HHS Public Access

Author manuscript

ACS Chem Biol. Author manuscript; available in PMC 2016 June 09.

Published in final edited form as:

ACS Chem Biol. 2016 February 19; 11(2): 319–323. doi:10.1021/acschembio.5b00966.

Boronic Acid for the Traceless Delivery of Proteins into Cells

Kristen A. Andersen[†,‡], **Thomas P. Smith**[§,‡], **Jo E. Lomax**[‖], and **Ronald T. Raines**[§,⊥,*]

[†]Graduate Program in Molecular and Cellular Pharmacology, University of Wisconsin–Madison, 1300 University Avenue, Madison, Wisconsin 53706, United States

[§]Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, Wisconsin 53706, United States

[‖]Graduate Program in Cellular and Molecular Biology, University of Wisconsin–Madison, 1525 Linden Drive, Madison, WI 53706, United States

[⊥]Department of Biochemistry, University of Wisconsin–Madison, 433 Babcock Drive, Madison, Wisconsin 53706, United States

Abstract

The use of exogenous proteins as intracellular probes and chemotherapeutic agents is in its infancy. A major hurdle has been the delivery of native proteins to an intracellular site of action. Herein, we report on a compact delivery vehicle that employs the intrinsic affinity of boronic acids for the carbohydrates that coat the surface of mammalian cells. In the vehicle, benzoxaborole is linked to protein amino groups via a “trimethyl lock”. Immolation of this linker is triggered by cellular esterases, releasing native protein. Efficacy is demonstrated by enhanced delivery of green fluorescent protein and a cytotoxic ribonuclease into mammalian cells. This versatile strategy provides new opportunities in chemical biology and pharmacology.

Graphical abstract

The delivery of proteins and other macromolecules to an intracellular site is made difficult by cellular membranes.[1] Extensive efforts have led to the development of effective delivery systems that invoke cell-penetrating peptides,[2–5] antibodies,[6] ligands for natural receptors,[7] dendrimers,[8] functionalized polymers,[9,10] liposomes,[11] or nanoparticles.[12,13] Extant

*Corresponding Author: rtraines@wisc.edu.

‡These authors contributed equally to this work and are listed alphabetically.

The authors declare no competing financial interest.

Supporting Information

Procedures for the synthesis of B-TML–NHS ester and Ac-TML–NHS ester, their use in protein modification, analyses of cellular internalization, and Figures S1–S6. This material is available free of charge via the Internet at http://pubs.acs.org.

strategies can, however, lead to adducts that are inapplicable *in vivo*, unstable in a physiological context, recalcitrant to biodegradation, or immunogenic.[14]

Boronic acids are physiologically benign Lewis acids that react spontaneously and reversibly with 1,2- and 1,3-diols to form five- and six-membered cyclic boronic esters, respectively.[15,16] The dynamic covalent bonding of boronic acids/esters can facilitate the delivery of cargo into cells, which are coated with a diol-rich glycocalyx. To exploit that attribute, polymers, nanoparticles, and noncovalent assemblies have been decorated with phenylboronic acid and other arylboronic acids.[17,18]

Recently, we showed that boronic acids can be advantageous when conjugated *directly* to a protein.[19] The ensuing formation of transient boronate esters with the glycocalyx enhances cellular delivery. To date, this approach has relied on the irreversible modification of the target protein, which can compromise activity[20,19,10,21] or lead to immunogenicity.[22,23] An ideal delivery system based on boronic acids (or any moiety) is “traceless” in its delivery of cargo.

We sought to use a boronic acid and an immolative linker to promote the delivery of native proteins into a cell. As a boronic acid, we chose 2-hydroxymethylphenylboronic acid (benzoxaborole), which has higher affinity than does phenylboronic acid for the glycopyranosides that are abundant in the glycocalyx.[24,19,18] As an immolative linker, we chose the *o*-hydroxydihydrocinnamic acid derivative known as the trimethyl lock (TML). After being triggered, the TML exhibits extremely high lactonization rates to release a cargo of interest (Scheme 1).[25–29] The TML has been used for a wide variety of applications in chemistry and pharmacology,[30] but not as an immolative linker on a protein. We chose ester hydrolysis as the means to trigger lactonization of the TML, as esterases are abundant inside, but not outside, of human cells[31–33] and underlie the action of numerous prodrugs.[34] We equipped our TML scaffold with an *N*-hydroxysuccinimide ester for chemoselective conjugation to amino groups,[20] such as those at the N terminus and on the side chain of lysine residues, which have a ~6% abundance in proteins.[35] Thus, our delivery vehicle (B-TML–NHS ester) has three modules: benzoxaborole, an esterase-activated TML linker, and an NHS ester (Figure 1A).

We synthesized B-TML–NHS ester convergently in 10 steps by extending a known procedure.[36] Then, we characterized its ability to enhance the cellular internalization of a green fluorescent protein (GFP) (Scheme 2), which has distinctive fluorescence and an inability to enter mammalian cells.[37] Overnight incubation at ambient temperature with 100-fold excess of B-TML–NHS ester in 3:1 PBS/acetonitrile yielded 3 ± 1 labels per protein (Figures 1B and S1). The number of labels in the B-TML–GFP conjugate did not decrease after a month of storage in PBS (Figure S2), consistent with the stability observed for other TML conjugates.[38–40] Labeling was, however, “bioreversible”. Incubation with a lysate from Chinese hamster ovary (CHO) K1 cells removed all of the labels from B-TML–GFP (Figure 1B).

Next, we compared the uptake of B-TML–GFP and unlabeled GFP by CHO K1 cells. After a 4-h incubation, we observed a dramatic increase in the cellular uptake of B-TML–GFP

(Figure 1C). The fluorescence in microscopy images was largely punctate, suggesting that B-TML–GFP was taken up via an endosomal pathway (Figure 1D). Co-localization of this bright punctate staining with a stain for transferrin was consistent with this conclusion (Figure S3). After a 24-h incubation, some cytosolic staining was observed, suggestive of endosomal escape (Figure S4).

To confirm that the boronic acid moiety was responsible for the difference in cellular entry, we performed two control experiments. First, we modified GFP with a vehicle (Ac-TML–NHS ester) that lacks the benzoxaborole functionality (Figure 1A), yielding a level of labeling similar to that from B-TML–NHS ester (Figure S1). When incubated with cells for 4 h, Ac-TML–GFP was taken up comparably to unlabeled GFP rather than to B-TML–GFP (Figures 1C and 1D). These data indicate that the enhanced delivery upon treatment with B-TML–NHS ester is not due to the mere modification of lysine residues or to interactions with the TML portion of B-TML. Next, we repeated the cellular uptake experiments with B-TML–GFP in the presence of fructose, which has a K_a of 336 M^{-1} for benzoxaborole.[19] We observed a significant decrease in GFP uptake in the presence of fructose, apparent with both confocal microscopy and flow cytometry (Figures 2A and 2B). Again, these data indict the boronic acid portion of B-TML–GFP as being responsible for cellular uptake.

Finally, we sought to test the efficacy of B-TML as a delivery vehicle to the cytosol. To do so, we employed an enzymic cytotoxin—the G88R variant of ribonuclease A, which must reach cytosolic RNA to manifest its toxic activity.[41,42] After labeling the ribonuclease by the same procedure used to label GFP, we observed an average of 1.6 ± 0.7 labels per molecule of protein (Figure S5). This lower labeling is consistent with GFP (19 lysine residues) having more amino groups than does the ribonuclease (12 lysine residues). Again, we found that the labeling was bioreversible, as incubation with a CHO K1 cell lysate removed all of the labels (Figure S6). Finally, we assayed the ability of B-TML–ribonuclease and unlabeled ribonuclease to inhibit the proliferation of K-562 cells, which are derived from a human myelogenous leukemia line. We found that the pendant boronic acids resulted in a decrease in the IC_{50} value (Figure 3), indicative of more cytotoxin reaching the cytosol.

We conclude that covalent modification of proteins with B-TML–NHS ester can increase their ability to enter mammalian cells. Importantly, this modification is traceless, as cellular esterase activity restores the proteins to their unmodified state. This bioreversibility of our delivery vehicle provides new opportunities. The sulfhydryl groups of cysteine residues have long been used for this purpose because their mixed disulfides suffer reduction within the cytosol.[43] Recently, we found that appropriately tuned diazo compounds can esterify protein carboxyl groups, providing a second type of bioreversible modification.[44,45] In this work, we report on a bioreversible modification of protein amino groups that is distinct from others[46–48] in its reliance on enzymatic catalysis. With its traceless utility in promoting cellular uptake, B-TML–NHS ester provides new opportunities in chemical biology and pharmacology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Ismet Çağlar Tanrikulu (University of Wisconsin–Madison) for technical advice. K.A.A. was supported by a predoctoral fellowship from the PhRMA Foundation and Molecular and Cellular Pharmacology Training Grant T32 GM008688 (NIH). J.E.L. was supported by a National Science Foundation Graduate Research Fellowship. This work was supported by grants R01 GM044786 and R01 CA073808 (NIH), and made use of the National Magnetic Resonance Facility at Madison, which is supported by Grant P41 GM103399 (NIH), and the University of Wisconsin Carbone Cancer Center, which is supported by Grant P30 CA014520 (NIH).

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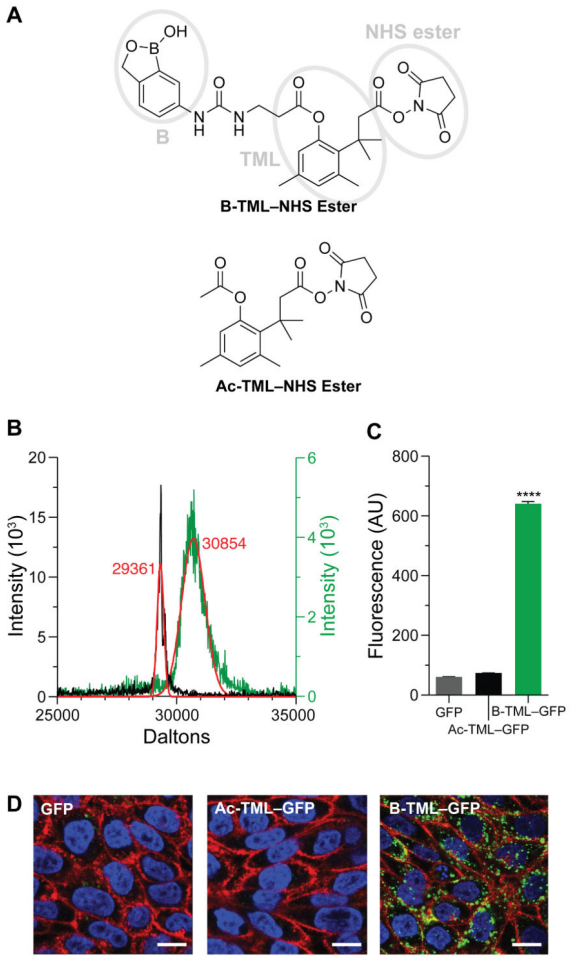


Figure 1.

Cellular internalization of B-TML–labeled GFP. (A) Structures of B-TML–NHS ester and Ac-TML–NHS ester. Ellipses denote the three distinct modules within B-TML–NHS ester. (B) MALDI–TOF mass spectra of B-TML–GFP (green), conjugated to ~3 boronic acid moieties per molecule, and the same protein after exposure to CHO K1 cell lysate and purification (gray). Expected *m*/*z*: GFP, 29361; each B-TML moiety, 450. (C) Flow cytometry analysis of CHO K1 cells incubated with 10 μM unlabeled GFP, GFP labeled with a control vehicle (Ac-TML), or GFP labeled with the boronate vehicle (B-TML) for 4 h ($p < 0.0001$). (D) Confocal microscopy of CHO K1 cells grown as in panel C. Cells were stained with WGA-594 (red) and Hoechst 33342 (blue). Scale bars: 10 μm.

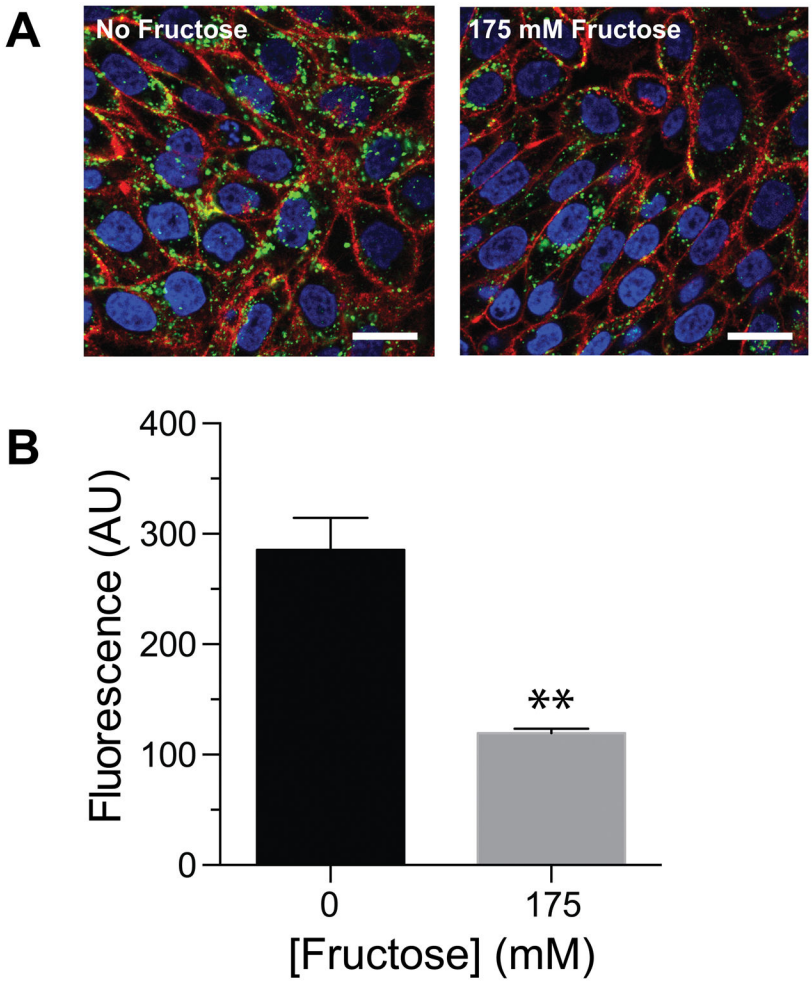


Figure 2.

Effect of fructose on the cellular internalization of B-TML–labeled GFP. (A) Confocal microscopy of B-TML–GFP (10 μM) preincubated with PBS or 175 mM fructose for 30 min, then used to treat CHO K1 cells for 4 h. Cells were stained with WGA-594 (red) and Hoechst 33342 (blue). Scale bars: 20 μm. (B) Flow cytometry analysis of CHO K1 cells treated as in panel A ($p < 0.01$).

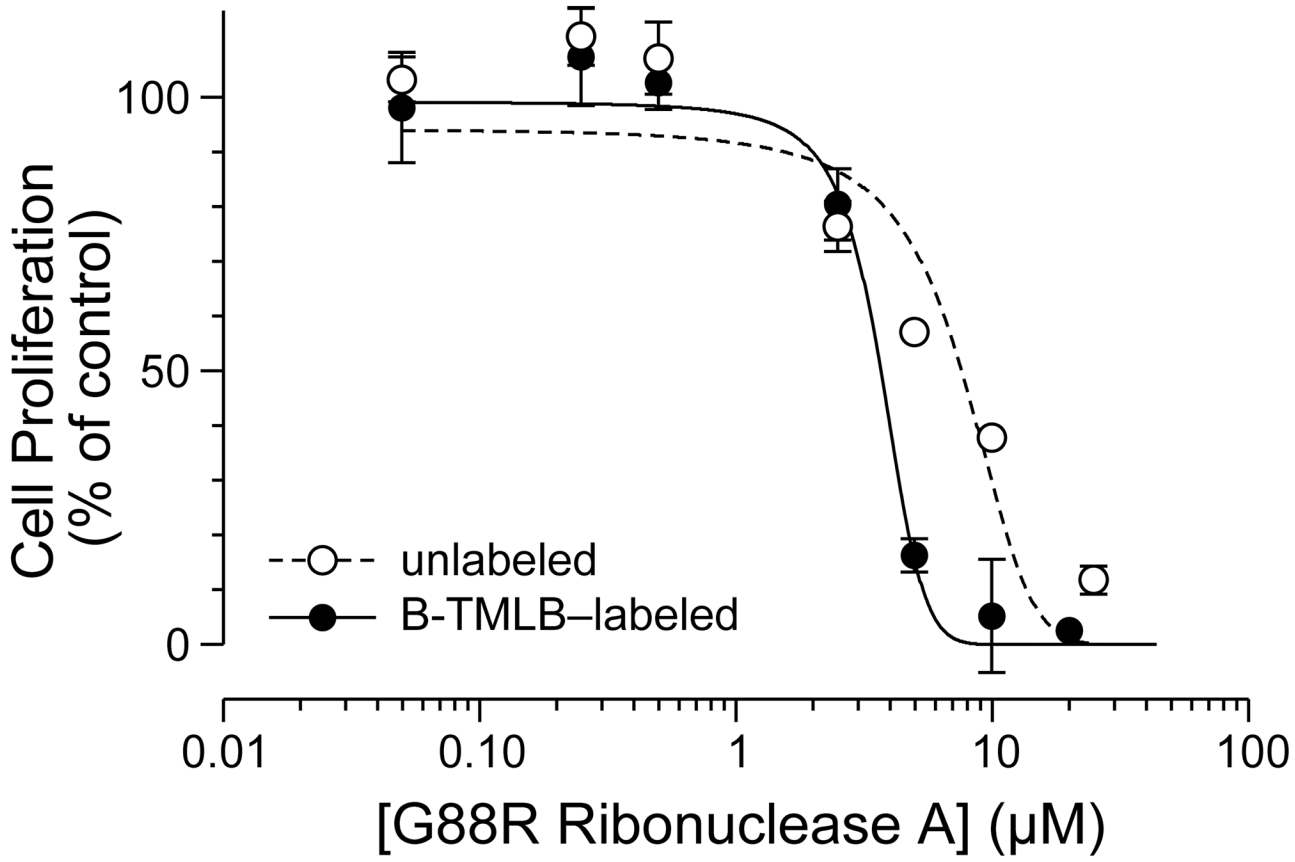
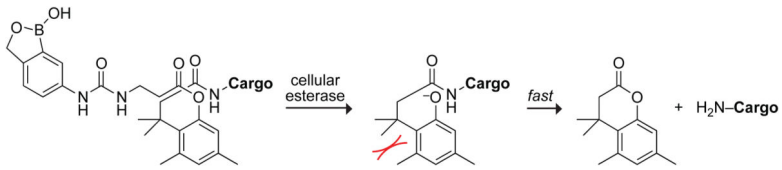
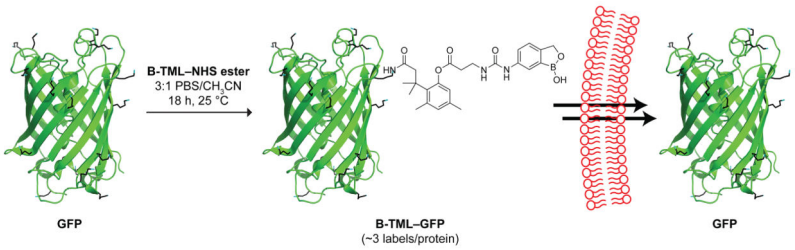


Figure 3.

Effect of B-TML–labeling on the inhibition of K-562 cell proliferation by a ribonuclease. Unlabeled G88R ribonuclease A, IC_{50} = (6.4 ± 0.1) μM; B-TML–labeled G88R ribonuclease A, IC_{50} = (3.5 ± 0.8) μM. Each data point represents the mean ± SE for three separate experiments, each performed in duplicate.



Scheme 1.



Scheme 2.