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A Boronic Acid Conjugate of Angiogenin that Shows ROS-Responsive Neuroprotective Activity

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Abstract

Angiogenin (ANG) is a human ribonuclease that is compromised in patients with amyotrophic lateral sclerosis (ALS). ANG also promotes neovascularization, and can induce hemorrhage and encourage tumor growth. The causal neurodegeneration of ALS is associated with reactive oxygen species, which are also known to elicit the oxidative cleavage of carbon–boron bonds. We have developed a synthetic boronic acid mask that restrains the ribonucleolytic activity of ANG. The masked ANG does not stimulate endothelial cell proliferation but protects astrocytes from oxidative stress. By differentiating between the two dichotomous biological activities of ANG, this strategy predicates a viable pharmacological approach for the treatment of ALS.

Activatable growth factor

Spatiotemporal control of protein function can lead to new therapeutic modalities. Angiogenin is damaged in ALS patients. A semisynthetic angiogenin has a key active-site residue masked with a boronic acid, vitiating deleterious side effects. Hydrogen peroxide, which is linked to ALS, unmasks the angiogenin, which then protects astroglia.

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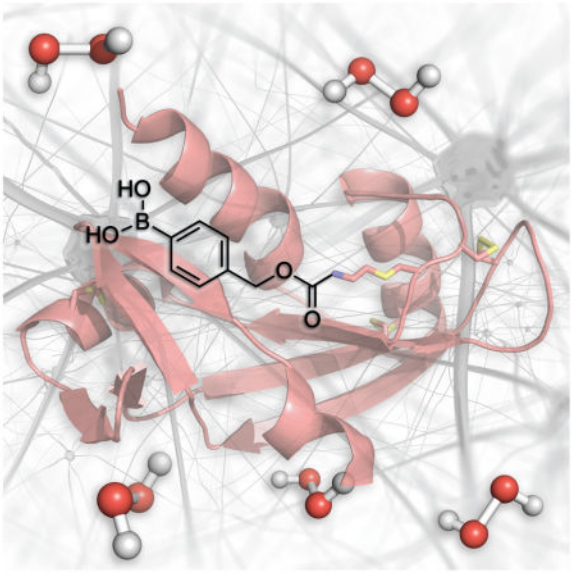

Keywords

amyotrophic lateral sclerosis; angiogenesis; prodrug; reactive oxygen species; ribonuclease

Amyotrophic lateral sclerosis (ALS) is an aggressive, fatal disease that is characterized by the selective destruction of motor neurons in the motor cortex, brain stem, and spinal cord.[1] Although the fundamental cause of ALS is not clear, its pathogenesis arises from several mechanisms, including oxidative stress.[2] The only approved chemotherapeutic agent for ALS is a sodium-channel-blocking agent, riluzole (Rilutek), which was approved for human use in 1995, extends survival by only 2–3 months, and does not improve motor function.[3]

Loss-of-function mutations in the human gene encoding a secretory ribonuclease, angiogenin (ANG), are associated with the progression of ALS.[4] In accord, the administration of human ANG increases the lifespan and improves the motor function of ALS-like transgenic mice.[5] Nevertheless, ANG has a well-known adverse effect as a potential chemotherapeutic agent for ALS. As its name implies, ANG induces the proliferation of endothelial cells to form new blood vessels by a mechanism uncovered recently.[6] Accordingly, long-term treatment with ANG could engender hemorrhage and tumor growth.[7]

The neurodegeneration that is characteristic of ALS correlates with an abundance of reactive oxygen species (ROS), which are cytotoxic.[2a,8] Moreover, ALS is linked to the hyperactivity of superoxide dismutase (SOD1).[9] This enzyme catalyzes the conversion of superoxide ion (O_2^-) to hydrogen peroxide (H_2O_2), which is the major physiological ROS.

The chemical reactivity of H_2O_2 can be exploited in a physiological context. For example, H_2O_2 has long been known to effect the oxidative cleavage of the boron–carbon bond in phenylboronic acid, leading to phenol and boric acid ($B(OH)_3$).[10] This reaction has served as the basis of chemoselective probes for H_2O_2 and in cancer prodrug strategies.[11,12]

ANG relies on the intracellular manifestation of its ribonucleolytic activity to mediate neuroprotection.[13] We envisioned the oxidative cleavage of a boronic acid as a means to generate active ANG *only* in cells suffering from ROS-mediated toxicity. To install an ROS-sensitive trigger in ANG, we chose to target a key active-site residue: Lys40 (Scheme 1). This residue is essential for the ribonucleolytic activity of ANG.[14] We used recombinant DNA methods to replace Lys40 with a cysteine residue. We reasoned that its S^{γ} could serve as a reactive handle for conjugation of a boronic acid containing a latent amino group that is poised to reconstitute catalytic activity. We synthesized the boronic acid (**1**), as well as a control molecule lacking the boronic acid moiety (**2**), from an azide precursor via a Curtius rearrangement.

An ROS-activatable phenylboronic acid conjugate (B-thiaK40 ANG) or an inactivatable phenyl conjugate (P-thiaK40 ANG) were made by employing a radical-initiated thiol-ene reaction (Scheme 1).[15] Notably, the classic method of generating γ-thialysine derivatives by *S*-alkylation with a haloethylamine[16] failed with K40C ANG despite working with ribonuclease A, which is an ANG homolog.[17,18] The integrities of the K40C variant and its conjugation products were confirmed with LC-MS/MS after trypsin digestion (Figure S1).

Exposure to an ROS reconstitutes the enzymatic activity of B-thiaK40 ANG *in vitro*. A zymogram assay of ribonucleolytic activity revealed that P-thiaK40 ANG was inactive, even after treatment with H_2O_2 (Figure 1A). In contrast, this ROS did elicit activity from B-thiaK40 ANG (Figure 1A), which was quantified to be (21 ± 6)% that of the wild-type enzyme (Figure 1B). This value is indistinguishable from the relative value of k_{cat}/K_M, which was determined to be (16 ± 4)% in solution with a fluorescence-based assay (Figure 1C).[19]

The intrinsic catalytic activity of ANG is low.[20] In the three-dimensional structure of ANG, the side chain of Gln117 obstructs a nucleobase-binding pocket in the active site.[21] A Q117G substitution increases the catalytic activity of ANG toward conventional substrates by 30-fold.[22] Accordingly, we generated an ROS-activatable phenylboronic acid conjugate with K40C/Q117G ANG, and we observed enhanced catalytic activity upon its unmasking with H_2O_2, both in a zymogram assay (Figures 1A and 1B) and in solution (Figure 1C). Moreover, in the context of Q117G ANG, having a lysine or γ-thialysine as residue 40 affects catalytic activity by only twofold.

ANG promotes the proliferation of human endothelial cells, unlike its P-thiaK40, P-thiaK40/Q117G, B-thiaK40, or B-thiaK40/Q117G variants (Figure 2). Thus, conjugation eliminates this biological activity of ANG. Exposure to H_2O_2 does enable B-thiaK40 ANG and B-thiaK40/Q117G ANG to induce cell proliferation. The unmasked variants that have a γ-thialysine as residue 40 are, however, less potent than their isosteres with lysine as residue 40, consistent with relative enzymatic activities observed *in vitro* (Figure 1).

Next, we asked the key question: Do ROS-activatable masked ANG conjugates elicit a phenotype that could benefit an ALS patient? Astrocytes are prevalent glial cells in the central nervous system that support neuronal plasticity and recovery after injury.[9,25] Under stress like that imposed by ALS, motor neurons secrete ANG, which is taken up selectively

by astrocytes.[26] Within astrocytes, ANG stimulates pro-survival signals, which are transmitted to motor neurons to afford protection from oxidative damage.[27]

Oxidative stress was imposed upon astrocytes by treatment with either phorbol 12-myristate 13-acetate (PMA) or H_2O_2. PMA activates protein kinase C, stimulating the catalytic production of O_2^- by nicotinamide adenine dinucleotide phosphate oxidase.[28] To begin, we determined the toxicity of each agent to human astrocytes (Figure S2A), and we found a dose–response correlation between doses that led to 25%, 50%, and 75% cell survival and ROS levels within astrocytes (Figure S2B).

Like wild-type ANG, B-thiaK40 ANG and B-thiaK40/Q117G ANG protect human astrocytes from ROS-mediated toxicity. This protection was evident for cells challenged with all three doses of PMA or H_2O_2 (Figure 3). In contrast, no benefit was observed upon treatment of astrocytes with P-thiaK40 ANG or P-thiaK40/Q117G ANG. Importantly, our data indicate that the neuroprotection afforded by B-thiaK40/Q117G ANG is comparable to that from the wild-type enzyme. Thus, B-thiaK40/Q117G ANG has attributes of a biologic prodrug for ALS.

Finally, we assessed the cytotoxicity of the byproducts that form upon unmasking of the ANG conjugates. The immolative mechanism of unmasking produces carbon dioxide, boric acid, and *p*-quinone methide, which can react with water to form 4-hydroxybenzyl alcohol (Scheme 1).[29] We found that millimolar levels of boric acid or 4-hydroxybenzyl alcohol, alone or in combination, did not lead to detectable toxicity for human astrocytes (Figure S3). Notably, boric acid is common in the environment as well as in a normal diet.[30]

In summary, we have used a thiol-ene reaction to create a semisynthetic ANG that is inactive under normal physiological conditions but becomes active in the presence of the most prevalent ROS—H_2O_2. ALS is an incurable disease that is linked to hypoactive ANG[4] and hyperactive SOD1,[9] which catalyzes the formation of H_2O_2. The accumulating H_2O_2 could serve to unmask our semisynthetic ANG selectively in contexts relevant for the treatment of ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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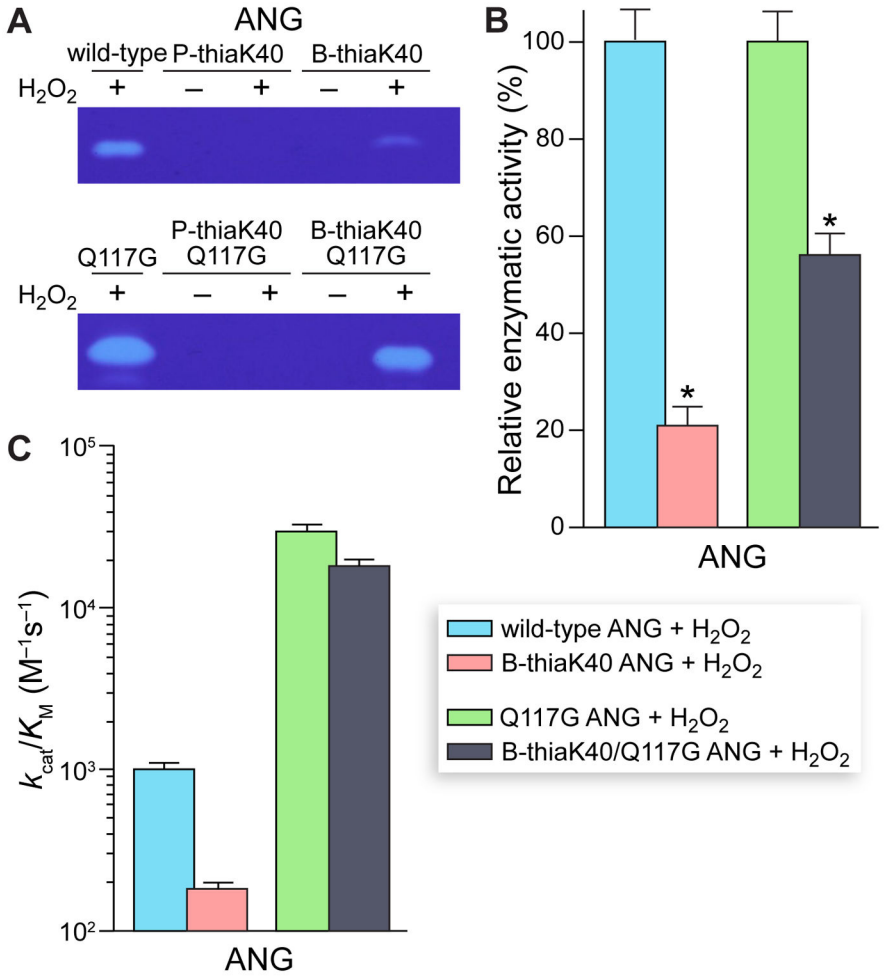


Figure 1.

An ROS restores the ribonucleolytic activity of B-thiaK40 ANG and its Q117G variant. (A) Representative zymograms of ANG, Q117G ANG, and their conjugates. Proteins were exposed to H_2O_2 (1.0 mM for 3 h at 37 °C), and assayed for their ability to cleave poly(cytidylic acid) by negative-staining with toluidine blue.[23] (B) Graph of quantified data from all zymograms of ANG, Q117G ANG, and their boronated conjugates (all exposed to H_2O_2). Values are the mean ± SE ($n = 4$, technical replicates). *, $p < 0.05$. (C) Graph of the values of k_{cat}/K_M for the cleavage of 6-FAM–dArUdAdA–6-TAMRA[24] by ANG, Q117G ANG, and their boronated conjugates (all exposed to H_2O_2). Values are the mean ± SE ($n = 3$, technical replicates).

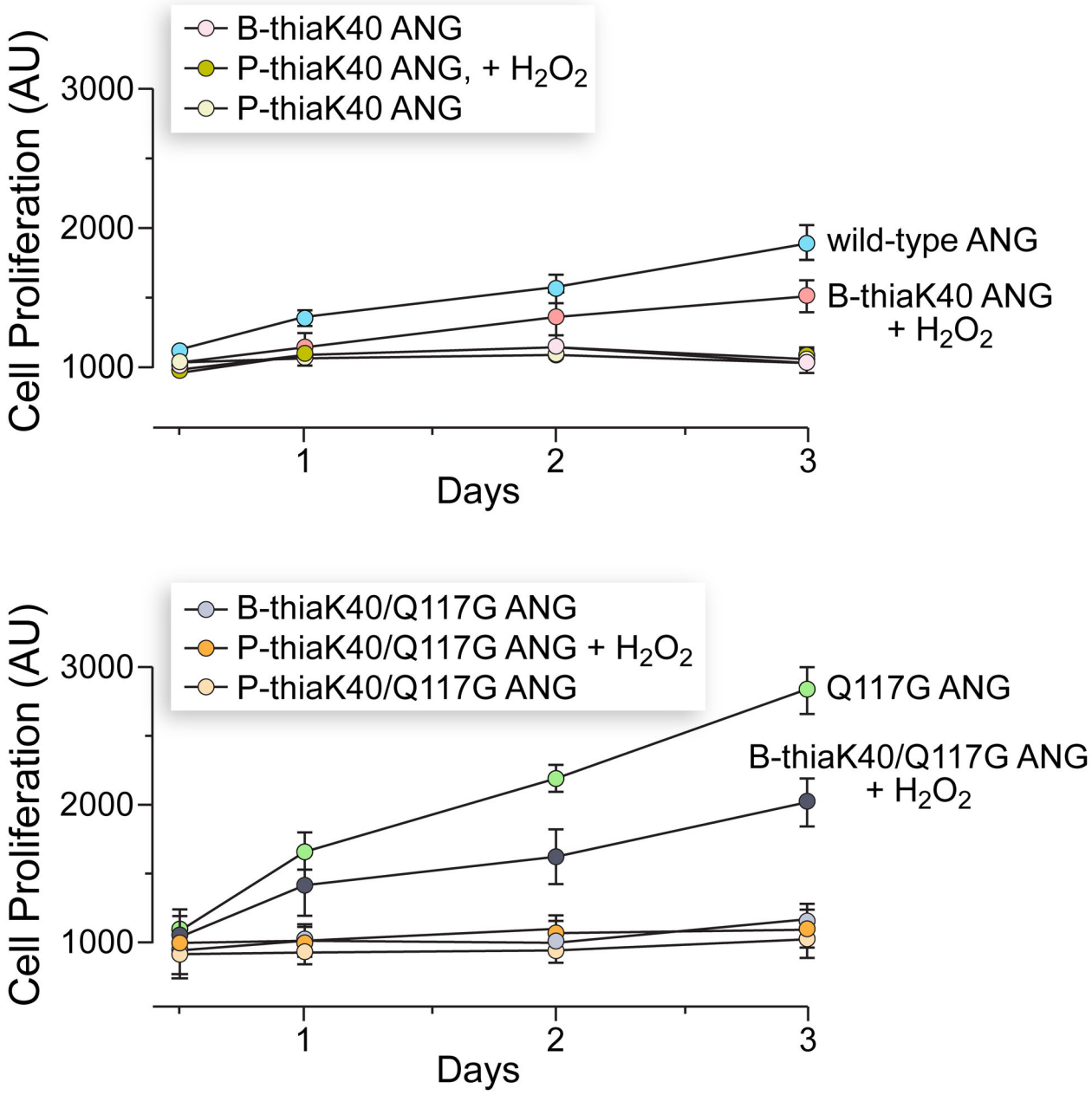


Figure 2.

Masked ANG conjugates do not promote the proliferation of human endothelial cells. Graphs showing that B-thiaK40 ANG and B-thiaK40/Q117G ANG promote the growth of HUVEC cells only after exposure to H_2O_2 (1.0 mM for 3 h at 37 °C). Neither P-K40 ANG nor its P-K40/Q117G variant affects growth. Values represent the mean ± SE (n = 3, biological replicates).

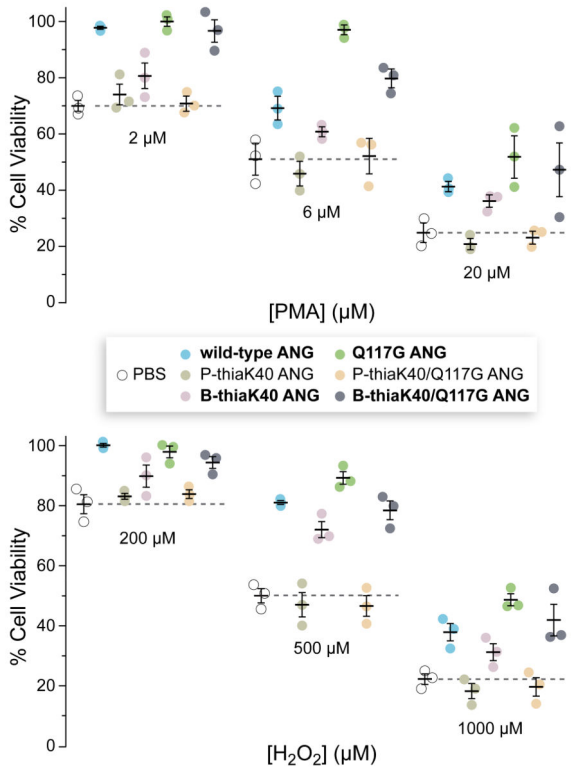
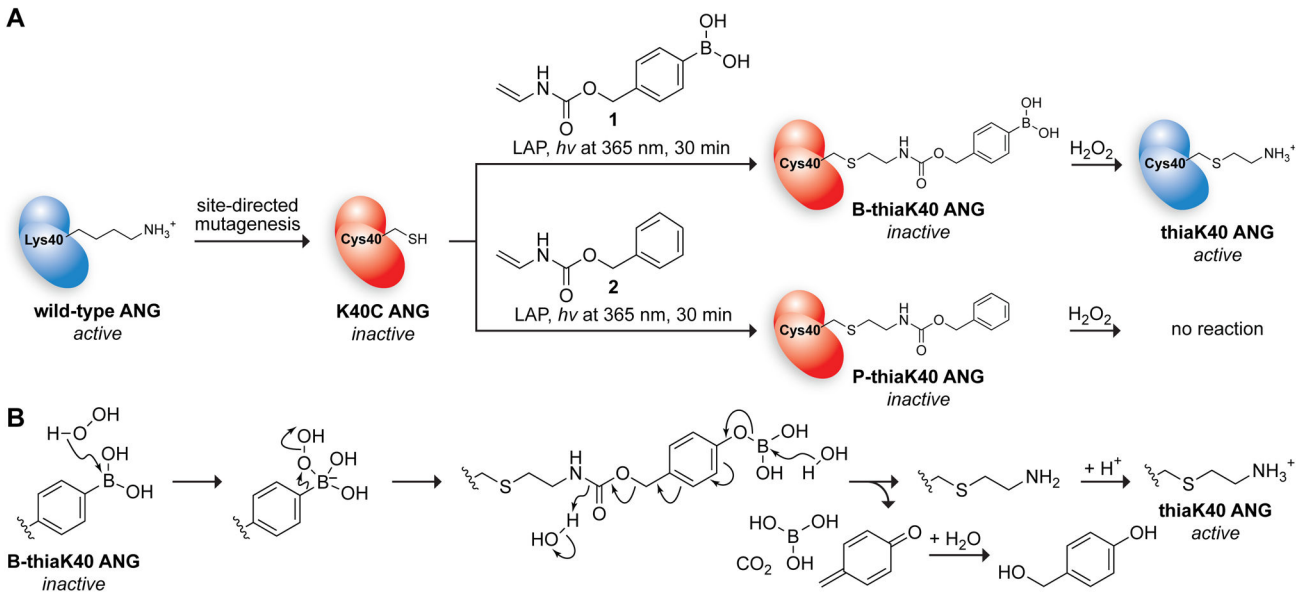


Figure 3.

B-thiaK40 ANG and its Q117G variant protect human astrocytes from oxidative stress. Cells were pre-treated for 24 h with ANG, Q117G ANG, or their conjugates (1.0 μg/mL) prior to exposure to PMA or H_2O_2. The cytotoxicity of these agents was assessed 24 h later. Only ANG with the native lysine or activatable γ-thialysine residue at position 40 protected cells from oxidative stress. Values represent the mean ± SEM (n = 3, biological replicates).



Scheme 1.

Synthesis and unmasking of an ROS-responsive conjugate of human ANG. (A) Lys40 is a key residue in the active-site of ANG. The K40C variant, which lacks biological activity, was modified by a radical-initiated thiol-ene reaction with a boronic acid that contains a latent amino group (**1**). The ensuing B-thiaK40 ANG is also inactive, except in environments with high levels of H_2O_2, which unmask the γ-thialysine residue and restore the biological activity of ANG. P-thiaK40 ANG lacks the boronic acid moiety and is not responsive to H_2O_2. (B) Putative mechanism for the oxidative cleavage of the boron–carbon bond that unmasks the γ-thialysine residue, converting B-thiaK40 ANG into thiaK40 ANG.