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CHEMMEDCHEM Communications

A Double Prodrug with Improved Membrane Permeability over the Parent Chelator HBED Provides Superior Cytoprotection against Hydrogen Peroxide

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The clinical use of *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED) has been hindered by its lack of bioavailability. *N*,*N*′-bis(2-boronic pinacol ester benzyl)ethylenediamine-*N*,*N*′-diacetic acid methyl, ethyl, and isopropyl esters **7a–c**, respectively, and their dimesylate salts **8a–c**, are double prodrugs that mask the two phenolate and two carboxylate donors of HBED as boronic esters and carboxylate esters, respectively. Their activation by chemical hydrolysis and oxidation, their passive diffusivity, and their cytoprotective capabilities have been investigated here. **8a–c** hydrolyzed in minimum essential medium at 37 °C with half-lives of 0.69, 0.81, and 2.28 h, respectively. The intermediate formed, **9** [*N*,*N*′-bis(2-boronic acid benzyl)ethylenediamine-*N*,*N*′-diacetic acid], then underwent oxidative deboronation by H_2O_2 to give HBED (k = 1.82 M^{-1} min^{-1}). Solubility measurements in mineral oil and in phosphate buffer indicated that **7a** had a better balance between lipid and aqueous solubilities than did HBED. **7a** was also able to passively diffuse across a lipid-like silicone membrane (log flux = −0.36), whereas HBED-HCl was not. **8c** provided better protection to retinal cells than did HBED against a lethal dose of H_2O_2 (84% vs. 28% protection, respectively, at 44 μM). These results suggest that the double prodrugs have better membrane permeability than does HBED, and therefore could be therapeutically useful for improving the delivery of HBED.

N,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED), a hexadentate iron chelator, was investigated some time ago as an orally administered alternative to desferrioxamine, which is given by subcutaneous infusion, for the treatment of transfusional iron overload.[1] Unfortunately, HBED was found to be relatively ineffective at removing excess iron from iron-loaded primates[2] and from humans with β-thalassemia,[3] probably because it is not well absorbed from the gastrointestinal tract. In an effort to improve oral bioavailability, simple alkyl ester prodrugs of HBED were prepared,[1b,4] but these too failed to be effective in iron-loaded primates[2,5] because they were unable to chemically[4] or enzymatically[6] hydrolyze to give the active chelator in vivo. Recently, we described a series of novel HBED double prodrugs of the general form **7** that mask the two phenolate and two carboxylate donors of HBED as boronic acids/esters and simple alkyl carboxylate esters, respectively (Scheme 1).[7] Three of the double prodrugs were confirmed to undergo activation at physiological pH by chemically mediated hydrolysis to give intermediate **9** (Scheme 1), which is itself a prodrug because of its weak affinity for Fe^{3+} and Cu^{2+}. Subsequent oxidation of **9** by H_2O_2 generated the active parent chelator HBED (Scheme 1). These double prodrugs may be useful therapeutic agents for diseases where focal iron accumulation and oxidative stress are implicated in disease progression (e.g., Parkinson's disease and other neurodegenerative diseases, skin photo-aging, and age-related macular degeneration). In the present work, we describe the rates of activation of the double prodrugs in cell culture medium, which more closely mimics physiological conditions; the passive diffusivity of a double prodrug across a lipid-like membrane; and the protection afforded by the double prodrugs to retinal pigment epithelial cells exposed to a lethal dose of H_2O_2.

As previously described,[7] double prodrugs **7a–c** were synthesized as their dimesylate salts **8a–c** over four steps (Scheme 2). Briefly, **3** was synthesized via reductive amination and then condensed with pinacol to give **5**. The bis acetic acid esters were installed onto **5** using a simple N-alkylation reaction, which gave **7a–c** as oils. Conversion of **7a–c** to their corresponding dimesylate salts afforded **8a–c** as pure solids. In studies requiring **7a**, **8a** was simply re-converted to its free base using diisopropylethylamine (see Supporting Information).

We have previously shown that the double prodrugs **8a–c** convert to the parent iron chelator, HBED, by chemical hydrolysis and H_2O_2 oxidation in N-methylmorpholine and phosphate buffers at pH 7.4 with methanol added as a cosolvent.[7] Here, before investigating the effectiveness of the double prodrugs in cell culture, we wanted to verify that the conversion to HBED occurs in cell culture medium. Consecutive hydrolysis and H_2O_2 oxidation of the double prodrugs was monitored by UV in minimum essential medium (MEM) at 37 °C. **8a**, **8b**, and **8c** hydrolyzed with pseudo first-order rate constants of 0.0167 ± 0.003 min^{-1}, 0.0143 ± 0.0019 min^{-1}, and 0.0051 ± 0.0014 min^{-1}, respectively, which correspond to half-lives of 0.69, 0.81, and 2.28 h. The final spectrum obtained from each prodrug's hydrolysis matched that of an authentic sample of **9** (Figure S1). The rate-limiting step for the hydrolysis of **8a–c** most likely is the hydrolysis of the carboxylate esters, since pinacol ester hydrolysis is very rapid.[7] Thus, the more sterically hindered the carboxylate esters were (IPr > Et > Me), the

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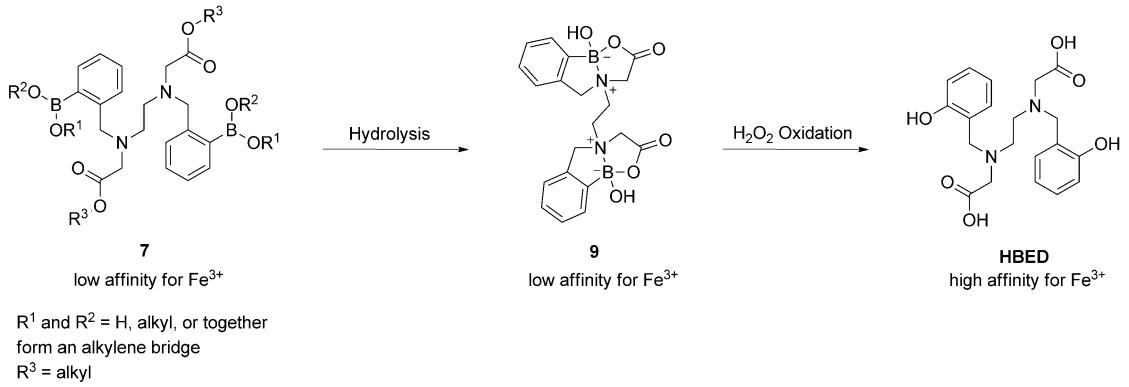
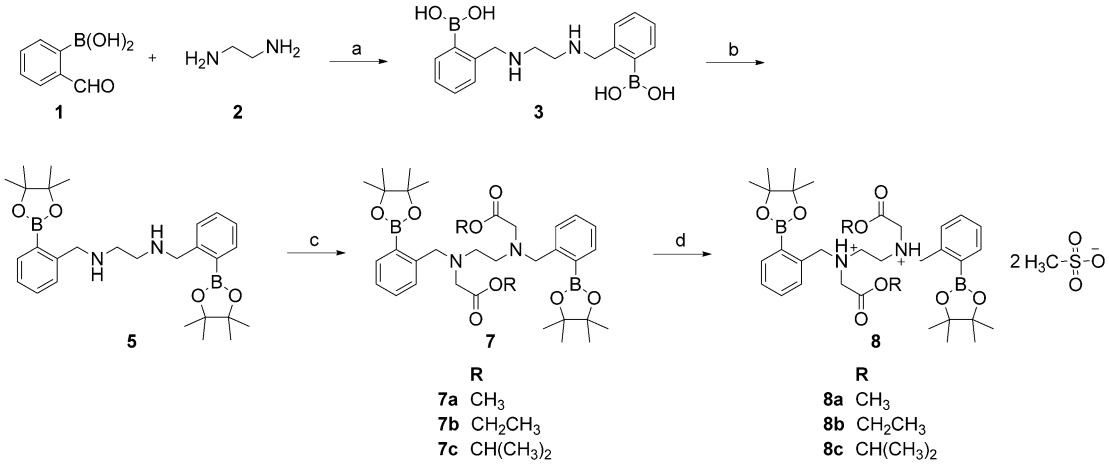


Scheme 1. Double prodrugs of the general form **7** undergo activation by nonenzymatic hydrolysis to form **9**, which then is oxidatively deboronated by H_2O_2 to generate the parent chelator HBED.



Scheme 2. Synthesis of HBED double prodrugs as their dimesylate salts **8a–c**. a) MeOH, RT, 2 h, then $NaBH_4$, 0 °C→RT, 1.5 h, 87%; b) pinacol, toluene, reflux, 22 h; c) alkyl bromoacetate, diisopropylethylamine, dry ACN, relux, o/n; d) methanesulfonic acid, dry ether, RT, 2 h, 59–81% over three steps.

longer were the macro half-lives of the double prodrugs (**8c** > **8b** > **8a**). Interestingly, these half-lives in MEM are substantially shorter than those obtained for **8a–c** in pH 7.4 phosphate buffer containing 50% MeOH (half-lives of 3.8, 5.9, and 26.3 h, respectively),[7] probably due to catalysis by amino acids such as glutamine present in high concentrations in MEM.[8]

To a solution of **9** formed from the hydrolysis of **8a** in MEM was then added 7.5-30 mM of H_2O_2 (50–200× excess). UV spectra obtained during a representative oxidation reaction are given in Figure S2. The final spectrum for each oxidation reaction matched that of an authentic sample of HBED. A second-order rate constant (k) of 1.82 $M^{-1}min^{-1}$ for the macro oxidation of **9** to HBED was obtained from the slope of the linear fit of the plot of k_{obs} versus H_2O_2 concentration. The results of the serial hydrolysis and oxidation study provides proof of activation of the double prodrugs in MEM to the parent chelator HBED.

In order to access the interior of a cell to express its activity, a new drug or the prodrug of that new drug must be able to passively diffuse through the cell membrane. One physicochemical property that may facilitate passive diffusion is adequate lipid solubility. Double prodrugs **7b** and **7c**, the free bases of **8b** and **8c**, were not available in sufficient quantities to determine their lipid solubilities. However, double prodrug **7a**, the free base of **8a**, was found to possess a high solubility in mineral oil, a lipid-like solvent: 115 ± 3.5 mM. In contrast, the parent chelator, HBED, was insoluble as its monohydrochloride salt in mineral oil. This lack of lipid solubility was anticipated since HBED lacks oral availability due to its polar functional groups and to its ionization at physiological pH. Aqueous solubility is also important for passive diffusivity. HBED-HCl displayed substantial aqueous solubility (> 10 mM) at pH 7.4 in phosphate buffer, while the double prodrugs **8a**, **8b**, and **8c** had more moderate aqueous solubilities of 618 μM, 171 μM, and 54 μM, respectively.[7] Due to the rapid hydrolysis of the boronic pinacol esters of **8a–c**, the aqueous solubilities of these prodrugs are probably reflective of the corresponding boronic acid species present in solution. Taken together, these solubility studies suggest that the membrane permeability of the double prodrugs may be improved compared to that of HBED because they possess both good lipid and good aqueous solubilities,[9] while HBED lacks lipid solubility.

Passive diffusion studies of **7a** and HBED-HCl using Franz diffusion cells equipped with silicone membranes further sup-

port the improved membrane permeability of the double prodrugs. Silicone membranes, which are lipid-like, have been validated as a surrogate for flux across a biological membrane: namely, the skin.[10] Mineral oil was used as the donor vehicle in which the compounds were dissolved so that **7a** would remain intact in the donor phase throughout the experiment. Use of a protic solvent would most likely have led to its decomposition. The receptor chambers contained phosphate buffer. All receptor phase samples taken from diffusion cells to which **7a** was applied were allowed to hydrolyze to **9** before analysis because **7a** and **9** do not have sufficiently different UV spectra to enable one to calculate the amount of each in a mixed sample. The amount of **9** in receptor phase samples presumably corresponds to the amount of **7a** that was able to diffuse intact through the silicone membranes before subsequently hydrolyzing in the receptor chambers. **7a** showed excellent diffusion across the silicone membranes, having a maximum flux of 0.43 ± 0.04 μmol cm^2h^{-1} (log flux $= -0.36$). By contrast, no flux was observed for HBED-HCl through the silicone membranes from a mineral oil vehicle. Once again, this suggests that the double prodrugs may have better membrane permeability than the parent chelator. While a better comparison could have been made if the flux of HBED as the free base had been investigated, it was not readily available.

Attention was then turned to investigating the abilities of the double prodrugs **8a**–**c** and prodrug **9** to undergo activation in vitro to afford cytoprotection against H_2O_2-induced death, compared with the cytoprotective ability of the parent chelator, HBED. H_2O_2 has been found to oxidize ferritin, the major iron storage protein, leading to a release of iron that can catalyze free radical damage.[11] Protection by the prodrugs will require their entry into cells and their timely activation to HBED so that the freed iron can be sequestered. A spontaneously-arising human retinal pigment epithelial cell line (ARPE-19) was chosen for its relevance to age-related macular degeneration. This incurable disease is the most common cause of vision loss for people over the age of 50 and increased labile iron is implicated in its pathogenesis.[12]

Confluent cells were pretreated with **8a**–**c**, **9**, or HBED (as its HCl salt) in MEM for 15 h so that all compounds would have sufficient time to enter the cells if they are membrane permeable, and so that the double prodrugs would also have time to hydrolyze. The cells were then treated for 8 h with a lethal dose of 500 μM of H_2O_2, which decreased cell viability by approximately 79% in a preliminary kill curve experiment (Figure 1). Percent protection afforded by the compounds was calculated from the results of an MTT cell viability assay (see Supporting Information). The results of these cytoprotection studies are given in Figure 2. Cytoprotection was dose dependent for all compounds tested. At their highest concentrations of 150 μM, **8a**, **8b**, **9**, and HBED all provided moderate protection to cells from the lethal dose of H_2O_2 relative to cells exposed to H_2O_2 but given no prodrug or HBED (62–68% protection, $p < 0.001$ by t-test). In contrast, despite applying it at lower concentrations due to solubility limitations, **8c** gave the highest cytoprotection: at only 44 μM, **8c** afforded 84% protection against H_2O_2 relative to cells exposed to H_2O_2 but given no prodrug or HBED ($p < 0.001$). Additionally, while neither **8a**, **8b**, nor **9** showed enhanced protection over that of HBED at any equivalent dose, **8c** significantly out-protected HBED at concentrations of 13, 20, 30, and 44 μM ($p < 0.001$). At 44 μM, **8c** provided 3-fold higher protection to cells compared to HBED. **8c** was also not toxic to cells at this dose (Figure S3).

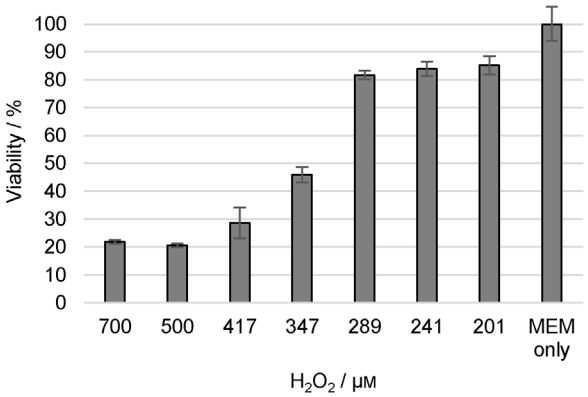


Figure 1. H_2O_2 kill curve for ARPE-19 cells. After cells were grown to 100% confluence in growth medium (DMEM:F12 with 10% FBS), the medium was removed and MEM was applied. After 15 h, various concentrations of H_2O_2 were added to the wells, and the plates were incubated for another 8 h. Cell viability was determined by an MTT assay, and is reported as the average of triplicate wells ±1 standard deviation for each concentration of H_2O_2 applied.

From these cytoprotection studies, several points can be highlighted. First, despite the indication that **8a** and **8b** may have improved membrane permeability from the results of the solubility and diffusion cell studies, they did not perform as well as **8c. 8c** may provide better protection to cells than **8a** or **8b** because it remains intact the longest— its carboxylate esters are the slowest to hydrolyze—in MEM, which facilitates its entry into cells. Second, **8c** has enhanced membrane permeability compared to HBED. Presumably, 44 μM of **8c** was converted by hydrolysis and oxidation to 44 μM HBED. If **8c** and HBED have similar membrane permeability, **8c** would have provided protection similar to that of HBED, or even less protection because the prodrugs are not able to instantly protect the cells upon exposure to H_2O_2: they first must be oxidized. Therefore, the superior protection by **8c** compared with HBED further supports the superior membrane permeability of **8c**. Indeed, **8a** (as the free base **7a**), the methyl ester double prodrug, is much more lipophilic than HBED, so **8c**, as the isopropyl ester double prodrug, is even more lipophilic: partition coefficients increase monolithically with the sequential addition of CH_2 groups.[13] The third point that can be made is that the primary mode of protection of **8c** is not consumption of H_2O_2 during its unmasking. The oxidation of 44 μM of **8c** (presumably present as **9** after the 15 h pretreatment period) by H_2O_2 will consume a total of 88 μM of H_2O_2, leaving 412 μM of the 500 μM dose of H_2O_2 to challenge the cells. According to Figure 1, exposure to a similar amount of H_2O_2 (417 μM) decreased cell viability by ~71%. But in the presence of 44 μM of **8c**, cell viability was only decreased by 22% (not shown). Therefore, while cytoprotection by **8c** may have been slightly improved by the consumption of H_2O_2, it is more likely that

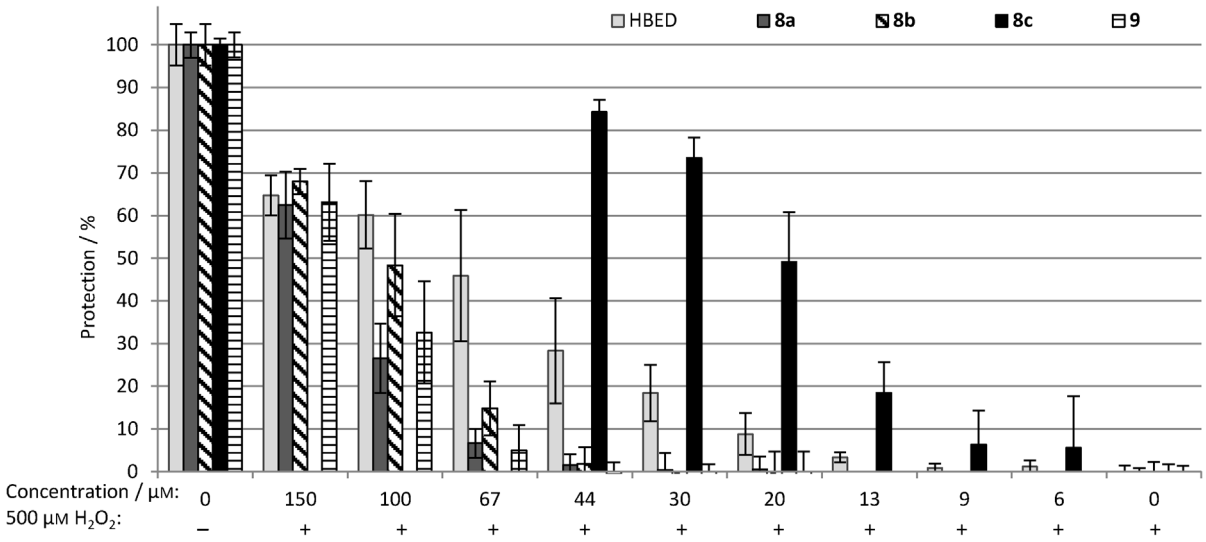


Figure 2. Protection of ARPE-19 cells against 500 μM H_2O_2 by double prodrugs **8a**–**c**, prodrug **9**, and HBED. After cells were grown to 100% confluence in growth medium (DMEM:F12 with 10% FBS), the medium was removed and the cells were pretreated with various concentrations of prodrug or HBED in MEM for 15 h. This was followed by an 8 h challenge with 500 μM H_2O_2. Percent protection was calculated from the results of an MTT cell viability assay and is reported as the mean ±1 standard deviation ($n=6$) for each concentration of drug applied.

the primary mechanism of protection by **8c** against H_2O_2-induced cell death is from chelation of free iron upon the activation of **8c** to HBED.

In summary, we have shown here that selected double prodrugs of HBED can be activated under physiologically relevant conditions in vitro to give HBED, the parent iron chelator, and can ultimately provide cells with protection against oxidative stress promoted by the application of exogenous H_2O_2. The studies suggest that at least one double prodrug, **8c**, has improved membrane permeability and cytoprotective capacity over HBED. Therefore, double prodrugs of this kind may prove useful in improving the oral bioavailability of HBED and its delivery to specific tissues where an iron chelator is needed to curtail Fenton chemistry. This has implications for the treatment of a broad range of diseases that are promoted by oxidative stress.

Supporting Information

The Supporting Information for this article contains the complete Experimental Section for the work described, which includes synthesis, solubility determination, activation studies, diffusion cell studies, and biological assay protocols.

Abbreviations

HBED (*N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid); MEM (minimum essential medium).

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Keywords: boronic acids · chelation therapy · hydrogen peroxide · iron · prodrugs

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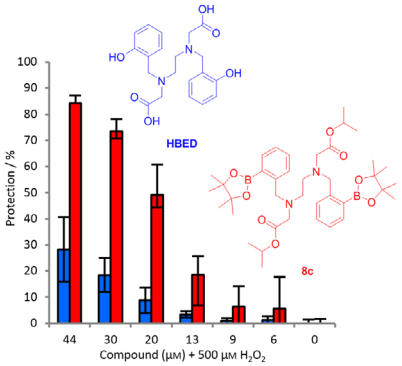
Double your prodrug, double your fun! *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED), an iron chelator, has poor oral bioavailability that limits its clinical use. Here, double prodrugs of HBED that are activated by hydrolysis and oxidation were studied. Solubility, diffusivity, and cytoprotection studies all suggest that the double prodrugs may have improved membrane permeability over HBED that could lead to better delivery in vivo.



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