Reactive Oxygen Species

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Hydrogen Peroxide Induced Activation of Gene Expression in Mammalian Cells using Boronate Estrone Derivatives**

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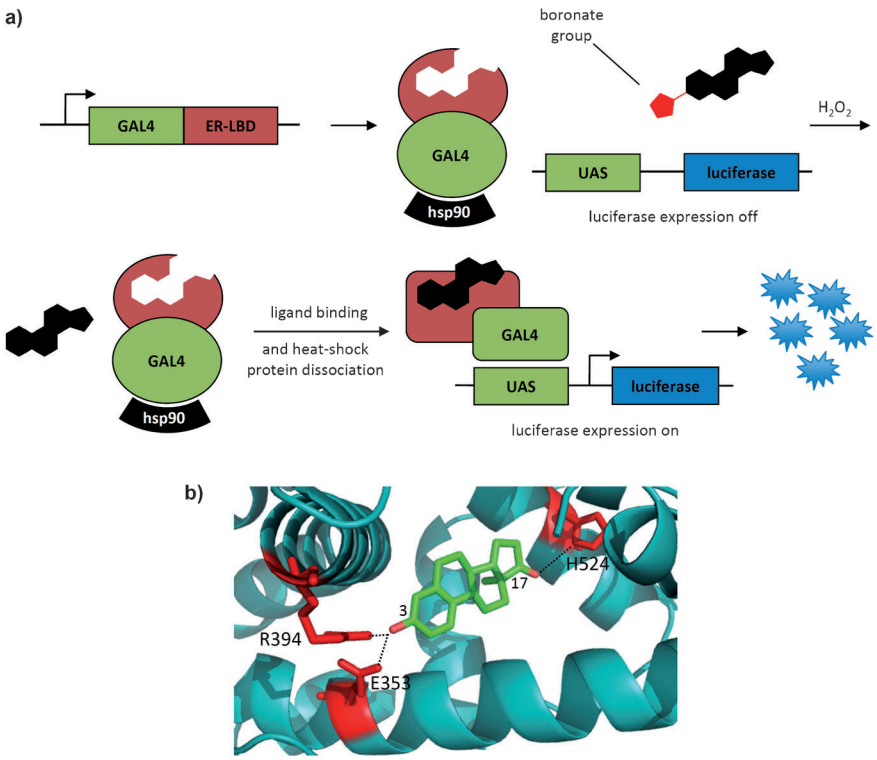
Hydrogen peroxide (H_2O_2) plays important roles in biological and cellular processes. H_2O_2 is a reactive oxygen species (ROS) that was originally thought of as only an oxidative stress marker in diseases,[1] but recently has been shown to be an important secondary messenger in biological systems.[2] H_2O_2 is involved in several biological processes, including cell signaling,[3] embryogenesis,[4] apoptosis,[5] aging, and diseases, such as cancer[6] and neurodegenerative diseases.[7] Herein, we describe the development of a genetic switch that enables the induction of gene expression in response to H_2O_2. Because of the modularity of the system, any gene of interest can be placed under the control of H_2O_2. Importantly, this genetic switch allows for the sensitive and selective detection of H_2O_2 in live mammalian cells.

The H_2O_2-triggered genetic switch relies on a GAL4-UAS (upstream activating sequence) system, in which the DNA-binding domain of GAL4 is fused to an α-estrogen-receptor ligand-binding domain (ER). In the absence of a suitable ER-ligand (e.g., estrone), the GAL4-ER fusion protein is tightly bound to a complex of heat-shock chaperone proteins (e.g., hsp90) that keeps the ER in an inactive state.[8] Upon ligand binding, the GAL4-ER undergoes a conformational change that displaces the hsp90 complex.[9] This active GAL4-ER-ligand complex translocates into the nucleus,[10] binds to the UAS located upstream of the gene of interest, in this case of luciferase, and induces transcription (Scheme 1a). The GAL4-UAS system was changed into a H_2O_2-responsive system by taking advantage of a boron-oxidation reaction.[11] Boronated small molecules have previously been applied in the fluorescent detection of H_2O_2,[12,13] but not in the activation of gene expression. Based on the results of structure activity relationship studies[14–16] and X-ray structures (Scheme 1b),[17,18] a boronate ester group was introduced at either the 3-hydroxy or the 17-carbonyl position (or both) of estrone, to inhibit binding to the ER. In the presence of H_2O_2, the boronate group will be oxidized, resulting in the native phenol or ketone, and thus activating gene expression through ER binding.



Scheme 1. a) Activation of gene expression by H_2O_2 through the oxidation of boronate estrone derivatives. b) X-ray structure of estrone (green) bound to ERα. PDB: 3M1.

A series of boronated estrones were synthesized and investigated as H_2O_2-responsive molecules (Scheme 2). It was previously shown that removal of the 17-carbonyl group reduced the relative binding affinity of estrone by 84%,[14] and that removal of the 3-hydroxy group reduced the relative binding interaction by two orders of magnitude.[15] Based on an X-ray structure of the ER (Scheme 1b), the 17-carbonyl group of estrone interacts with His^{524}, which then forms a hydrogen bond with the peptidic carbonyl group of Glu^{419}, creating a hydrogen-bonding cascade.[17] The 3-hydroxy group

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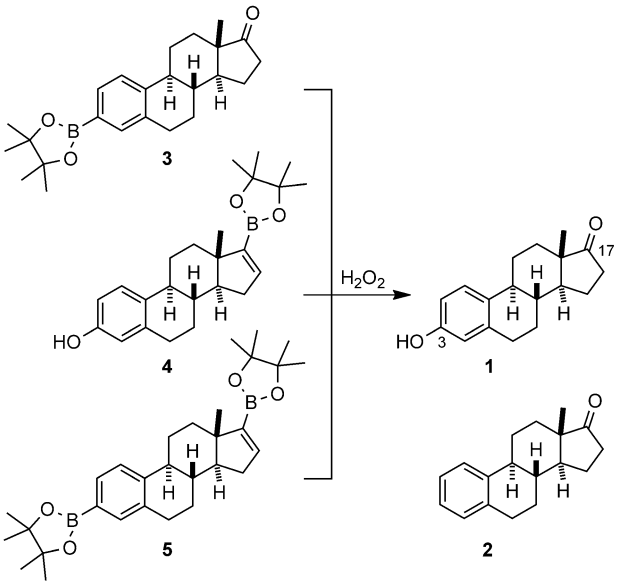
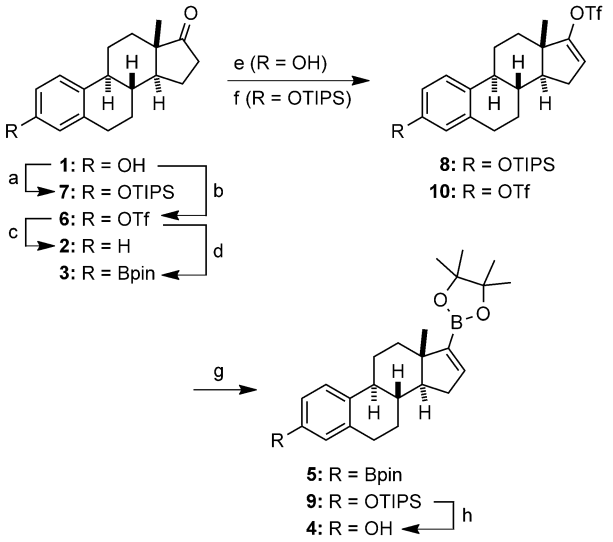


Scheme 2. Structures of boronated estrone derivatives **3**–**5** and their oxidation to estrone (**1**) by hydrogen peroxide. Dehydroxy estrone (**2**) served as a negative control.

interacts through hydrogen bonds with Glu^{353}, Arg^{394}, and a water molecule within the binding pocket.[18] Therefore, the replacement of either one of these groups with a phenyl or vinyl boronic acid ester derivative should render the estrone molecule biologically inactive.[14,15] If the boronation of either the 3-position (**3**), or 17-position (**4**), or both (**5**) completely inactivates the binding of the estrone derivative to the ER, then estrone activity should be restored through H_2O_2-mediated oxidation to a functional estrone molecule. In addition, the dehydroxy estrone **2** was used as a negative control compound.

The dehydroxy estrone **2** was prepared in two steps starting from estrone (**1**). The phenolic hydroxy group was transformed into a triflate using trifluoromethanesulfonic anhydride in the presence of triethylamine, yielding **6** in 88%.[19] The triflate was subsequently removed in a Pd-$(OAc)_2$-catalyzed reduction to give **2** in 67% yield (Scheme 3).[20] Pinacolborane was introduced using Suzuki-coupling conditions in the presence of $Pd(dppf)Cl_2$ (dppf = 1,1′-bis(diphenylphosphino)ferrocene) to give the boronic acid ester **3** from the common triflate intermediate **6** in good yield.[21] Synthesis of the boronate estrone **4** was completed in four steps starting with triisopropylsilyl (TIPS) ether protection of the phenolic hydroxy group in **1**, giving **7** in quantitative yield.[22] The carbonyl group in **7** was then converted into the enol triflate **8** using trifluoromethanesulfonic anhydride in the presence of 2,6-lutidine. The triflate **8** was then subjected to a Suzuki coupling reaction yielding the TIPS-protected boronate estrone **9**. Removal of the TIPS group with tetra-*n*-butylammonium fluoride (TBAF) produced the boronate estrone **4** in 77% yield (Scheme 3).[22] The diboronate estrone **5** was assembled in a similar manner as the boronate estrone **4**. Using 4-dimethylaminopyridine (DMAP) as the base in the triflate-forming step lead to a readily separable mixture of the estrone triflate and the estrone ditriflate **10** in 57% yield.[23] A palladium-mediated Suzuki



Scheme 3. Synthesis of the estrone derivatives **2**–**5**. a) TIPSCl, imidazole, dimethylformamide (DMF), quantitative yield. b) Tf_2O, triethylamine (TEA), CH_2Cl_2, 88%. c) $Pd(OAc)_2$, dppf, TEA, HCO_2H, DMF, 60°C, 67%. d) Pinacolborane, $Pd(dppf)Cl_2/CH_2Cl_2$, TEA, dioxane, 90°C, 71%. e) Tf_2O, DMAP, CH_2Cl_2, 57%. f) Tf_2O, 2,6-lutidine, DCM, 69%. g) Pinacolborane, $Pd(dppf)Cl_2/CH_2Cl_2$, TEA, dioxane, 90°C, 46–48%. h) TBAF, tetrahydrofuran (THF), 77%. pin = pinacol; Tf = triflate.

coupling reaction with the ditriflate **10** and pinacolborane led to the diboronated estrone **5** in 46% yield (Scheme 3).

To investigate the activity of estrone and its boronate derivatives, human epithelial carcinoma (A431) cells were transfected with a plasmid expressing GAL4-ER (pBind-ERα) and a plasmid with a UAS-driven luciferase gene (pGL4.35; see Experimental Section). Following transfection, the cells were treated for 2 h with estrone (**1**) or estrone derivatives **2**–**5** at a concentration of 50 nM. H_2O_2 (100 nM) was added to the cells and luciferase expression was assayed after 48 hours (Figure 1). Treatment with estrone (**1**) resulted in a 43-fold increase in firefly luciferase expression relative to

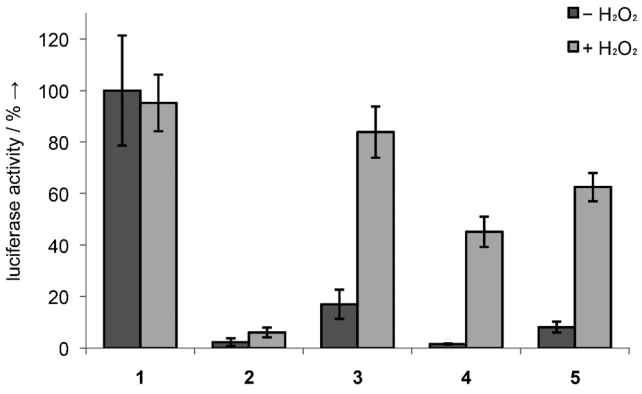


Figure 1. Hydrogen peroxide-induced activation of gene expression in the presence of boronated estrone analogues. A431 cells (10000) were transfected with pBind-ERα and pGL4.35, treated with estrone derivatives **2**–**5** (50 nM), followed by exposure to H_2O_2 (100 nM). A luciferase assay was conducted and reporter gene activity was normalized to exposure to estrone (**1**). All experiments were performed in triplicate and error bars represent the standard deviation.

untreated cells. The dehydroxy estrone **2** was used as a negative control and indeed showed only a basal level of firefly luciferase expression. Moreover, luciferase induction by both estrone (**1**) and dehydroxy estrone (**2**) was not significantly affected by addition of H_2O_2.

The estrone derivative **3**, with a boronic acid ester at the 3-position, showed a slightly higher background level of luciferase expression than the negative control **2**, but is still mostly inactive compared to estrone (**1**). Upon addition of H_2O_2, the boronate group of **3** is oxidized and the resulting estrone is able to bind to the ER inducing a fivefold increase in luciferase expression, a level that approximates that of treatment with **1**. The estrone boronated at the 17-position (**4**), displayed a lower background level of luciferase expression than **3** and exposure to H_2O_2 resulted in a 28-fold increase in gene expression. Even though the level of luciferase expression was only 48% of the natural estrone (**1**), the signal-to-background ratio was excellent. Similar results were found with the diboronated estrone **5**, which displayed a low background level of luciferase activity before exposure to H_2O_2 and an eightfold increase in gene expression after H_2O_2 addition.

The ability of the sensor to detect endogenously produced H_2O_2 in mammalian cells was tested. Cells produce H_2O_2 when stimulated with external cytokines such as transforming growth factor-β1, interleukin-1, or epidermal growth factor (EGF).[24] Here, EGF was used to stimulate H_2O_2 production in A431 cells. These assays were conducted as described above but instead of adding H_2O_2 to the media, the cells were treated with EGF (1 μg mL^{-1}). As seen in Figure 2, the intracellular generation of H_2O_2 can be detected as efficiently as when it is added externally. In comparison to the positive and negative controls (**1** and **2**, respectively), the boronate estrone **3** showed only a moderate level of background luciferase expression before addition of EGF and a fivefold increase in luciferase after EGF addition. Importantly, the boronate estrone **4** and the diboronate estrone **5** showed further reduced levels of background activity, and addition of EGF resulted in a dramatic 33-fold increase in luciferase activity for the boronate estrone **4** and a fivefold increase for the diboronate estrone **5**. The lower activation from **5** is presumably the result of an incomplete conversion into **1** by intracellularly generated H_2O_2. Further increase in the concentration of EGF added to the cell culture media led to a linear increase in luciferase signal, because cellular H_2O_2 production increases with increasing EGF exposure[25] (see Supporting Information).

Thus, the developed H_2O_2 reporter provides a substantially greater dynamic range than previously reported H_2O_2 sensors. The higher signal-to-background ratio (up to 33-fold) of this system may be the result of two linked catalytic processes: gene transcription induced by H_2O_2 and subsequent bioluminescence through conversion of luciferin into oxyluciferin catalyzed by luciferase. Intracellular detection of H_2O_2 through fluorescence measurements have been reported using boronate fluorophores[13] and by a genetically encoded protein that emits fluorescence when oxidized by H_2O_2.[26,27] However, only two- to sixfold changes in fluorescence were measured with these systems. Importantly, since any coding or non-coding genetic sequence can be cloned downstream of the UAS, the developed system reported herein can also be used as a transcriptional switch for the activation of any gene of interest by H_2O_2.

One challenge in creating a cellular H_2O_2 reporter is to ensure that it is sensitive and selective to H_2O_2 over other competing cellular ROS, such as hydroxyl radicals and hypochlorite ions.[27,28] In this regard, the selectivity of the gene activation system was tested in cell culture by treatment with several ROS: H_2O_2, *tert*-butyl hydroperoxide (TBHP), hypochlorite (OCl^-), hydroxyl radical (•OH, generated from H_2O_2 and $FeSO_4$), and *tert*-butoxy radical (•O*t*Bu, generated from TBHP and $FeSO_4$). The boronate estrone **3** was used because it showed the highest recovery of gene expression after addition of H_2O_2, relative to native estrone (see Figure 1). No reporter gene expression was detected in response to any ROS except H_2O_2 (Figure 3). Following addition of H_2O_2 to cells treated with the boronate estrone **3**,

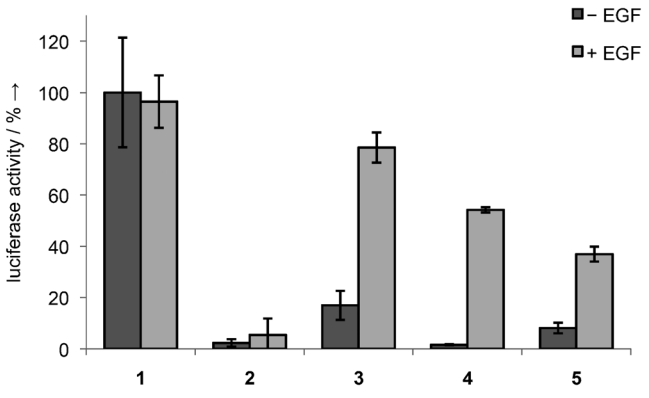


Figure 2. Intracellular detection of hydrogen peroxide. A431 cells (10000) were transfected with pBind-ERα and pGL4.35, and treated with estrone analogues **2**–**5** (50 nM) and EGF (1 μg mL^{-1}). A firefly luciferase assay was performed and reporter gene activity was normalized to exposure to estrone (**1**). All experiments were performed in triplicate and error bars represent the standard deviation.

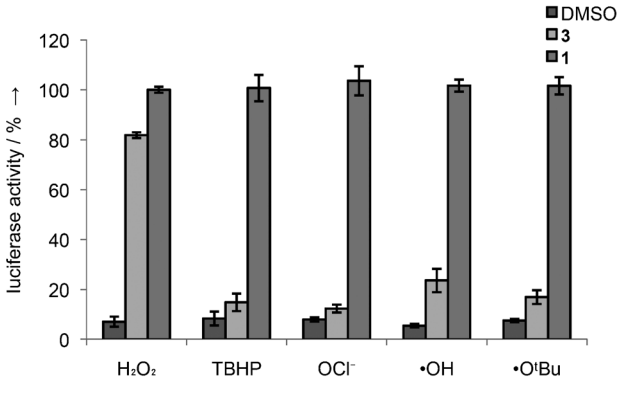


Figure 3. The boronated estrone derivative **3** is selective for H_2O_2. A431 cells (10000) were transfected with pBind-ERα and pGL4.35, treated with estrone (**1**, 50 nM), boronate estrone **3** (50 nM), or DMSO only, then exposed to the ROS (100 nM) shown under the graph, and a luciferase assay was performed. Reporter gene activity is normalized to treatment with estrone (**1**). All experiments were performed in triplicate and error bars represent the standard deviation.

a luciferase response almost identical to that of native-estrone-induced levels was detected. However, exposure to TBHP, OCl^-, ·OH, or ·O*t*Bu, instead of H_2O_2, only resulted in background levels of gene expression. To confirm that these results are the consequence of a highly selective oxidation of the boronate estrone **3**, *in vitro* oxidation reactions were analyzed by GC (see Supporting Information, Figure S5). Furthermore, a modified cell-based assay was performed where the estrone and boronate estrone **3** were incubated with the ROS reagents prior to addition to the cells. In this assay, if the ROS oxidizes **3**, it should do so before being introduced into the cell, regardless of its lifetime. Confirming our previous results, selective activation of luciferase activity was detected exclusively in the presence of H_2O_2 and no other ROS reagent (see Supporting Information, Figure S6). Together, these results indicate that the estrone derivative **3**, in conjunction with a genetically encoded reporter, is highly specific for H_2O_2 and can differentiate it from other ROS with an exceptionally high signal-to-background ratio.

In summary, we have developed a genetically encoded gene activation system that selectively responds to H_2O_2. This method can be used for the activation of any gene of interest. A central component of this system is a novel boronate estrone "cofactor" that is cell permeable but inactive until oxidized by H_2O_2. The oxidation step converts the inactive boronate estrone into estrone, which induces transcriptional activation of the gene of interest, for example, a luciferase reporter gene. The sensor was able to detect H_2O_2 that was either added to the cellular medium or generated endogenously through growth factor-induced cellular H_2O_2 production. Importantly, the system is highly specific for H_2O_2 and is not activated by any other reactive oxygen species. In contrast to previously reported intracellular H_2O_2 sensors, this system displays a substantially larger dynamic range of output signal. Moreover, it is conceivable that this system could be adapted to other orthogonal, ligand-induced transcription factors to activate genes of interest in response to an H_2O_2 stimulus. For example, in addition to transcriptional activators, fusion proteins of the ER have been used in the conditional control of Cre recombinase,[29] the I-Sec1 restriction enzyme,[30] Flpe recombinase,[10] and interferon regulatory factor-3.[31] Thus, these proteins and others could also be regulated by intracellular H_2O_2 levels using boronate estrone derivatives.

Experimental Section

Estrone-induced gene expression in mammalian cells: A431 human epithelial carcinoma cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Hyclone), supplemented with 10% fetal bovine serum (Hyclone) and 10% streptomycin/penicillin (MP Biomedicals). Cells were passaged into a 96-well plate (200 μL per well, 10000 cells per well) and transfected with pBind-ERα (0.15 μg, Promega) and pGL4.35 (0.15 μg, Promega) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. All transfections were performed in triplicate. After a 16 h incubation, the medium was replaced with DMEM growth media containing the estrone derivatives. The cells were then treated with H_2O_2 (100 nM) or EGF (1 μg mL^{-1}) and incubated for 48 h at 37 °C and 5% CO_2. Luciferase expression was determined with a Bright Glo-Luciferase Reporter Assay system (Promega) using a Biotek Synergy 4 microplate reader. For each of the triplicates, the data were averaged and standard deviations were calculated.

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