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A bicyclic peptide scaffold promotes phosphotyrosine mimicry and cellular uptake

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ABSTRACT

While peptides are promising as probes and therapeutics, targeting intracellular proteins will require greater understanding of highly structured, cell-internalized scaffolds. We recently reported **BC1**, an 11-residue bicyclic peptide that inhibits the Src homology 2 (SH2) domain of growth factor receptor-bound protein 2 (Grb2). In this work, we describe the unique structural and cell uptake properties of **BC1** and similar cyclic and bicyclic scaffolds. These constrained scaffolds are taken up by mammalian cells despite their net neutral or negative charges, while unconstrained analogs are not. The mechanism of uptake is shown to be energy-dependent and endocytic, but distinct from that of Tat. The solution structure of **BC1** was investigated by NMR and MD simulations, which revealed discrete water-binding sites on **BC1** that reduce exposure of backbone amides to bulk water. This represents an original and potentially general strategy for promoting cell uptake.

1. Introduction

Constrained peptides are an important class of biologically-inspired molecules with utility for inhibiting protein-protein interactions. A growing body of work has shown that precisely designed covalent constraints can improve peptide affinity, selectivity, metabolic stability, and in some cases, cell penetration.[1] Cell internalization via endocytosis is frequently correlated to amphipathic character and positive charge, both of which promote association with the plasma membrane and induce various endocytic pathways.[2,3] By contrast, there are strikingly few reports of conformationally constrained peptides with neutral or net negative charge that are taken up by mammalian cells.[4–6] While the mechanisms of internalization for polycationic sequences, including the HIV-derived Tat peptide, have been richly explored,[3,7–9] comparatively little is known about the precise mechanisms by which non-cationic, constrained peptides enter the cell.[5,10,11] Herein, we report a series of neutral and negatively charged constrained peptides that are internalized by mammalian cells. We also report details of uptake mechanism and identify unique structural features that may promote cellular uptake.

The head-to-tail cyclic peptide **HT1** and bicyclic peptide **BC1** (Fig. 1) were designed as inhibitors of the SH2 domain of Grb2.[12] They were derived from disulfide- and thioether-bridged macrocycles originally described by Roller and co-workers.[13,14] **BC1**, which has an IC_{50} of 350 nM for Grb2 inhibition in biochemical assays, did not show anti-proliferative activity up to 30 μM (Supplementary Fig. S1). This was not surprising, since small molecules that target Grb2-SH2 produced by Burke and colleagues inhibited the protein with potencies in the 1–20 nM range, but only affected cells in the 0.8–25 μM range.[15,16] Some inhibitors of the SH2 domain have shown low-nanomolar affinity in vitro and yet were unable to disrupt the Grb2–pErbB2 interaction in live cells.[15] This lack of cellular activity may be attributed to the high degree of negative charge characteristic of these phosphotyrosine isosteres and mimetics. Because our peptides did not demonstrate Grb2-dependent effects on cultured cells, we were interested in whether the primary barrier was uptake of **BC1** by mammalian cells or subsequent endosomal release. If **BC1** was taken up by mammalian cells, even to a small extent, it would be surprising since most cell-internalized peptides are highly cationic. Among peptides with less overall charge, such as amphipathic helices, a single negative charge is typically enough to completely prevent internalization.[5,17–19] Thus, we designed a series of experiments to explicitly test the uptake of **BC1** and analogs in cultured cells, in order to begin to elucidate

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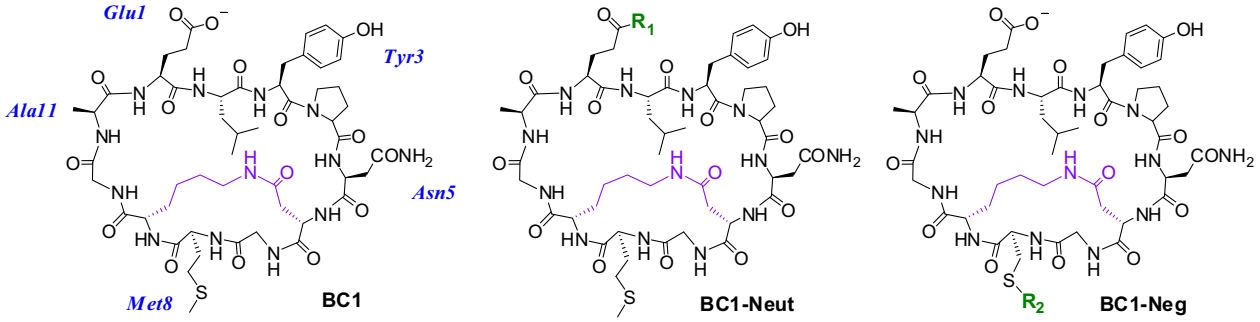

Figure 1. Chemical structures of the **BC1** scaffold and BODIPY-FL-labeled analogs. R_1 is the adduct of hydrazine-functionalized BODIPY-FL, and R_2 is the adduct of maleimide-functionalized BODIPY-FL. Fluorescein-labeled peptides were also prepared and tested to rule out dye-dependent effects on cell uptake. **HT1-Neut** and **HT1-Neg** are head-to-tail cyclic analogs of **BC1-Neut** and **BC1-Neg**, and **Lin-Neut** and **Lin-Neg** are linear analogs of **BC1-Neut** and **BC1-Neg**. See Supplementary information for complete structural and synthesis details.

how structured peptides could be designed to maximize cell uptake.

2. Results and discussion

2.1. Cell uptake of dye-labeled cyclic and bicyclic peptides

Our first variants were designed to explore the role of the negative charge. Specifically, we prepared variants of **HT1** and **BC1** with BODIPY-FL conjugated to Glu1 to eliminate the negative charge (**HT1-Neut** and **BC1-Neut**), or with the dye conjugated to a Cys residue substituted for Met8 to maintain a net charge of −1 (**BC1-Neg**; see Fig. 1 and Supplementary Fig. S2). To explore the role of conformational constraint, we also prepared dye-labeled linear analogs, as well as a dye-labeled bicyclic scaffold with a different lactam cross-link (**BC3-Neut**, Supplementary Fig. S2). BODIPY-FL-labeled Tat peptide (**Tat-Bdy**) was used as a positive control. We used confocal fluorescence microscopy to investigate the cellular uptake of these dye-labeled peptides using MDA-MB-453 cells, a metastatic human epithelial cancer line. Confocal microscopy revealed that both **BC1-Neg** and **BC1-Neut** were internalized by MDA-MB-453 cells (Fig. 2). Uptake was quantified using two independent measurements: ImageJ analysis of confocal microscopy images, and direct fluorometry of cell lysates following incubation and washing (Fig. 3). Both techniques showed similar trends. The neutral **BC1-Neut** was internalized as effectively as **Tat-Bdy**, and, surprisingly, the anionic **BC1-Neg** was internalized almost as effectively. Interestingly, **BC3-Neut** was internalized to a similar extent, implying that multiple bicyclic structures can be efficiently taken up by cells. The less rigid, monocyclic peptide **HT1-Neut** was taken up by cells to a similar extent as **BC1-Neut**, but linear peptides labeled at Glu1 (**Lin-Neut**) or at the N-terminus (**Lin-Neg**) were not effectively. In the case of these linear peptides, the discrepancy between the fluorescence exhibited by cell lysates and the quantification of confocal microscopy images suggests that some fluorescence was associated with cell lysates at high concentrations (5 μM peptide). In fact, visualization by confocal microscopy shows dim fluorescence localized to the exterior of the cell (experiments performed with 10 μM peptide). However, this association diminishes dramatically at lower peptide concentrations. As shown in Figure 3b and Supplementary Figure S8, cell lysate fluorescence decreases very rapidly for these linear peptides at lower concentrations compared to lysates from cells incubated with bicyclic and cyclic scaffolds. Thus, for these scaffolds we

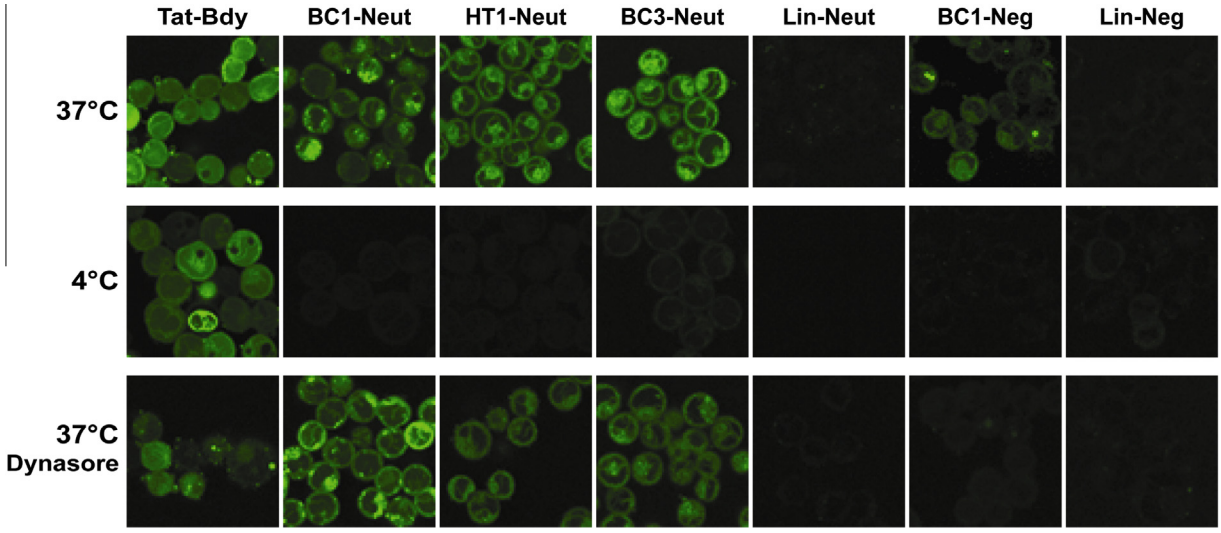

Figure 2. Analysis of peptide internalization by confocal fluorescence microscopy. Mammalian breast epithelial cancer cells (MDA-MB-453) were grown to 95% confluence in a 24-well glass-bottom plate and incubated with 10 μM fluorescently-labeled peptide in PBS for 1 h at 37 °C or 4 °C.[8] Low-temperature conditions were used to demonstrate the extent to which the uptake process was energy-dependent. Alternatively, cells were pre-treated with 80 μM dynasore (a dynamin inhibitor that prevents clathrin-mediated endocytosis)[20] for 30 min at 37 °C prior to incubation with dye-labeled peptides at the same temperature. Cells were washed thoroughly in PBS prior to imaging by confocal microscopy. While peptides were incubated in PBS, we also verified their stability in buffered human serum as described previously (Supplementary Fig. S3).[12]