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Achieving cell penetration with distance-matching cysteine cross-linkers: a facile route to cell-permeable peptide dual inhibitors of Mdm2/Mdmx†

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Abstract

We report the design of bisarylmethylene bromides as a new class of rigid, distance-matching cysteine cross-linkers. By cross-linking a peptide dual inhibitor of Mdm2/Mdmx containing cysteines at *i,i*+7 positions, dramatic enhancement in cell permeability was achieved, along with increased helicity and biological activity.

A growing number of chemical strategies have been developed recently to convert peptides into cell-permeable ligands targeting intracellular protein-protein interactions.¹ These include the use of alternative backbones such as β -peptides,² the incorporation of α,α -dialkylamino acids such as Aib,³ the side chain-to-side chain cross-linking *via* the lactam,⁴ hydrocarbon,⁵ and heterocyclic bridges,⁶ the backbone-to-side chain cross-linking,⁷ and the low molecular weight peptidomimetics such as terphenyl-based helix mimics.⁸ Though these strategies have generated peptide analogs with improved cell permeability, nonnatural amino acids were typically required. For example, hydrocarbon-based cross-linking requires the introduction of two chiral α,α -dialkylamino acids carrying olefin side chains and subsequent ruthenium-catalyzed ring closing metathesis produces a mixture of *Z*- and *E*-olefin linkages.^{5a,5b,9}

One effective strategy to impart cell permeability is to fuse the peptide with cell penetrating peptide sequences;¹⁰ however, significant mass is added to the peptide. In search of an atom-economical strategy, we were intrigued by an earlier report by Woolley and co-workers¹¹ in which alkylation of dicysteines located at *i,i*+11 positions of a peptide with distance-matching dichloroacetamides resulted in a substantial increase in peptide helicity; however, the effect of cysteine cross-linking on cell permeability was not demonstrated. Since the increased helicity generally leads to improved cell penetration,¹² we envisioned that with a proper choice of rigid, hydrophobic cross-linkers we could obtain the cross-linked peptides with improved cell permeability. Herein, we report the design of a pair of bisarylmethylene

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bromides as rigid *i,i*+7 cysteine cross-linkers, and their effect on cell permeability of a bioactive peptide (Fig. 1).

In order to evaluate the effect of cysteine cross-linking on cell permeability, we decided to use a peptide dual inhibitor of p53-Mdm2/Mdmx interactions (PDI) as our model peptide because (i) PDI has shown to be an excellent dual inhibitor of the p53-Mdm2 and the p53-Mdmx interactions,¹³ two validated cancer targets; and (ii) the crystal structures of PDI and its analogs in complexes with Mdm2/Mdmx have been solved recently.¹⁴ However, PDI was found to be cell impermeable,¹³ diminishing its therapeutic potential. Since PDI (sequence = LTFEHYWAQLTS) does not encode cysteine, we reasoned that we could replace two residues at the solvent exposed face of the helix with cysteines followed by the cysteine-specific cross-linking. Inspection of the PDI-Mdm2/Mdmx complex structures revealed that Glu-4 and Thr-11 are solvent exposed and could be substituted without substantial loss of inhibitory activity.¹⁴ Besides, a heterocyclic cross-linker has been successfully applied to these two sites.^{6c} Thus, we prepared L,L-dicysteine substituted PDI analog **1** and found that the substitutions were largely tolerated based on the ELISA data (compare **1** to PDI in Table 1). We also prepared a D,L-dicysteine analog **3** by placing D-Cys at position 4 with an aim to examine configurational effect.^{5b,15} ELISA data showed that D,L-dicysteine substitution was very well tolerated; indeed, more than 3-fold increase in Mdm2 activity was observed (Table 1).

To identify suitable rigid cross-linkers that selectively alkylate dicysteines at the *i,i*+7 positions of PDI with a distance of 11.9 Å between two β-carbons for the L,L-configuration (Fig. 1) and 10.9 Å for the D,L-configuration, we explored various aryl methylene bromides and found 4,4'-bis-bromomethyl-biphenyl (Bph) and 6,6'-bis-bromomethyl-[3,3']bipyridine (Bpy) provide nearly perfect matches. In a Langevin dynamic simulation study, the sulfur-to-sulfur distances range from 10.5 to 12.2 Å for Bph and Bpy scaffolds, respectively, with the median distances center around 11.6 Å (Fig. S1 in ESI). Bph is commercially available while Bpy can be conveniently prepared through a two-step procedure (Scheme S1). To evaluate the efficiency of the cross-linking reaction, we incubated the fully deprotected peptide **1** or **3** at 1 mM concentrations with 1.5 equiv of Bph or Bpy in a mixed solution of acetonitrile/30 mM ammonium bicarbonate, pH 8.5. HPLC traces of the crude products showed that the reactions proceeded to completion within 2 h with greater than 95% conversion (Fig. S2).

While the initial cross-linked peptides (**1a**, **1b**, **3a**, **3b**) were charge-neutral, we also prepared the positively charged analogs (**2a**, **2b**, **4a**, **4b**) by placing two arginines at the solvent exposed side. Then, we performed ELISA with these compounds to gauge the cross-linking effect on their inhibitory activity (Table 1 and Fig. S3). All charge-neutral cross-linked peptides showed modest increase in Mdm2 activity, but variable effect on Mdmx activity relative to their linear counterparts (compare **1a/1b** to **1**; **3a/3b** to **3**). Interestingly, cysteine cross-linked peptides **3a** and **3b** with D,L-configuration exhibited a 15- and 24-fold increase in Mdmx activity, respectively (Table 1). In a molecular modeling study, the D,L-dicysteine-linked Bpy in **3b** appears to bind snugly onto a nearby hydrophobic pocket while the L,L-dicysteine-linked Bpy does not (Fig. S4). This type of cross-linker-protein surface inter-action was observed recently in the crystal structure of Mcl-1 bound to a hydrocarbon

cross-linked peptide.¹⁶ Meanwhile, all positively charged peptides showed significantly reduced activities compared to their neutral counterparts (compare **2a** to **1a**, **2b** to **1b**, **4a** to **3a**, and **4b** to **3b** in Table 1), indicating that arginine substitutions at positions 5 and 9 were not well tolerated.

Next, we examined the effect of cysteine cross-linking on cell permeability using fluorescence activated cell sorting (FACS). Because Bpy cross-linked peptides showed superior water solubility and higher bioactivities than Bph cross-linked ones, we prepared the N-terminally fluorescein-conjugated **1b**, **2b**, **3b**, and **4b** along with PDI, **1**, and **2** as controls. As shown in Fig. 2, linear fluorescein-labeled PDI and **1** exhibited minimal cell penetration relative to the DMSO control. As expected,¹⁷ substituting two arginines into PDI led to a 2.5-fold increase in mean fluorescence (compare **2** to **1**). On the other hand, cysteine cross-linking by Bpy caused greater increase in mean fluorescence (~6-fold for **1b** over **1**, ~5-fold for **2b** over **2**), indicating that the effect of cross-linking on enhancing the cell permeability is greater than that of positive charge. Moreover, these two effects appear additive as the positively charged, Bpy cross-linked **2b** showed additional 2-fold increase compared to **1b**. Similar enhancement was observed for peptides **3b** and **4b** with the D,L-configuration (Fig. 2b). To determine the extent by which structural change upon Bph/Bpy cross-linking contributes to cell permeability, we performed the CD measurement of the cross-linked peptides. Compared to the linear 12-mer peptides PDI, **1**, and **3** which showed 11–13% helicity, cysteine cross-linking caused modest increase in percent helicity (up to 20%, see Fig. S5). Overall, a positive correlation between helicity and permeability appears to exist as peptides with higher helicity generally showed higher cell permeability.

To corroborate the FACS result, a confocal microscopy study was carried out and the data were collected in Fig. 3a. In the FITC channel, the positively charged, Bpy cross-linked peptides **2b** and **4b** showed the strongest cellular fluorescence, in excellent agreement with the FACS data; however, cellular debris was detected for **2b** and **4b** treated cells in the DIC channel, indicating apparent cytotoxicity. This cytotoxicity was further confirmed by the ATP assay (Fig. 3b), which was attributed to the positive charge. To gain an insight into the possible mechanism of cell permeation, we examined the subcellular localization of **3b**, the most potent, cell-permeable, non-toxic peptide in our series, in live HeLa cells by confocal microscopy after treatment with **3b**, followed by Alexa Fluor 647 labeled transferrin and Hoescht 33342 to visualize the recycling endosomes¹⁸ and nuclei, respectively (Fig. 4). We found that while **3b** showed a more diffusive distribution in the cytosol (panel a), it predominantly co-localized with transferrin in the recycling endosomes (panels b and d), suggesting that a likely mode of cell penetration for **3b** is *via* the endosome-mediated active transport. Consistent with this mode of transport, **3b** showed a modest 70% increase in luciferase activity compared to linear peptide **3** in PA1 cells stably expressing a p53-dependent luciferase reporter (Fig. S6).

In summary, we have designed bisarylmethylene bromides as a new class of rigid cysteine cross-linkers. By cross-linking PDI analogs containing two cysteines at *i,i+7* positions, significant enhancement in cell permeability was achieved without apparent cytotoxicity, along with modest increases in helicity and bioactivity. Since dicysteines can be readily

introduced into proteins, this cysteine cross-linking chemistry could also be potentially useful for improved delivery of cross-linked proteins into cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. (a) Yin H, Hamilton AD. *Angew Chem, Int Ed.* 2005; 44:4130.(b) Verdine GL, Walensky LD. *Clin Cancer Res.* 2007; 13:7264. [PubMed: 18094406]
2. Kritzer JA, Lear JD, Hodsdon ME, Schepartz A. *J Am Chem Soc.* 2004; 126:9468. [PubMed: 15291512]
3. (a) Balaram P. *Curr Opin Struct Biol.* 1992; 2:845.(b) Roy RS, Karle IL, Raghothama S, Balaram P. *Proc Natl Acad Sci U S A.* 2004; 101:16478. [PubMed: 15546995]
4. (a) Bracken C, Gulyas J, Taylor JW, Baum J. *J Am Chem Soc.* 1994; 116:6431.(b) Phelan CJ, Skelton NJ, Braisted AC, McDowell RS. *J Am Chem Soc.* 1997; 119:455.
5. (a) Blackwell HE, Grubbs RH. *Angew Chem, Int Ed.* 1998; 37:3281.(b) Schafmeister CE, Po J, Verdine GL. *J Am Chem Soc.* 2000; 122:5891.(c) Sharp DA, Kratowicz SA, Sank MJ, George DL. *J Biol Chem.* 1999; 274:38189. [PubMed: 10608892] (d) Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. *Science.* 2004; 305:1466. [PubMed: 15353804]
6. (a) Cantel S, Isaad ALC, Scrima M, Levy JJ, DiMarchi RD, Rovero P, Halperin JA, D'Ursi AM, Papini AM, Chorev M. *J Org Chem.* 2008; 73:5663. [PubMed: 18489158] (b) Madden MM, Vera CI, Rivera, Song W, Lin Q. *Chem Commun.* 2009:5588.(c) Madden MM, Muppidi A, Li Z, Li X, Chen J, Lin Q. *Bioorg Med Chem Lett.* 2011; 21:1472. [PubMed: 21277201]
7. Patgiri A, Jochim AL, Arora PS. *Acc Chem Res.* 2008; 41:1289. [PubMed: 18630933]
8. Orner BP, Ernst JT, Hamilton AD. *J Am Chem Soc.* 2001; 123:5382. [PubMed: 11457415]
9. (a) Blackwell HE, Sadowsky JD, Howard RJ, Sampson JN, Chao JA, Steinmetz WE, O'Leary DJ, Grubbs RH. *J Org Chem.* 2001; 66:5291. [PubMed: 11485448] (b) Boal AK, Guryanov I, Moretto A, Crisma M, Lanni EL, Toniolo C, Grubbs RH, O'Leary DJ. *J Am Chem Soc.* 2007; 129:6986. [PubMed: 17497781]
10. (a) Fonseca SB, Pereira MP, Kelley SO. *Adv Drug Delivery Rev.* 2009; 61:953.(b) Myrberg H, Zhang L, Mae M, Langel U. *Bioconjugate Chem.* 2008; 19:70.
11. Zhang F, Sadovski O, Xin SJ, Woolley GA. *J Am Chem Soc.* 2007; 129:14154. [PubMed: 17960932]
12. Kim YW, Verdine GL. *Bioorg Med Chem Lett.* 2009; 19:2533. [PubMed: 19332370]
13. Hu B, Gilkes DM, Chen J. *Cancer Res.* 2007; 67:8810. [PubMed: 17875722]
14. (a) Czarna A, Popowicz GM, Pecak A, Wolf S, Dubin G, Holak TA. *Cell Cycle.* 2009; 8:1176. [PubMed: 19305137] (b) Phan J, Li Z, Kasprzak A, Li B, Sebt SM, Guida W, Schonbrunn E, Chen J. *J Biol Chem.* 2010; 285:2174. [PubMed: 19910468]
15. Jackson DY, King DS, Chmielewski J, Singh S, Schultz PG. *J Am Chem Soc.* 1991; 113:9391.
16. Stewart ML, Fire E, Keating AE, Walensky LD. *Nat Chem Biol.* 2010; 6:595. [PubMed: 20562877]
17. Smith BA, Daniels DS, Coplin AE, Jordan GE, McGregor LM, Schepartz A. *J Am Chem Soc.* 2008; 130:2948. [PubMed: 18271592]
18. Hopkins CR, Gibson A, Shipman M, Strickland DK, Trowbridge IS. *J Cell Biol.* 1994; 125:1265. [PubMed: 7515888]

Fig. 1. Structures of the rigid cysteine cross-linkers Bph and Bpy (left), and a model of Bpy cross-linked peptide dual inhibitor (right) with two cysteines separated by a distance of 11.9 Å.

Fig. 2.

Flow cytometry analysis of HeLa cells after treatment with 10 μ M fluorescein-conjugated peptides. (a) Representative histograms; (b) Bar graph showing normalized mean fluorescence.

Fig. 3.

(a) Confocal images of fixed HeLa cells after treatment with 10 μ M of fluorescein-conjugated peptides for 2 h. Scale bar = 50 μ m. (b) Cell viability assay using the CellTiter-Glo reagent.

Fig. 4. Confocal fluorescence microscopy of live HeLa cells after treatment with 10 μM of fluorescein-conjugated **3b** for 2 h followed by incubation with 10 $\mu\text{g mL}^{-1}$ Alexa Fluor 586 labeled transferrin and 1.5 μM Hoescht 33342 dye for 30 min: (a) FITC channel; (b) Fluor 585 channel; (c) Hoescht channel; (d) merged image of channels a–c.

Table 1Sequence and inhibitory activity of the cross-linked peptides^a

Name	Sequence	Charge	Mdm2 IC ₅₀ (nM)	Mdmx IC ₅₀ (nM)
PDI ^b	LTFEHYWAQLTS	−1	44	500
1	LTFC ^c HYWAQLCS	0	57 ± 4.6	1800 ± 180
1a	LTFC ^c HYWAQLC ^c S ^c	0	39 ± 3.2	4800 ± 1100
1b	LTFC ^c HYWAQLC ^c S ^d	0	35 ± 4.5	890 ± 120
2	LTFCRYWARLCS	0	ND	ND
2a	LTFC ^c RYWARLC ^c S	+2	240 ± 13	5500 ± 980
2b	LTFC ^c RYWARLC ^c S	+2	240 ± 16	1500 ± 150
3	LTFC ^e HYWAQLCS ^e	0	13 ± 1.3	340 ± 27
3a	LTFC ^c HYWAQLC ^c S	0	8.3 ± 0.5	22 ± 4.0
3b	LTFC ^c HYWAQLC ^c S	0	5.4 ± 0.4	14 ± 2.0
4a	LTFC ^c RYWARLC ^c S	+2	210 ± 19	4500 ± 890
4b	LTFC ^c RYWARLC ^c S	+2	100 ± 8.2	1200 ± 87

^aELISA was repeated three times to derive average IC₅₀ values along with standard deviations.^bIC₅₀ values were taken from ref. 13 where the same assay was performed.^cC^c denotes Bph-linked cysteine.^dC^d denotes Bpy-linked cysteine.^ec denotes D-cysteine. ND, no data.