CXCR4 Ligands

DOI: 10.1002/anie.201202090

A Conformationally Frozen Peptoid Boosts CXCR4 Affinity and Anti-HIV Activity**

*Oliver Demmer, Andreas O. Frank, Franz Hagn, Margret Schottelius, Luciana Marinelli, Sandro Cosconati, Ruth Brack-Werner, Stephan Kremb, Hans-Jürgen Wester, and Horst Kessler**

The chemokine receptor subtype CXCR4 belongs to the G-protein coupled receptors (GPCRs) and is, together with its natural ligand CXCL12 (or SDF-1), a central part of the signaling system in the human body. Its functions range from stem-cell trafficking during embryogenesis, through cardiovascular, hematopoietic, and brain development, to signaling in the nervous and immune system.[1–6] Furthermore, CXCR4 is one of two major coreceptors used by the human immunodeficiency virus (HIV) for cell entry and CXCR4-using viruses are critical for the pathogenesis of AIDS.[7,8] Hence, CXCR4 represents a valuable therapeutic option for multiple diseases, such as inflammation, cancer,[9] and HIV/AIDS.[10] The recent approval of the CXCR4 antagonist AMD3100 (Mozobil) as a drug for stem-cell mobilization paves the way for development of further compounds that target CXCR4 related diseases.[1,11] However at present, none of the FDA-approved anti-HIV drugs target the CXCR4 receptor.

[*] Dr. O. Demmer, Dr. A. O. Frank, Dr. F. Hagn, Prof. Dr. H. Kessler
Institute for Advanced Study at the Department Chemie
Technische Universität München
Lichtenbergstrasse 4, 85748 Garching (Germany)
and
Chemistry Department, Faculty of Science
King Abdulaziz University
P.O. Box 80203, Jeddah 21589 (Saudi Arabia)
E-mail: kessler@tum.de
Homepage: http://www.org.chemie.tu-muenchen.de
Dr. M. Schottelius, Prof. Dr. H.-J. Wester
Lehrstuhl für Pharmazeutische Radiochemie
Garching (Germany)
Prof. Dr. L. Marinelli
Dipartimento di Chimica Farmaceutica e Tossicologica
Università di Napoli "Federico II", Napoli (Italy)
Dr. S. Cosconati
Dipartimento di Scienze Ambientali
Seconda Università di Napoli, Caserta (Italy)
Prof. Dr. R. Brack-Werner, Dr. S. Kremb
Helmholtz Zentrum München
Institute of Virology Neuherberg/München (Germany)

[**] We thank M. Wolff and B. Cordes for technical assistance and E. Gourni for the help in the cell binding assay. This work was supported by the Center of Integrated Protein Science Munich (CIPS). Financial support by the DFG (SFB824, Subproject B5) is acknowledged. The representation of the HI virus in the Table of Contents graphic was originally created by the US National Institute of Health (Permission: PD-USGov-HHS-NIH)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201202090.

One of the most interesting classes of CXCR4 antagonists is derived from the naturally occurring bicyclic peptide polyphemusin II which was modified in a stepwise fashion into monocyclic T140.[12] The monocyclic T140 and its derivatives are inverse agonists and therefore additionally offer the advantage of selectivity towards CXCR4 over other CXCR4 antagonists which function as partial agonists, for example, AMD3100.[13,14] In our efforts to develop high-affinity CXCR4 ligands as suitable probes for molecular imaging[15–17] we extended our studies through ligand-based design using conformational considerations and structure–activity relationships (SAR). Herein we describe CXCR4 antagonists with picomolar affinity, their binding mode, and their capacity to inhibit the HIV infection of cells.

The pioneering work of Fujii et al. demonstrated that the peptidic CXCR4 antagonist T140 can be downsized from 14 amino acids into a head-to-tail cyclized pentapeptide with a binding affinity (IC_{50}) of 8 nM (FC131; **1a**).[18] This peptide was further modified into the *N*-methylated analogue *cyclo*(-D-Tyr^1-D-[*N*Me]Arg^2-Arg^3-Nal^4-Gly^5-) (Nal = L-3-(2-naphthyl)alanine) **1b** resulting in even higher affinity (IC_{50} = 3 nM, Figure 1).[19] This is the highest CXCR4 binding affinity known to date for compounds with the cyclopentapeptide scaffold.

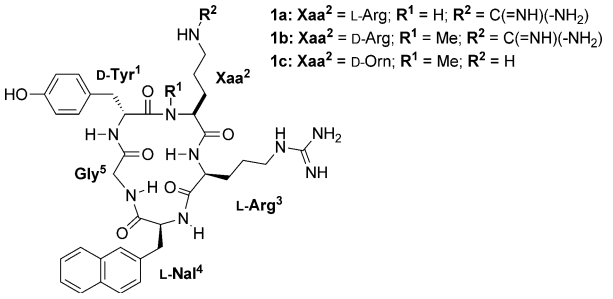

Figure 1. Constitutionally similar cyclic pentapeptides exhibiting different conformational behavior because of the introduction of an *N*-methyl group and different chirality in position 2.

Despite its high affinity, the NMR spectrum of this *N*-methylated peptide (**1b**) exhibits two conformations in slow equilibrium. The major one exhibits a conformation similar to **1a** and was assumed by Ueda et al.[19] to be the bioactive conformation. We determined the two conformations of **1c** ($IC_{50} = 6 \pm 1$ nM) in water by NMR spectroscopy to obtain detailed insight into the different binding modes with additional help of molecular modeling. For these experiments we used the ornithine^2 (Orn^2) derivative instead of Arg^2 as it was

previously modified for our molecular imaging study.[15–17] It can be assumed that this substitution has no influence on the backbone conformation; subsequently, we constructed ligands with even greater affinity by using a peptoid motif that exhibits one single conformation.

The structure of the major **1c** conformer, as investigated in water, is virtually the same as the one elucidated earlier for **1b** in DMSO (Figure 2).[19] The only major difference

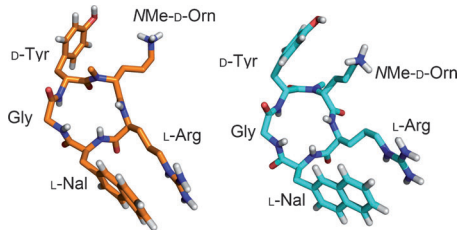

Figure 2. The major (*trans* amide bond between D-Tyr[1] and D-Orn[2], orange) and minor (*cis*, blue) conformations of **1c** exhibit a similar overall conformation of the peptide backbone except for the orientation at the *N*-methyl amide between D-Tyr[1] and D-Orn[2] which is flipped from *trans* to *cis*. Both structures are the most representative conformations taken from their unrestrained MD trajectories in explicit water (see Supporting Information for more details). All aliphatic protons were omitted for clarity.

between these two structures is the preferred orientation of the peptide bond between Nal[4] and Gly[5]. The minor conformation exhibits a *cis* peptide bond at the *N*-methylation site, which can be already concluded from the strong ROE intensity (ROE = rotating frame nuclear Overhauser effect) between the two H^{α} atoms of Tyr[1] to Orn[2] (see Supporting Information). This result demonstrates that the allylic strain induced by the introduction of the *N*-methyl group is strong enough to cause a flip from a *trans* to a *cis* peptide bond.[20]

As shown in Figure 2, the side chains of Tyr[1] and Orn[2] lie next to each other in solution and are inclined towards each other. A similar behavior had been observed in Somatostatin peptides where hydrophobic clustering is the main driving force for the preferred side-chain orientation.[21] These preliminary conformational studies prompted the design of new analogues in which the Orn (or Arg) side chain was shifted from the α-carbon to the adjacent nitrogen atom assuming that this change into a peptoid[22] can be assumed to result in a structure, where the peptide bond (*cis-trans*) would be *frozen* in its *trans* conformation. Such a conformational impact of the peptoid motif in combination with enhanced biological properties has been observed previously.[23]

For the synthesis of the peptoids, D-Orn was replaced by D-Ala and aminoalkyl residues were introduced to its N^{α} atom by the Fukuyama–Mitsunobu reaction on solid support in combination with standard Fmoc chemistry (Fmoc = 9*H*-fluoren-9-ylmethoxy)carbonyl).[24] Specifically, an *o*-nitrobenzensulfonyl (o-nosyl; Ns) protected amine is alkylated with an alcohol under Mitsunobu conditions. After deprotection of the Ns group the secondary amine is acylated using 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). The linear pentapeptides were then cyclized in dilute solution using diphenylphosphoryl azide (DPPA) and $NaHCO_3$. Deprotection with trifluoroacetic acid (TFA) and subsequent HPLC separation yielded the pure peptides (Table 1). Aminoalcohols were *tert*-butoxycarbonyl (Boc) protected prior to alkylation and 7-aminoheptan-1-ole was synthesized according to procedures described in literature.[25–27]

Table 1: Values for binding affinity to CXCR4 (IC_{50}) and anti-HIV activity (EC_{50}) of synthesized compounds.

Comp	*n*	R^1	R^2	IC_{50} [nM][a]	EC_{50} [nM][b]
1b	–	*N*-Me-D-Arg		3[c]	66 ± 5.7
2	4	Me	H	0.9 ± 0.1	n.d.
3	5	Me	H	1.6 ± 0.3	n.d.
4	6	Me	H	0.3 ± 0.2	53 ± 3.7
5	7	Me	H	2.4 ± 0.8	n.d.
6	6	H	H	100 ± 9	n.d.
7	6	H	Me	94 ± 38	979 ± 84
8[d]	6	Me	H	0.04 ± 0.02	29 ± 1.5
AMD 3100				651 ± 37[e]	365 ± 35.0

[a] Measured against [^{125}I]FC131. Mean value of at least three experiments. [b] Mean values ± standard deviations of 3–5 infection assays. [c] Taken from Ref. [19]. [d] The amine group in the *N*-alkyl chain is exchanged for a guanidine group. [e] Measured against [^{125}I]SDF1-α.[35]

Determination of the CXCR4 binding affinities of peptoids **2**–**8** (see Supporting Information) revealed that they all have at least twofold higher affinity towards CXCR4 than the parent peptide **1c** (Figure 3 and Table 1) regardless of their *N*-alkyl chain length, *n* in Table 1. D-Ala was exchanged with L-Ala (**7**) and Gly (**6**) in the backbone of the most active ligand **4**. Both derivatives exhibited two orders of magnitude lower affinity, indicating that the methyl group and its chirality in the D-Ala side chain is important for keeping the conformation close to the bioactive one.

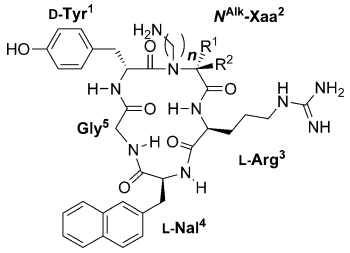

Figure 3. Peptoids with varying *N*-alkyl length and different chirality. See Table 1 for additional information.

Compound **4** has a subnanomolar affinity, which is nearly 20-fold higher than **1c**. On the NMR time scale **4** has a single conformation that corresponds to the all-*trans* form of **1c**; the alkylated amide bonds of **4** and **1c** have a similar orientation (Figure 4). This result confirms the assumption that the all-*trans* conformation of **1c** is the bioactive one,[19] and the Tyr[1] side chain and the *N*-alkyl group are arranged in a stacked conformation. The stacking leads to an upfield shift of the

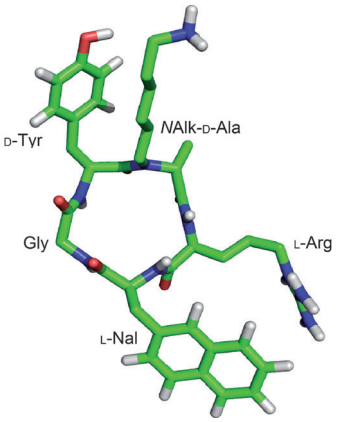

Figure 4. Compound **4** exhibits only one conformation in contrast to its *N*-methylated parent peptide **1c**. The Tyr^1 side chain and the *N*-alkyl residue form a hydrophobic interaction stabilizing the *trans* conformation. All aliphatic protons are omitted for clarity .

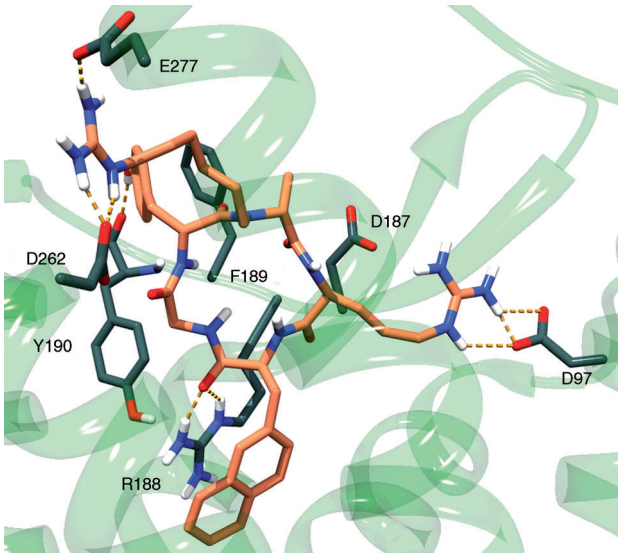

Figure 5. Binding conformation of peptoid **8** in the CXCR4 crystal structure as calculated by using the software Glide. The receptor is represented as light-green sticks and ribbons. The ligand is represented as orange sticks. Hydrogen bonds are represented as yellow dashed lines. For clarity, only interacting residues are shown.

proton signals on the second carbon from the backbone amide to $\delta = 0.58$ and 1.17 ppm compared to the signals of γ protons in an average arginine at $\delta = 1.56$ ppm. Furthermore, the peptide bond Nal–Gly in **4** is flipped so that the carbonyl oxygen atom points in the opposite direction to that of **1c** (Figure 4).

In a final optimization step the guanidine group was reintroduced in the peptoid aminoalkyl chain. This resulted in compound **8** having an additional almost 10-fold increase in affinity compared to **4** making it the most affine CXCR4 ligand reported to date.

To rationalize the ultra-high affinity displayed by the synthesized peptoids, docking studies (Figure 5) were carried out on the most active ligand **8** using the recently published X-ray structure of CXCR4 bound to the CVX15 peptide (protein data bank (PDB) code: 3OE0).[28] The best binding mode obtained with the docking program Glide places **8** in the receptor region occupied by CVX15 (See Figure 2a in Supporting Information for a comparison) with its Tyr^1 residue establishing a well oriented π–π interaction with F189 and a H-bond with the Y190 backbone CO (Figure 2 in Supporting Information). The adjacent *N*-alkylated Ala^2 is optimally oriented to place the long guanidinoalkyl chain towards a negatively charged region of the receptor (see Figure 3 in Supporting Information). This results in strong ionic interactions of the terminal guanidine group with D262 and E277. Interestingly, the same interaction pattern is established by Arg14 of the crystallized CVX15 peptide (see Figure 2a in Supporting Information). Consistent with the SAR data, the CXCR4 affinity of these peptoids depends on the length of the *N*-alkyl chain and on the nature of the terminal positively charged group. The almost 10-fold higher affinity of **8** in comparison with **4** should be ascribed to the ability of the guanidine group to establish a double salt bridge with the E277 and D262 residues. For the D262 residue, mutagenesis data outlined that its mutation abrogates the antagonistic activity of bicyclam compounds.[29] The hydrogen bonding and electrostatic interactions are also established by Arg^3 of **8** which makes polar contacts with D97 and D187 while Nal^4 is embedded in the inner portion of the receptor making a well-oriented cation–π interaction with R188. Noteworthy, such a contact is further reinforced by the establishment of an H-bond between the Nal^4 carbonyl oxygen atom and the R188 guanidine group of the receptor.

Although the peptoid structures can be considered close structural analogues of compound **1a–c**, the introduction of a well-defined (*trans*) conformation of the Tyr^1-*N*-alkylated Ala^2 amide bond allows the ligand to make contact with a receptor region that was previously unexplored by the parent compounds (see Figure 5 and 2b in Supporting Information). This contact most probably accounts for the enhanced affinity of **8**.

The extremely high binding affinity motivated us to explore the capacity of this compound to inhibit HIV infection. For this purpose we used a full HIV-replication assay validated for the discovery and the analysis of the efficacy of HIV inhibitory molecules.[30] This assay is based on HIV-permissive cells that express the HIV receptor CD4 and the CXCR4 co-receptor and contain a reporter gene activated by HIV infection to express a red fluorescent protein.

All tested compounds inhibited infection of the reporter cells by HIV-1(LAI) (Table 1), a prototypical virus strain that uses CXCR4 for entry.[31] Compounds **4** and **8**, which have the highest affinity to CXCR4 also showed strongest anti-HIV activities, with EC_{50} values of (53 ± 3.7) and (29 ± 1.5) nM, respectively. Compound **7**, which has the lowest affinity to CXCR4 also showed the lowest anti-HIV activity, which was over 30-limes lower than the anti-HIV activity of compound **8**. Compounds **4**, **8**, and **1b** all exhibited higher anti-HIV activity than AMD3100. All compounds failed to inhibit infection of CCR5-expressing reporter cells by HIV-1 (AD8), an HIV-1 isolate that enters cells through the CCR5 coreceptor,[32] when tested in HIV permissive cells that express the CCR5

coreceptor (see Figure 1 on page 6 of the Supporting Information). This result confirms specificity of the tested compounds for HIV-1 CXCR4-using HIV-1 variants.

In conclusion, we succeeded in restricting the conformation of a known CXCR4 ligand into a single active conformation by using a peptoid motif. In combination with structure–activity relationship studies the binding affinity was optimized to be 400 to 1500-fold higher than compounds currently under clinical development, such as KRH-1636 or AMD3100. AMD3100 is approved by the FDA for treatment of non-Hodgkin lymphoma and multiple myeloma.[33,34] Together with the advantages of modified cyclic peptides as drugs (e.g. biocompatible metabolism, high stability against enzymatic degradation, selectivity profile[35,36]) this molecule has the potential to be a promising candidate for future medical applications in CXCR4 related diseases.[29]

Received: March 16, 2012
Revised: May 2, 2012
Published online: July 3, 2012

Keywords: biological activity · drug design · medicinal chemistry · peptides · peptidomimetics

[1] W.-T. Choi, S. Duggineni, Y. Xu, Z. Huang, J. An, *J. Med. Chem.* **2012**, *55*, 977 – 994.
[2] C. C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, T. A. Springer, *Nature* **1996**, *382*, 829 – 833.
[3] E. Oberlin, A. Amara, F. Bachelerie, C. Bessia, J.-L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J.-M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, B. Moser, *Nature* **1996**, *382*, 833 – 835.
[4] C. Murdoch, *Immunol. Rev.* **2000**, *177*, 175 – 184.
[5] R. S. Klein, J. B. Rubin, *Trends Immunol.* **2004**, *25*, 306 – 314.
[6] M. Burger, T. Hartmann, M. Krome, J. Rawluk, H. Tamamura, N. Fujii, T. J. Kipps, J. A. Burger, *Blood* **2005**, *106*, 1824 – 1830.
[7] P. Lusso, *EMBO J.* **2006**, *25*, 447 – 456.
[8] P. R. Gorry, P. Ancuta, *Curr. HIV/AIDS Rep.* **2011**, *8*, 45 – 53.
[9] J. A. Burger, T. J. Kipps, *Blood* **2006**, *107*, 1761 – 1767.
[10] H. Tamamura, N. Fujii, *Expert Opin. Ther. Targets* **2005**, *9*, 1267 – 1282.
[11] J.-P. Levesque, I. G. Winkler, *Curr. Opin. Organ Transplant.* **2008**, *13*, 53 – 58.
[12] H. Tamamura, Y. Xu, T. Hattori, X. Zhang, R. Arakaki, K. Kanbara, A. Omagari, A. Otaka, T. Ibuka, N. Yamamoto, H. Nakashima, N. Fujii, *Biochem. Biophys. Res. Commun.* **1998**, *253*, 877 – 882.
[13] M. M. Rosenkilde, L.-O. Gerlach, J. S. Jakobsen, R. T. Skerlj, G. J. Bridger, T. W. Schwartz, *J. Biol. Chem.* **2004**, *279*, 3033 – 3041.
[14] E. De Clercq, *Biochem. Pharmacol.* **2009**, *77*, 1655 – 1664.
[15] O. Demmer, E. Gourni, U. Schumacher, H. Kessler, H.-J. Wester, *ChemMedChem* **2011**, *6*, 1789 – 1791.
[16] O. Demmer, I. Dijkgraaf, U. Schumacher, L. Marinelli, S. Cosconati, E. Gourni, H.-J. Wester, H. Kessler, *J. Med. Chem.* **2011**, *54*, 7648 – 7662.
[17] E. Gourni, O. Demmer, M. Schottelius, C. D′Alessandria, S. Schulz, I. Dijkgraaf, U. Schumacher, M. Schwaiger, H. Kessler, H.-J. Wester, *J. Nucl. Med.* **2011**, *52*, 1803 – 1810.
[18] N. Fujii, S. Oishi, K. Hiramatsu, T. Araki, S. Ueda, H. Tamamura, A. Otaka, S. Kusano, S. Terakubo, H. Nakashima, J. A. Broach, J. O. Trent, Z. X. Wang, S. C. Peiper, *Angew. Chem.* **2003**, *115*, 3373 – 3375; *Angew. Chem. Int. Ed.* **2003**, *42*, 3251 – 3253.
[19] S. Ueda, S. Oishi, Z.-X. Wang, T. Araki, H. Tamamura, J. Cluzeau, H. Ohno, S. Kusano, H. Nakashima, J. O. Trent, S. C. Peiper, N. Fujii, *J. Med. Chem.* **2007**, *50*, 192 – 198.
[20] J. Chatterjee, D. Mierke, H. Kessler, *J. Am. Chem. Soc.* **2006**, *128*, 15164 – 15172.
[21] B. H. Arison, R. Hirschmann, D. F. Veber, *Bioorg. Chem.* **1978**, *7*, 447 – 451.
[22] H. Kessler, *Angew. Chem.* **1993**, *105*, 572 – 573; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 543 – 544.
[23] S. C. Shankaramma, K. Moehle, S. James, J. W. Vrijbloed, D. Obrecht, J. A. Robinson, *Chem. Commun.* **2003**, 1842 – 1843.
[24] O. Demmer, I. Dijkgraaf, M. Schottelius, H.-J. Wester, H. Kessler, *Org. Lett.* **2008**, *10*, 2015 – 2018.
[25] J. M. Chong, M. A. Heuft, P. Rabbat, *J. Org. Chem.* **2000**, *65*, 5837 – 5838.
[26] C. C. Forbes, K. M. DiVittorio, B. D. Smith, *J. Am. Chem. Soc.* **2006**, *128*, 9211 – 9218.
[27] A. Nilsen, P. M. England, *J. Am. Chem. Soc.* **2007**, *129*, 4902 – 4903.
[28] B. Wu, E. Y. Chien, C. D. Mol, G. Fenalti, W. Liu, V. Katritch, R. Abagyan, A. Brooun, P. Wells, F. C. Bi, D. J. Hamel, P. Kuhn, T. M. Handel, V. Cherezov, R. C. Stevens, *Science* **2010**, *330*, 1066 – 1071.
[29] S. Hatse, K. Princen, L.-O. Gerlach, G. Bridger, G. Henson, E. De Clercq, T. W. Schwartz, D. Schols, *Mol. Pharmacol.* **2001**, *60*, 164 – 173.
[30] S. Kremb, M. Helfer, W. Heller, D. Hoffmann, H. Wolff, A. Kleinschmidt, S. Cepok, B. Hemmer, J. Durner, R. Brack-Werner, *Antimicrob. Agents Chemother.* **2010**, *54*, 5257 – 5268.
[31] K. Peden, M. Emerman, L. Montagnier, *Virology* **1991**, *185*, 661 – 672.
[32] E. O. Freed, G. Englund, M. A. Martin, *J. Virol.* **1995**, *69*, 3949 – 3954.
[33] K. Ichiyama, S. Yokoyama-Kumakura, Y. Tanaka, R. Tanaka, K. Hirose, K. Bannai, T. Edamatsu, M. Yanaka, Y. Niitani, N. Miyano-Kurosaki, H. Takaku, Y. Koyanagi, N. Yamamoto, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4185 – 4190.
[34] S. P. Fricker, V. Anastassov, J. Cox, M. C. Darkes, O. Grujic, S. R. Idzan, J. Labrecque, G. Lau, R. M. Mosi, K. L. Nelson, L. Qin, Z. Santucci, R. S. Y. Wong, *Biochem. Pharmacol.* **2006**, *72*, 588 – 596.
[35] P. Vlieghe, V. Lisowski, J. Martinez, M. Khrestchatisky, *Drug Discovery Today* **2010**, *15*, 40 – 56.
[36] J. Chatterjee, B. Laufer, H. Kessler, *Nat. Protoc.* **2012**, *7*, 432 – 444.

