Inhibitors

Deutsche Ausgabe: DOI: 10.1002/ange.201600190
Internationale Ausgabe: DOI: 10.1002/anie.201600190

Nonpeptidic Selective Inhibitors of the Chymotrypsin-Like (β5i) Subunit of the Immunoproteasome

*Izidor Sosič[+], Martina Gobec[+], Boris Brus, Damijan Knez, Matej Živec, Janez Konc, Samo Lešnik, Mitja Ogrizek, Aleš Obreza, Dušan Žigon, Dušanka Janežič, Irena Mlinarič-Raščan, and Stanislav Gobec**

Abstract: *Elevated expression of the immunoproteasome has been associated with autoimmune diseases, inflammatory diseases, and various types of cancer. Selective inhibitors of the immunoproteasome are not only scarce, but also almost entirely restricted to peptide-based compounds. Herein, we describe nonpeptidic reversible inhibitors that selectively block the chymotrypsin-like (β5i) subunit of the human immunoproteasome in the low micromolar range. The most potent of the reversibly acting compounds were then converted into covalent, irreversible, nonpeptidic inhibitors that retained selectivity for the β5i subunit. In addition, these inhibitors discriminate between the immunoproteasome and the constitutive proteasome in cell-based assays. Along with their lack of cytotoxicity, these data point to these nonpeptidic compounds being suitable for further investigation as β5i-selective probes for possible application in noncancer diseases related to the immunoproteasome.*

The eukaryotic 20S proteasome (the core particle, CP) represents the heart of the ubiquitin-proteasome system that is responsible for maintaining protein homeostasis and regulation for a variety of cellular processes, ranging from antigen processing and signal transduction, to cell differentiation and apoptosis.[1,2] The 20S proteasome has three enzymatically active subunits, namely the β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like) subunits, which all have distinct substrate specificities.[3] The constitutive proteasome (cCP) is expressed in all eukaryotic cells, whereas the immunoproteasome (iCP) is predominantly found in cells of hematopoietic origin.[4,5] In non-hematopoietic cells, expression of the iCP-active β subunits (designated β1i, β2i, β5i), which replace their constitutive counterparts, can be induced under the influence of tumor necrosis factor α and interferon-γ released by T cells during acute immune and stress responses.[6,7] Extensive research in the past decades has resulted in the successful development of bortezomib and carfilzomib as therapeutics for multiple myeloma and mantle-cell lymphoma that act as β5 and β5i inhibitors of cCP and iCP, respectively.[8,9] However, selective inhibition of the iCP is expected to cause fewer adverse effects, since its expression is induced only during the course of disease processes.[10,11] Current studies also indicate that β5i-selective compounds such as [PR-957 (ONX-0914)] provide more therapeutic potential than inhibition of the other two active sites of the iCP, especially for autoimmune and inflammatory diseases.[12,13] From a structural perspective, the majority of currently available inhibitors of the iCP have a peptidic backbone and are thus prone to poor metabolic stability and low bioavailability. Aside from improvements to the current array of peptide-like inhibitors of the iCP, it is imperative to develop inhibitors with different structural characteristics and improved physicochemical properties, and consequently improved pharmacokinetic and pharmacodynamic profiles.[14,15] To achieve this, options outside the classic "protease-inhibitor box" have to be considered. Various drug-discovery approaches[16] should enable the identification of compounds that are devoid of the peptidic backbone and can thus avoid the current limitations of peptide-based compounds. Currently, nonpeptidic inhibitors of the iCP are scarce, with only two recent examples known.[17,18] The crystal structure of the murine iCP (PDB ID: 3UNF) opens diverse possibilities for advanced computer-based discovery and design, as well as for rational optimization of selective iCP inhibitors.[19] In the present study, we describe reversible and irreversible nonpeptidic inhibitors of the human β5i subunit that were discovered through a structure-based virtual-screening approach (see the Supporting Information) using the ZINC drug-like subset of compounds. Irreversibly acting compounds show improved inhibition and better selectivity properties than PR-957, the established inhibitor of β5i, along with very low cytotoxicity.

In vitro evaluation of the best virtual screening hits (Table S1 in the Supporting Information) revealed a substituted psoralene derivative (Figure 1 a, virtual screening hit) as the most promising compound, since it showed good inhibition of the β5i subunit at 50 μM (residual activity = 0.5 %). This molecule was resynthesized by using a very efficient and straightforward synthesis (Scheme S1 in the Supporting

[*] Dr. I. Sosič,[+] Dr. M. Gobec,[+] Dr. B. Brus, D. Knez, Dr. M. Živec, Prof. Dr. A. Obreza, Prof. Dr. I. Mlinarič-Raščan, Prof. Dr. S. Gobec
Faculty of Pharmacy, University of Ljubljana
Aškerčeva 7, 1000 Ljubljana (Slovenia)
E-mail: stanislav.gobec@ffa.uni-lj.si
Dr. J. Konc, S. Lešnik, M. Ogrizek
National Institute of Chemistry
Hajdrihova 19, 1000 Ljubljana (Slovenia)
Dr. D. Žigon
Department of Environmental Sciences, Jožef Stefan Institute
Jamova 39, 1000 Ljubljana (Slovenia)
Prof. Dr. D. Janežič
Faculty of Mathematics
Natural Sciences and Information Technologies
University of Primorska, Glagoljaška 8, 6000 Koper (Slovenia)
[+] These authors contributed equally to this work.
Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201600190.

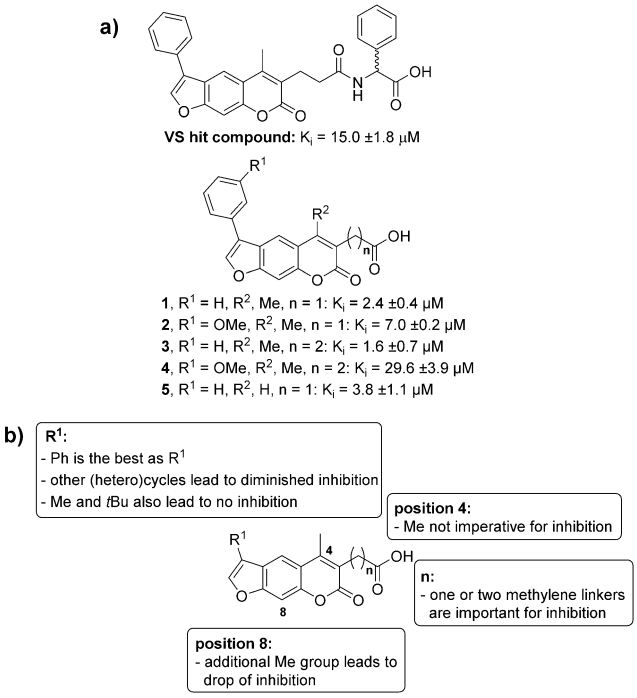

Figure 1. a) Structures of the virtual screening hit and the reversible inhibitors of β5i. b) SAR for the psoralene-based inhibitors.

Information) and its K_i was determined to be 15 μM. Its precursor in this synthesis (compound **3**) was also assayed, which attracted our attention because of its predicted similar interaction profile with the active-site residues of β5i. Compound **3** lacked the phenylglycine part of the virtual screening hit; however, it proved to be a 10-fold more potent inhibitor of the β5i subunit (K_i = 1.6 μM, Figure 1 a). Further structure–activity relationship (SAR) studies were enabled by the construction of a focused library of diversely substituted psoralenes (Table S2, Schemes S1–S3). In order not to over-interpret the SAR data, only the most important findings are shown in Figure 1 b.

To assess the selectivity of these psoralene-based inhibitors of β5i, compounds **1**–**5** (Figure 1 a), which demonstrated reasonable potency against the β5i subunit, were selected. These compounds showed preferential inhibition of β5i over the β2i and β1i subunits of human iCP, as well as over all three subunits of human cCP (Table S3). Compound **3** was the most selective; it showed more than 100-fold greater potency for the inhibition of β5i (K_i = 1.6 μM) than β5c (K_i = 172.2 μM) and negligible to modest activity against the β1 and β2 subunits of each of these CPs (Table S3).

To refine the potency of reversibly acting compounds, subsequent medicinal chemistry efforts were based on the design of covalent inhibitors. Sustained inhibition by covalent inhibitors can only be achieved when a compound first binds noncovalently to the target, followed by positioning of the electrophilic "warhead" near to the desired nucleophilic amino acid residue of the protein.[20,21] Since compound **3** already showed inhibitory activity through noncovalent interactions with the active site of β5i, incorporation of the correct "warhead" should improve the inhibitory potency. Docking studies revealed that the carboxylic group of the most potent inhibitor (**3**) from the noncovalent series is positioned in proximity to the catalytic Thr1 (Figure S1). Therefore, various electrophilic "warheads" were introduced on that portion of the molecule (Figure 2). The selection of the threonine-selective reactive groups used to date was summarized recently.[15] In this study, an alternate series of electrophilic analogues with the potential to react with catalytic Thr1 was prepared (Scheme S4) and evaluated biochemically (Table 1).

Interestingly, the nitrile-based (**31**–**36**) and acrylamide-based (**34**–**40**) compounds showed no improvement in β5i inhibition. However, our structure-based approach did yield compounds with significantly improved and irreversible inhibition of β5i. The succinimidyl esters **29** and **30** inhibited β5i activity with half-maximal inhibitory concentration (IC_{50}) values of 6.36 μM and 0.009 μM, respectively (Table 1, Figure S2). Very potent irreversible inhibition was also achieved with the oxathiazolone-bearing compounds **42**–**44**, with IC_{50} values ranging from 0.013 μM (**42**) to 0.20 μM (**44**; Table 1,

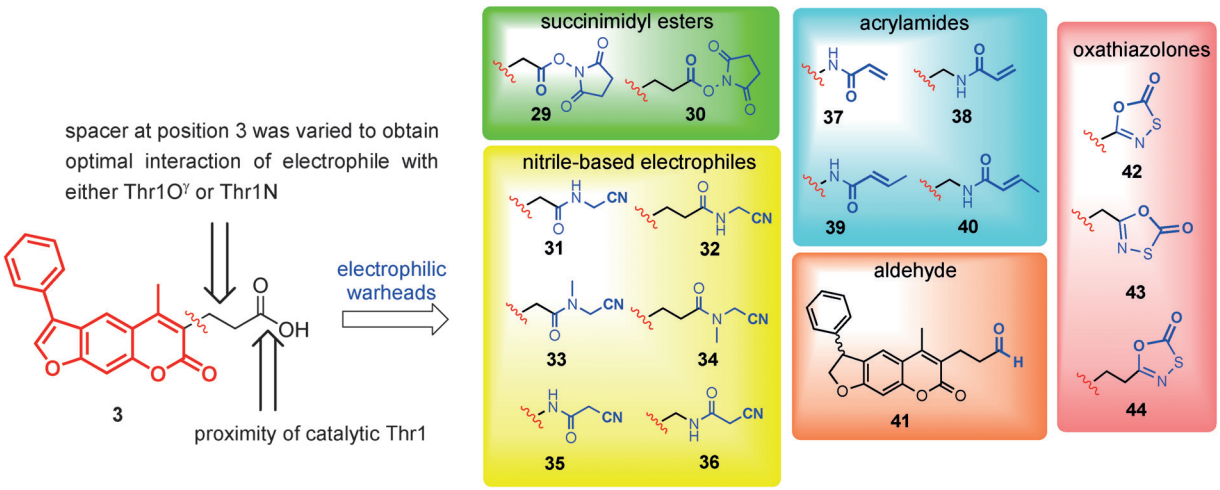

Figure 2. Structures of the designed electrophilic moieties coupled to the noncovalently acting scaffold of **3** (red).

Table 1: In vitro inhibitory potencies of covalently acting compounds derived from compound **3** against all subunits of the human cCP and iCP. Final concentrations of the cCP and iCP in the assays were 0.8 nM and 0.2 nM, respectively. The IC_{50} values were determined only for compounds that showed notable inhibition at 10 μM (RA < 60%). For comparison, the IC_{50} values for inhibition of the β5i subunit of commonly used peptidic inhibitors are presented (from Ref. [15]): bortezomib (0.004 μM), carfilzomib (0.033 μM), PR-924 (0.022 μM).

Compound	IC_{50} β5 i [μM]	IC_{50} β2 i [μM]	IC_{50} β1 i [μM]	IC_{50} β5 c [μM]	IC_{50} β2 c [μM]	IC_{50} β1 c [μM]	β5 c/ β5 i[b]
PR-957[a]	0.015 ± 0.002	0.90 ± 0.08	0.27 ± 0.04	0.16 ± 0.03	3.36 ± 0.26	6.59 ± 0.86	10.6
29	6.36 ± 0.61	21.6 ± 1.4	15.2 ± 1.4	6.39 ± 1.90	19.3 ± 5.5	10.5 ± 2.5	1.0
30	0.009 ± 0.002	7.58 ± 0.12	5.69 ± 0.24	0.22 ± 0.02	8.96 ± 0.35	6.13 ± 0.27	23.9
42	0.013 ± 0.004	NA	NA	1.07 ± 0.26	NA	NA	82.6
43	0.12 ± 0.05	NA	NA	NA	NA	NA	> 80
44	0.20 ± 0.04	NA	NA	NA	NA	NA	> 50

[a] The IC_{50} values correspond well to published data.[15] [b] A high IC_{50} β5c/β5i ratio indicates selectivity for β5i. NA: inactive at 10 μM.

Figure S2). Remarkably, both **30** and **42** turned out to be two-fold to eight-fold more selective for β5i than PR-957 is (Table 1). Oxathiazolones were recently described as a new threonine-selective "warhead".[22] Therefore, the results obtained for compounds **42**–**44** represent a clear proof of concept for this approach. Succinimidyl esters as "warheads", on the other hand, need to be considered with caution, since these are predominantly used as protein labeling or cross-linking reagents. Nonetheless, we showed that compound **30** is not promiscuous, since it showed a reasonable preference for β5i inhibition over β5c (23.9-fold), as well as over other iCP and cCP subunits (Table 1).

Although IC_{50} calculations are not the best method to accurately characterize irreversible and/or mechanism-based inhibitors,[21] these were used for comparisons with the established β5i inhibitor PR-957 (Table 1). We also characterized PR-957 fully by following the time course of the hydrolysis of Suc-LLVY-AMC by iCP and cCP in the presence of various concentrations of PR-957; the same was done for the two most potent irreversibly acting compounds, **30** and **42** (Table S4). PR-957 ($k_{inact}/K_i = 14\,324\ M^{-1}s^{-1}$) and **42** ($k_{inact}/K_i = 15\,448\ M^{-1}s^{-1}$) showed very similar kinetic parameters, but the β5i selectivity was significantly better for compound **42**, with a k_{inact}/K_i β5c/β5i ratio of 112, compared to 29 for PR-957 (Table S4). Additionally, the time-dependence and irreversibility of β5i inhibition by PR-957, **30**, and **42** were determined by IC_{50} shift assay (Table S5, Figure S3) and rapid-dilution assay (Table S6). To propose a mechanism for the covalent interaction of succinimidyl ester **30** with the active-site Thr1 of the β5i subunit, computational quantum mechanics/ molecular mechanics analysis was performed (see the Supporting Information). The simulation showed a very similar reaction mechanism to that proposed for sulfonyl fluorides.[23] However, although irreversible inhibition was clearly demonstrated by enzyme kinetics experiments, we could not substantiate this mechanism by time-resolved intact-protein mass spectrometry.

To determine whether reversible inhibitor **3** and its irreversibly acting counterparts **30** and **42** can inhibit iCP in the presence of other cytosolic components, cell lysates from HeLa and THP-1 cells were incubated with **3**, **30**, and **42**. THP-1 cells were used since they are derived from acute monocytic leukemia and express high levels of iCP, whereas HeLa cells predominantly contain cCP.[24] These compounds showed better inhibition of β5 activity in THP-1 cell lysates than in HeLa cell lysates (Table 2, Figure S4). Although approximately 10-fold reductions in potency were observed for compounds **30** and **42** when compared with in vitro assays with purified human iCP (Table 1), **30** and **42** showed about 10-fold and 40-fold higher inhibition, respectively, in the THP-1 cell lysates than in the HeLa cell lysates

Table 2: Cell-based IC_{50} values against β5 activity in THP-1 and HeLa cells and lysates.

Compound	IC_{50} THP-1 cells [μM]	IC_{50} HeLa cells [μM]	IC_{50} THP-1 lysates [μM]	IC_{50} HeLa lysates [μM]
PR-957[a]	0.011 ± 0.003	0.098 ± 0.021	0.007 ± 0.001	0.025 ± 0.004
3	11.6 ± 1.8	125 ± 16	1.31 ± 0.34	43.8 ± 4.1
30	27.8 ± 7.3	> 100	0.13 ± 0.01	1.45 ± 0.25
42	19.3 ± 3.1	> 100	0.22 ± 0.03	7.20 ± 0.83

[a] IC_{50} values determined are in good agreement with published data.[23]

(Table 2). On the other hand, despite being more active, PR-957 showed only around 3-fold difference in inhibition between these cell lysates (Table 2, Figure S4). Interestingly, the IC_{50} for reversible inhibitor **3** determined on THP-1 cell lysates ($IC_{50} = 1.3$ μM) was in perfect agreement with the IC_{50} on purified iCP ($IC_{50} = 1.6$ μM). Additionally, **3** showed 40-fold selectivity for the inhibition of β5 activity in THP-1 cell lysates over HeLa cell lysates.

The effects of **3**, **30**, and **42** on 26S proteasome activity in intact THP-1 and HeLa cells were also measured by using the Proteasome-Glo assay. These compounds retained good selectivity for THP-1 cells (Table 2, Figure S5). For **3**, the inhibitory potency determined with purified CPs corresponded well with the values from the Proteasome-Glo assays (Figure 1 a, Table 2). On the other hand, the IC_{50} values for the irreversible compounds **30** and **42** were higher in the Proteasome-Glo assays (Table 1 and Table 2). The data from Table 2 indicate that the noncovalently acting psoralene **3** is cell permeable and inhibits the immunoproteasome within cells, whereas the covalently acting counterparts **30** and **42** permeate membranes to a lesser extent, but can selectively inhibit iCP in the presence of other cytosolic components. Importantly, compounds **3**, **30**, and **42** did not significantly affect the viability of the THP-1 and HeLa cell lines at concentrations up to 50 μM (Figure S6).

This lack of cytotoxicity is probably indicative of low off-target binding. With off-target effects in mind, the structural similarity with known intercalators (e.g., bergapten)

prompted us to check possible intercalation-based interactions of these molecules with DNA. Using a DNA binding assay,[25] the randomly selected compounds **1**, **3**, and **30** did not intercalate between DNA base pairs, thus excluding intercalation as a possible unwanted interaction (Figure S7).

In summary, a focused library of nonpeptidic reversible β5i inhibitors was synthesized, followed by conversion into irreversibly acting compounds. The latter retain selectivity for β5i and provide more pronounced inhibition of β5i in THP-1 cell lysates than in HeLa cell lysates. We also showed that the reversible inhibitor **3** can enter human cells and inhibit the proteasomes within them. The very marginal cytotoxic effects of these compounds are an important feature for the future development of nonpeptidic and β5i-selective probes to study iCP involvement in diseases, such as inflammation or autoimmune disorders. Of note, intensive work on peptidic derivatives has recently culminated in very potent and β5i-selective compounds;[26] however, we believe that the described strategy of inhibitor design will aid in the future discovery of structurally diverse nonpeptidic compounds and will pave the way toward either more potent inhibitors or selective second-generation probes that can be used to further define the biology of the iCP and additionally uncover its potential as a therapeutic target.

Acknowledgements

This work was supported by the Slovenian Research Agency. We thank OpenEye Scientific Software, Inc (Santa Fe, NM, USA; http://www.eyesopen.com) for free academic licenses of their software. We also thank Chris Berrie for critical reading of the manuscript.

Keywords: drug design · immunoproteasome · inhibitors · irreversible inhibition · small-molecule inhibitors

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 5745–5748
Angew. Chem. **2016**, *128*, 5839–5842

[1] A. L. Goldberg, *Biochem. Soc. Trans.* **2007**, *35*, 12–17.
[2] N. Gallastegui, M. Groll, *Trends Biochem. Sci.* **2010**, *35*, 634–642.
[3] T. P. Dick, A. K. Nussbaum, M. Deeg, W. Heinemeyer, M. Groll, M. Schirle, W. Keilholz, S. Stevanović, D. H. Wolf, R. Huber et al., *J. Biol. Chem.* **1998**, *273*, 25637–25646.
[4] S. Murata, K. Sasaki, T. Kishimoto, S. Niwa, H. Hayashi, Y. Takahama, K. Tanaka, *Science* **2007**, *316*, 1349–1353.
[5] M. Groettrup, C. J. Kirk, M. Basler, *Nat. Rev. Immunol.* **2010**, *10*, 73–78.
[6] M. Groettrup, S. Khan, K. Schwarz, G. Schmidtke, *Biochimie* **2001**, *83*, 367–372.
[7] T. A. Griffin, D. Nandi, M. Cruz, H. J. Fehling, L. V. Kaer, J. J. Monaco, R. A. Colbert, *J. Exp. Med.* **1998**, *187*, 97–104.
[8] S. D. Demo, C. J. Kirk, M. A. Aujay, T. J. Buchholz, M. Dajee, M. N. Ho, J. Jiang, G. J. Laidig, E. R. Lewis, F. Parlati et al., *Cancer Res.* **2007**, *67*, 6383–6391.
[9] P. G. Richardson, T. Hideshima, K. C. Anderson, *Cancer Control* **2003**, *10*, 361–369.
[10] F. Parlati, S. J. Lee, M. Aujay, E. Suzuki, K. Levitsky, J. B. Lorens, D. R. Micklem, P. Ruurs, C. Sylvain, Y. Lu et al., *Blood* **2009**, *114*, 3439–3447.
[11] D. J. Kuhn, S. A. Hunsucker, Q. Chen, P. M. Voorhees, M. Orlowski, R. Z. Orlowski, *Blood* **2009**, *113*, 4667–4676.
[12] T. Muchamuel, M. Basler, M. A. Aujay, E. Suzuki, K. W. Kalim, C. Lauer, C. Sylvain, E. R. Ring, J. Shields, J. Jiang et al., *Nat. Med.* **2009**, *15*, 781–787.
[13] M. Basler, M. Dajee, C. Moll, M. Groettrup, C. J. Kirk, *J. Immunol.* **2010**, *185*, 634–641.
[14] A. F. Kisselev, M. Groettrup, *Curr. Opin. Chem. Biol.* **2014**, *23*, 16–22.
[15] E. M. Huber, M. Groll, *Angew. Chem. Int. Ed.* **2012**, *51*, 8708–8720; *Angew. Chem.* **2012**, *124*, 8838–8850.
[16] G. Sliwoski, S. Kothiwale, J. Meiler, E. W. Lowe, *Pharmacol. Rev.* **2014**, *66*, 334–395.
[17] V. Kasam, N.-R. Lee, K.-B. Kim, C.-G. Zhan, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3614–3617.
[18] P. Beck, M. Reboud-Ravaux, M. Groll, *Angew. Chem. Int. Ed.* **2015**, *54*, 11275–11278; *Angew. Chem.* **2015**, *127*, 11428–11431.
[19] E. M. Huber, M. Basler, R. Schwab, W. Heinemeyer, C. J. Kirk, M. Groettrup, M. Groll, *Cell* **2012**, *148*, 727–738.
[20] A. S. Kalgutkar, D. K. Dalvie, *Expert Opin. Drug Discovery* **2012**, *7*, 561–581.
[21] J. Singh, R. C. Petter, T. A. Baillie, A. Whitty, *Nat. Rev. Drug Discovery* **2011**, *10*, 307–317.
[22] H. Fan, N. G. Angelo, J. D. Warren, C. F. Nathan, G. Lin, *ACS Med. Chem. Lett.* **2014**, *5*, 405–410.
[23] C. Dubiella, H. Cui, M. Gersch, A. J. Brouwer, S. A. Sieber, A. Krüger, R. M. J. Liskamp, M. Groll, *Angew. Chem. Int. Ed.* **2014**, *53*, 11969–11973; *Angew. Chem.* **2014**, *126*, 12163–12167.
[24] D. Niewerth, G. J. L. Kaspers, Y. G. Assaraf, J. van Meerloo, C. J. Kirk, J. Anderl, J. L. Blank, P. M. van de Ven, S. Zweegman, G. Jansen et al., *J. Hematol. Oncol.* **2014**, *7*, 7.
[25] V. Pierroz, T. Joshi, A. Leonidova, C. Mari, J. Schur, I. Ott, L. Spiccia, S. Ferrari, G. Gasser, *J. Am. Chem. Soc.* **2012**, *134*, 20376–20387.
[26] G. de Bruin, E. M. Huber, B.-T. Xin, E. J. van Rooden, K. Al-Ayed, K.-B. Kim, A. F. Kisselev, C. Driessen, M. van der Stelt, G. A. van der Marel et al., *J. Med. Chem.* **2014**, *57*, 6197–6209.

Received: January 7, 2016
Revised: February 12, 2016
Published online: April 1, 2016

