VIP Cellular Uptake Very Important Paper

International Edition: DOI: 10.1002/anie.201502358
German Edition: DOI: 10.1002/ange.201502358

Ring Tension Applied to Thiol-Mediated Cellular Uptake**

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Abstract: *The objective of the study was to explore the potential of ring tension in cyclic disulfides for thiol-mediated cellular uptake. Fluorescent probes that cannot enter cells were equipped with cyclic disulfides of gradually increasing ring tension. As demonstrated by flow cytometry experiments, uptake into HeLa Kyoto cells increased with increasing tension. Differences in carbon-sulfur-sulfur-carbon (CSSC) dihedral angles as small as 8° caused significant changes in uptake efficiency. Uptake with high ring tension was better than with inactivated or activated linear disulfides or with thiols. Conversion of thiols on the cell surface into sulfides and disulfides decreased the uptake. Reduction of exofacial disulfides into thiols increased the uptake of transporters with disulfides and inactivated controls with thiols. These results confirm the occurrence of dynamic covalent disulfide-exchange chemistry on cell surfaces. Mechanistic and colocalization studies indicate that endocytosis does not fully account for this cellular uptake with ring tension.*

Ring tension is not only useful for explosives;[1] in many inspired variations, it has been beneficial for chemically influencing biological systems in the broadest sense. Highlights include β-lactam antibiotics, such as penicillin, and antitumor natural products such as mitomycin C, neocarcinostatin, dynemicin A, and (+)-CC-1065.[2] More recent milestones concern bioorthogonal chemistry in cells[3] or the modulation of the secondary structure of β-peptide antimicrobials.[4] In this report, we apply ring tension to cellular uptake.

Cells express thiols on their surface as protection against oxidation.[5,6] Disulfide exchange[5–7] with these thiols or their disulfide counterparts (Figure 1a) has been associated with the facilitated uptake of disulfides, maleimides,[8] and thiols attached to natural products, fluorescent probes, peptides (including cell-penetrating peptides), proteins, and oligonucleotides.[6,8–10] Thiol-mediated uptake has been proposed recently[11] to account at least in part for the excellent performance of the multivalent substrate-initiated cell-penetrating polydisulfides (siCPDs, for example, **1**; Figure 1c).[6,11,12] During our research on disulfide-exchange polymerizations, we realized that asparagusic acid derivatives polymerize much more easily than lipoic acid derivatives.[13,14] This difference was remarkable because the carbon-sulfur-sulfur-carbon (CSSC) dihedral angles of asparagusic acid at 27° and lipoic acid at 35° are quite similar compared to the 90° of strain-free disulfides (Figure 1d).[15] Dramatically different reactivity caused by such small changes in strain suggested that ring tension could be equally decisive for disulfide exchange on cell surfaces. We show herein that this is the case.

To explore ring tension for cellular uptake, a fluorescent probe, here carboxyfluorescein (CF), was equipped with thiols and disulfides with different tension and leaving groups (Figure 1b,d). Details of the synthesis and spectroscopic and analytical data for the focused collection of transporters **2**–**8** can be found in the Supporting Information.

Cellular uptake was measured in HeLa Kyoto cells. After incubation, external probes were washed away and the

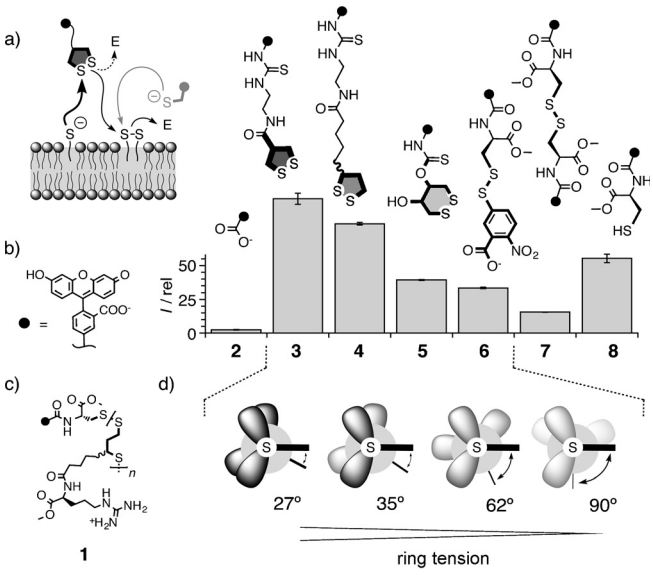

Figure 1. a) Exchange between external strained disulfides (bold, dotted) and thiolates (grey) with thiolates and disulfides on cell surfaces (E = electrophiles, including protons). b) Flow cytometry data showing the fluorescence of HeLa Kyoto cells after incubation with fluorophores **2**–**8** (results are calibrated for the different emission intensities *I* of the different fluorophores). c) Structure of cell-penetrating poly(disulfide) **1**. d) CSSC dihedral angles in derivatives of asparagusic acid (**3**), lipoic acid (**4**), DTT (**5**) and relaxed disulfides (**6**, **7**).

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[**] We thank Aurélien Roux and Guillaume Molinard for assistance with cell culture and colocalization analysis; the NMR, Mass Spectrometry (SMS), and Bioimaging platforms for services, and the University of Geneva, the European Research Council (ERC Advanced Investigator), the National Centre of Competence in Research (NCCR) Chemical Biology, the NCCR Molecular Systems Engineering, and the Swiss NSF for financial support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201502358.

fluorescence intensity of individual cells was recorded by flow cytometry. Quite remarkably, cellular uptake increased with increasing ring tension in the transporter (Figure 1 d). The best results were obtained with the asparagusic acid derivative **3**, which has a CSSC angle of 27°. The 8° decrease in tension in the lipoic acid derivative **4** resulted in less uptake. The residual tension in the dithiothreitol (DTT) derivative **5** gave much less uptake. Interestingly, a good leaving group for disulfide exchange present in the "Ellman" disulfide **6** was even less effective than the very modest ring tension in **5**. However, tension-free disulfide **7**, which does not have activated leaving groups was still less active (but much better than CF (**2**) without any sulfur atom). Control **8**, which has a thiol in place of the disulfides in **3**–**7**, was significantly less active than disulfides **3** and **4**, which have high tension, but more active than disulfides **5**–**7**, which have low or no tension. This respectable activity of **8** was in agreement with the presence of exofacial disulfides at significant concentrations for the uptake of thiols by reversed disulfide exchange (Figure 1 a, grey arrows).

Comparisons of the absolute uptake values from flow cytometry have to be considered with caution because of the different quantum yields of the fluorophores, which were calibrated and corrected but might change again upon disulfide exchange at the cell surface. The comparison of changes in uptake upon modification of the cell surface, however, is straightforward and free of any uncertainty (Figure 2). Preincubation of the cells with iodoacetamide **9** reduced the uptake of all of the transporters (Figure 2 g, empty bars). Inactivation of disulfides **3**–**7** in response to the conversion of thiols at the cell surface into thioethers showed that dynamic covalent disulfide exchange at the cell surface is decisive for uptake (Figure 2 a, d, bold arrows). Additional inactivation of thiol **8** suggested that the disulfides present at the cell surface are removed as well, thus hinting at enzymatic disulfide reduction in response to thiol removal with iodoacetamide **9**, possibly by protein disulfide isomerases (PDIs, Figure 2 d).[5,6]

More interesting than inactivation with iodoacetamide **9** was selective activation with either Ellman's reagent (DTNB) or DTT (**10**). Preincubation of cells with DTNB converts exofacial thiols into activated disulfides (Figure 2 e). As a result, disulfides **3**–**6** were reliably inactivated and thiol **8** was strongly activated (Figure 2 g, filled bars). The reduction of exofacial disulfides to thiols with DTT (**10**; Figure 2 f) activated disulfides **3**, **6**, and **7** and inactivated thiol **8** (Figure 2 g, hatched bars). However, the uptake efficiency of disulfide **6**, which has activated leaving groups, increased more than that of the otherwise superior disulfide **3**, which has the highest ring tension. This finding could suggest that the release of ring tension at the cell surface benefits from exofacial disulfides as well. The conclusion would be that ring-opening disulfide exchange initiated by exofacial thiolates (Figure 2 a, f, bold arrows) continues with a reaction of the obtained thiolate with an exofacial disulfide (Figure 2 a, solid arrow). The result would be an active structure with two mixed disulfides between the transporter and the cell (Figure 2 b). However, uptake of the best-performing asparagusic acid derivative **3** still increased in response to cell-surface

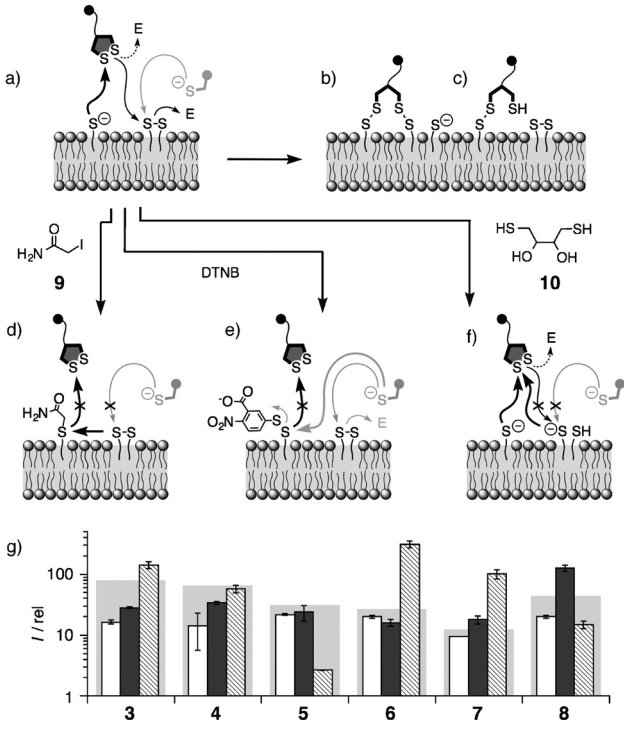

Figure 2. Thiolate–disulfide exchange reactions on the surface of a) untreated cells (with likely reaction products (b, c)), d) iodoacetamide-treated cells, e) DTNB-treated cells, or f) DTT-treated cells. g) Flow cytometry results for the uptake of **3**–**8** into HeLa Kyoto cells after preincubation with iodoacetamide **9** (empty bars), DTNB (filled bars) or DTT **10** (hatched bars) compared to untreated cells (background grey bars).

reduction with DTT (Figure 2 g). This suggests that uptake under the highest ring tension is also operational if the thiolate resulting from ring-opening disulfide exchange reacts with another electrophile, most likely a proton (Figure 2 a, f, dashed arrow). As a result, active structures with one (Figure 2 c) or two disulfides between the transporter and the cell (Figure 2 b) are likely to coexist.

Confocal laser scanning microscopy (CLSM) images of HeLa Kyoto cells incubated for 1 hour with the asparagusic acid derivative **3** and the lipoic acid derivative **4** were in agreement with the results from flow cytometry. Most remarkably, they provide corroborative evidence that a small difference in ring tension makes a big difference to cellular uptake: Uptake with transporter **3**, which has the highest ring tension, was clearly better than with **4** (Figure 3 a vs b). This hypersensitivity to small changes in ring tension echoes previous findings on the surface-initiated polymerization of multicomponent photosystems.[13]

Colocalization studies were performed with trackers for the mitochondria (Figure S2 and S3 in the Supporting Information), lysosomes (Figure 3 d), and endosomes (Figure 3 c). Quantitative colocalization analysis using both Pearson's correlation coefficient (PCC, Figure S4) and Manders' colocalization coefficients (Figure S5) confirmed that transporters **3** and **4** enter the endosomes only partially (PCC = 0.65, 0.74, respectively) and avoid the lysosomes

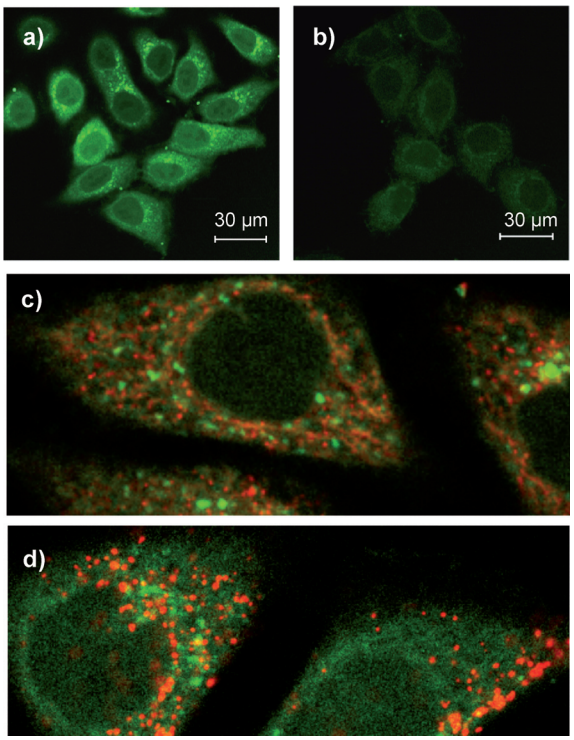

Figure 3. CLSM images of HeLa Kyoto cells after 1 hour incubation with **3** (a) and **4** (b, 10 μM in Leibovitz's medium at 37°C), and enlarged colocalization CLSM images for **3** with c) Dextran Red to track endosomes and d) LysoTracker Red DND-99 to track lysosomes.

(PCC = 0.44, 0.26) and mitochondria (PCC = 0.51, 0.43). The fluorescence images further indicate that ring tension is compatible with cytosolic delivery of the transporters, whereas fluorescence from the nucleus was very weak (Figure 3). Moreover, transporters **3** and **4** were insensitive to standard endocytosis inhibitors such as chlorpromazine, wortmannin, and methyl-β-cyclodextrin (Figure S7). However, at 4°C, their activity was clearly reduced (Figure S7). These mechanistic observations are most intriguing. Access to the cytosol but neither the nucleus nor the lysosomes, high temperature dependence, insensitivity to endocytosis inhibitors, and incomplete colocalization with endosomes are all compatible with the possibility that cellular uptake with ring tension for dynamic covalent chemistry on the cell surface could provide access to a new, energy-dependent uptake pathway. The existence of such entry into cells is further supported by indications that the redox state of exofacial thiols and cytosolic glutathione are coupled[5,6] and that simple covalent binding to cell surfaces (by boronic acids, for example) often seems to rather hinder than help internalization.[11] Mechanistic studies are ongoing, together with efforts to explore the scope and limitations of cellular uptake under tension, particularly with regard to practical biological applications. It should be emphasized that the uptake efficiency of fully developed siCPDs such as **1** remains far superior to that of monomeric disulfides, even at high ring tension. The recharging of CPDs with ring tension thus emerges as one of the next big concepts to explore.

Keywords: cell-penetrating peptides · cellular uptake · dihedral angles · disulfides · ring tension

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 7328–7331
Angew. Chem. **2015**, *127*, 7436–7439

[1] M.-X. Zhang, P. E. Eaton, R. Gilardi, *Angew. Chem. Int. Ed.* **2000**, *39*, 401–404; *Angew. Chem.* **2000**, *112*, 422–426.
[2] a) C. Walsh, *Antibiotics: Actions, Origins and Resistance*, Washington, ASM Press, **2003**; b) S. M. Hecht, *Bioorganic Chemistry: Nucleic Acids*, Oxford University Press, Oxford, **1996**.
[3] E. M. Sletten, C. R. Bertozzi, *Acc. Chem. Res.* **2011**, *44*, 666–676.
[4] R. P. Cheng, S. H. Gellman, W. F. DeGrado, *Chem. Rev.* **2001**, *101*, 3219–3232.
[5] A. G. Torres, M. J. Gait, *Trends Biotechnol.* **2012**, *30*, 185–190.
[6] D. Oupický, J. Li, *Macromol. Biosci.* **2014**, *14*, 908–922.
[7] a) N. K. P. Samuel, M. Singh, K. Yamaguchi, S. L. Regen, *J. Am. Chem. Soc.* **1985**, *107*, 42–47; b) J. Li, P. Nowak, S. Otto, *J. Am. Chem. Soc.* **2013**, *135*, 9222–9239; c) S. P. Black, J. K. M. Sanders, A. R. Stefankiewicz, *Chem. Soc. Rev.* **2014**, *43*, 1861–1872; d) A. Wilson, G. Gasparini, S. Matile, *Chem. Soc. Rev.* **2014**, *43*, 1948–1962.
[8] M. M. Fretz, N. A. Penning, S. Al-Taei, S. Futaki, T. Takeuchi, I. Nakase, G. Storm, A. T. Jones, *Biochem. J.* **2007**, *403*, 335–342.
[9] a) I. D. Alves, A. Walran, C. Bechara, S. Sagan, *Curr. Protein Pept. Sci.* **2012**, *13*, 658–671; b) T. Li, S. Takeoka, *Int. J. Nanomed.* **2014**, *9*, 2849–2861; c) E. P. Feener, W. C. Shen, H. J. P. Ryser, *J. Biol. Chem.* **1990**, *265*, 18780–18785; d) S. Aubry, F. Burlina, E. Dupont, D. Delaroche, A. Juliot, S. Laveille, G. Chassaing, S. Sagan, *FASEB J.* **2009**, *23*, 2956–2967.
[10] a) E. G. Stanzl, B. M. Trantow, J. R. Vargas, P. A. Wender, *Acc. Chem. Res.* **2013**, *46*, 2944–2954; b) F. Sgolastra, B. M. Deronde, J. M. Sarapas, A. Som, G. N. Tew, *Acc. Chem. Res.* **2013**, *46*, 2977–2987; c) R. Brock, *Bioconjugate Chem.* **2014**, *25*, 863–868; d) E. Koren, V. P. Torchilin, *Trends Mol. Med.* **2012**, *18*, 385–393; e) M. Li, S. Schlesiger, S. K. Knauer, C. Schmuck, *Angew. Chem. Int. Ed.* **2015**, *54*, 2941–2944; *Angew. Chem.* **2015**, *127*, 2984—2987; f) M. Whitney, E. N. Savariar, B. Friedman, R. A. Levin, J. L. Crisp, H. L. Glasgow, R. Lefkowitz, S. R. Adams, P. Steinbach, N. Nashi, Q. T. Nguyen, R. Y. Tsien, *Angew. Chem. Int. Ed.* **2013**, *52*, 325–330; *Angew. Chem.* **2013**, *125*, 343–348.
[11] G. Gasparini, E.-K. Bang, G. Molinard, D. V. Tulumello, S. Ward, S. O. Kelley, A. Roux, N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2014**, *136*, 6069–6074.
[12] a) C. Lin, J. F. J. Engbersen, *Expert Opin. Drug Delivery* **2009**, *6*, 421–439; b) E.-K. Bang, M. Lista, G. Sforazzini, N. Sakai, S. Matile, *Chem. Sci.* **2012**, *3*, 1752–1763; c) T.-I. Kim, S. W. Kim, *React. Funct. Polym.* **2011**, *71*, 344–349; d) H. Zeng, H. C. Little, T. N. Tiambeng, G. A. Williams, Z. Guan, *J. Am. Chem. Soc.* **2013**, *135*, 4962–4965; e) C. A. Drake, A. Aissaoui, O. Argyros, M. Thanou, J. H. G. Steinke, A. D. Miller, *J. Controlled Release* **2013**, *171*, 81–90; f) L. Brülisauer, N. Kathriner, M. Prenrecaj, M. A. Gauthier, J.-C. Leroux, *Angew. Chem. Int. Ed.* **2012**, *51*, 12454–12458; *Angew. Chem.* **2012**, *124*, 12622–12626; g) E.-K. Bang, G. Gasparini, G. Molinard, A. Roux, N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2013**, *135*, 2088–2091; h) S. Son, R. Namgung, J. Kim, K. Singha, W. J. Kim, *Acc. Chem. Res.* **2012**, *45*, 1100–1112; i) M. Balakirev, G. Schoehn, J. Chroboczek, *Chem. Biol.* **2000**, *7*, 813–819.
[13] A. Carmine, Y. Domoto, N. Sakai, S. Matile, *Chem. Eur. J.* **2013**, *19*, 11558–11563.
[14] a) N. Sakai, M. Lista, O. Kel, S. Sakurai, D. Emery, J. Mareda, E. Vauthey, S. Matile, *J. Am. Chem. Soc.* **2011**, *133*, 15224–15227; b) M. Lista, J. Areephong, N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2011**, *133*, 15228–15231; c) N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2011**, *133*, 18542–18545; d) H. Hayashi, A. Sobczuk, A.

Bolag, N. Sakai, S. Matile, *Chem. Sci.* **2014**, *5*, 4610 – 4614; e) N. Sakai, P. Charbonnaz, S. Ward, S. Matile, *J. Am. Chem. Soc.* **2014**, *136*, 5575 – 5578.

[15] a) R. Singh, G. M. Whitesides, *J. Am. Chem. Soc.* **1990**, *112*, 1190 – 1197; b) E. Morera, G. Lucente, G. Ortar, M. Nalli, F. Mazza, E. Gavuzzo, S. Spisani, *Bioorg. Med. Chem.* **2002**, *10*, 147 – 157.

Received: March 13, 2015
Published online: April 29, 2015

