yields. The elongation, cyclization and deprotection steps allowed the formation of the peptides in crude form with a yield of 50% and a purity of 70%. The cyclization reaction was quantitative, as confirmed by IR spectroscopy and the modified Kaiser test. In order to dispose of pure peptides for biological studies, a RP-HPLC purification was performed. This purification step was low yielding and explains the low quantity of the isolated cyclic peptides. The most interesting aspect of this study is that this solid-phase cyclization approach led exclusively to the formation of cyclomonomeric products, as observed by HPLC and mass spectroscopy.

In order to experimentally confirm the exclusive formation of monomeric cyclic peptides, we proceeded to the cyclization of two peptides differing by the nature of their N-terminal azido-acid N_3FYDEPLEPra-NH_2 **6a** and N_3GYDEPLEPra-NH_2 bound on a same resin. Briefly, the first seven amino acids were coupled on a rink amide resin and the last residue was introduced as an equimolar mixture of tyrosine and glycine whose amine moieties were converted to an azide by on-resin diazo-transfer. Then we performed as previously the on resin cyclization by exposure to 0.5 equivalent of Cu(I). After cleavage and deprotection of a sample of resin, an HPLC analysis pursued before or after cyclization showed a relatively clean profile presenting two major peaks corresponding respectively to the two linear azido-peptides synthesized or to the cyclic ones (Fig. 2).

Therefore, as revealed by HPLC traces and MS analysis the major products formed correspond to the cyclomonomeric products and no other significant peak

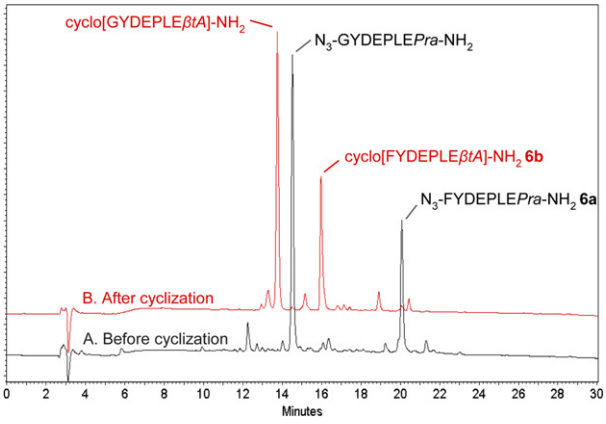


Figure 2. RP-HPLC analysis of crude products before (A) and after (B) cyclization of the peptide **6a** and its analogue bearing an N-terminal glycine residue, supported on a same resin.

that might correspond to the formation of a cycloheterodimeric product has been detected. Given the fact that we used almost the same reaction conditions than Punna et al. for performing the on-resin cyclization of peptides, we suggest that the difference of reactivity observed might be explained by the peptide sequences. Indeed, the linear peptides synthesized by Punna et al.[7] were characterized by the presence of an amino acid residue separating the propargylglycine from the resin, and an azide moiety supported by a relatively long alkyl chain. As a result, the linear peptide may be sufficiently long and flexible to allow the preferential formation of cyclodimeric products following the mechanism they proposed. By contrast, in our sequences, the propargylglycine was directly linked to the resin and the azide was supported by the N-terminal carbon-α. Thus, in this case, the cyclization process may truly benefit from the pseudo-dilution effect of the resin, and consequently promote efficiently the formation of cyclomonomeric products. In addition, the introduction of a proline in the peptide sequence may facilitate the intramolecular triazole formation by bringing the azide and alkyne moieties in close proximity.

The peptides affinity for VEGFR1 was determined thanks to a competition assay previously described.[20] Briefly, biotinylated $VEGF_{165}$ (131 pM) was incubated with the tested compound in presence of recombinant human VEGFR1 adsorbed on a microtiterplate. The biotinylated $VEGF_{165}$ remaining after wash steps was detected by chemiluminescence thanks to HRP-conjugated streptavidin. Table 1 summarizes the obtained results. As an internal control, we tested the peptide **SP5.2**, identified by phage-display screening and described as a VEGFR1-specific antagonist able to inhibit a broad-range of VEGF-mediated events.[21] Evaluated in our assay, **SP5.2** presented an IC_{50} of 28 μM. The linear peptides **1a** and **2a** proved to be potent ligands of the receptor 1 with respectively, an IC_{50} of 19 and 23 μM. The series of linear peptides mimicking the VEGF residues Y21, Y25, D63, E64, G65, L66 and E67 displayed strong differences of activity depending on the nature of the N-terminal azido-acids introduced. Indeed, only peptides containing a phenylalanine (**6a**) or a lysine (**9a**) exhibited significant activity at 100 μM.

Unfortunately, the corresponding cyclic peptides appeared either as active (**2b**, **6b**, **9b**), or less active (**1b**) than the corresponding linear peptides. We assume that the cyclization of these short peptides limited strongly their backbone flexibility and therefore, prevented the peptides from adapting to the VEGFR1 surface or that the selected sequence did not allow a proper alignment on the receptor surface.

In summary, the similar activities on VEGFR1 of the peptides **1a** and **2a** compared to SP5.2 is encouraging and they could be useful as a template for designing new VEGF antagonist. The evaluation of their biological activities will be investigated. Furthermore, we have demonstrated, for the first time, that CuI-catalysed 1,3-dipolar cycloaddition of azides and alkynes can be

applied to the specific synthesis of cyclomonomeric peptides.

Acknowledgments

Financial support for this work was provided by the Ligue nationale contre le cancer and the University Paris Descartes 'Bonus-Qualité-Recherche' grant. The authors thank Dr. Wang-Qing Liu for mass spectral data.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.07.087.

References and notes

1. Wu, P.; Fokin, V. V. *Aldrichim. Acta* **2007**, *40*, 7.
2. van Maarseveen, J. H.; Horne, W. S.; Ghadiri, M. R. *Org. Lett.* **2005**, *7*, 4503.
3. Bock, V. D.; Speijer, D.; Hiemstra, H.; van Maarseveen, J. H. *Org. Biomol. Chem.* **2007**, *5*, 971.
4. Brik, A.; Alexandratos, J.; Lin, Y. C.; Elder, J. H.; Olson, A. J.; Wlodawer, A.; Goodsell, D. S.; Wong, C. H. *Chembiochem* **2005**, *6*, 1167.
5. Angell, Y.; Burgess, K. *J. Org. Chem.* **2005**, *70*, 9595.
6. Choi, W. J.; Shi, Z. D.; Worthy, K. M.; Bindu, L.; Karki, R. G.; Nicklaus, M. C.; Fisher, R. J.; Burke, T. R. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5265.
7. Punna, S.; Kuzelka, J.; Wang, Q.; Finn, M. G. *Angew. Chem. Int. Ed.* **2005**, *44*, 2215.
8. Ferrara, N.; Kerbel, R. S. *Nature* **2005**, *438*, 967.
9. Takahashi, H.; Shibuya, M. *Clin. Sci. (Lond.)* **2005**, *109*, 227.
10. Luttun, A.; Autiero, M.; Tjwa, M.; Carmeliet, P. *Biochim. Biophys. Acta* **2004**, *1654*, 79.
11. Goncalves, V.; Gautier, B.; Lenoir, C.; Garbay, C.; Vidal, M.; Inguimbert, N. *Pharmachem* **2006**, 15.
12. Wiesmann, C.; Fuh, G.; Christinger, H. W.; Eigenbrot, C.; Wells, J. A.; de Vos, A. M. *Cell* **1997**, *91*, 695.
13. Christinger, H. W.; Fuh, G.; de Vos, A. M.; Wiesmann, C. *J. Biol. Chem.* **2004**, *279*, 10382.
14. Muller, Y. A.; Li, B.; Christinger, H. W.; Wells, J. A.; Cunningham, B. C.; de Vos, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7192.
15. Keyt, B. A.; Nguyen, H. V.; Berleau, L. T.; Duarte, C. M.; Park, J.; Chen, H.; Ferrara, N. *J. Biol. Chem.* **1996**, *271*, 5638.
16. Chang, C. D.; Meienhofer, J. *Int. J. Pept. Prot. Res.* **1978**, *11*, 246.
17. Lundquist, J. T.; Pelletier, J. C. *Org. Lett.* **2001**, *3*, 781.
18. Punna, S.; Finn, M. G. *Synlett* **2004**, 99.
19. Rijkers, D. T. S.; van Vugt, H. H. R.; Jacobs, H. J. F.; Liskamp, R. M. J. *Tetrahedron Lett.* **2002**, *43*, 3657.
20. Goncalves, V.; Gautier, B.; Garbay, C.; Vidal, M.; Inguimbert, N. *Anal. Biochem.* **2007**, *366*, 108.
21. El-Mousawi, M.; Tchistiakova, L.; Yurchenko, L.; Pietrzynski, G.; Moreno, M.; Stanimirovic, D.; Ahmad, D.; Alakhov, V. *J. Biol. Chem.* **2003**, *278*, 46681.