Enzyme Activatable Photodynamic Therapy Agent Targeting Melanoma

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1. General Methods

All reagents and solvents were purchased from commercial sources and used without further purification. Column chromatography was carried out using silica stationary phase (230–400 mesh, SiliCycle Inc., Canada). Analytical thin layer chromatography was performed on 0.25 mm thick precoated silica gel plates (60F254, Merck, Germany). All $^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker instrument (400 MHz) at Necmettin Erbakan University, Science and Technology Research Center (BITAM). Chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak. Coupling constants (J) are reported in hertz (Hz). Standard abbreviations indicating multiplicities are given: d = doublet, m = multiplet, s = singlet, t = triplet. High-resolution mass spectrometry (HRMS) was carried out using Agilent 6530 Accurate-Mass Q-TOF LC/MS of the Eastern Anatolia Advanced Technology Research and Application Centre (DAYTAM, Erzurum, Turkey). For cell culture experiments human breast adenocarcinoma cell line (MCF7), Human hepatocellular carcinoma (HEP3B) and mouse melanoma cell line B16-F10 (ATCC) were used. Cells were visualized with Zeiss Fluorescence Microscopy. Cell culture experiments were performed at Research and Development Centre for Diagnostic Kits (KİT-ARGEM). For UV-Vis absorbance and fluorescence analysis of BOD1 and BOD2 solutions, Cary Eclipse 60 Agilent spectrophotometers were used. For cell viability analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) absorbance values are recorded using Quant ELISA Plate Reader. For singlet oxygen generation and photodynamic activity measurements in the cell, light emitting diode (LED) light source with a maximum peak at 506 nm was used (Bright Light Electronics Corp. BL-BG43V4V). Tyrosinase (Mushroom, 1000 U/mg, Enzyme Commission Number:1.14.18.1) and esterase (Porcine Liver, 15 U/mg, Enzyme Commission Number: 3.1.1.1) enzymes were obtained from Sigma-Aldrich.
2. Additional Figures

**Figure S1.** Normalized UV-Vis absorbance spectra of **BOD1** (orange) and **BOD2** (blue) in DMSO.

**Figure S2.** Change in the UV-Vis absorption spectra of DPBF (50 μM) in DMSO. Sample was kept in dark for 8 min, then irradiated with 506 nm light for 22 min.
**Figure S3.** Change in UV-Vis absorption spectra of DPBF (50 μM) in the presence of **BOD1** (0.5 μM) in DMSO. Sample was kept in dark in for 8 min, then irradiated with 506 nm light for 22 min.

**Figure S4.** Change in the UV-Vis absorption spectra of DPBF (50 μM) in the presence of **BOD2** (0.5 μM) in DMSO. Sample was kept in dark for 8 min, then irradiated with 506 nm light for 22 min.
**Figure S5.** Change in the UV-Vis Absorbance value of DPBF at 411 nm upon irradiation with 506 nm LED light in the presence of either 0.5 μM RB (orange), BOD1 (gray) or BOD2 (blue) in acetonitrile. Slope of the curves are used to calculate $\phi_\Delta$ using Formula 1.

**Figure S6.** UV-Vis Absorbance (top, left) and fluorescence spectra (top, right) of BOD2 (50 μM) in high glucose DMEM culture medium before and after incubation with tyrosinase (100 U/ml) and esterase (100 U/ml) for 3 h. Fluorescence spectra of BOD1 (50 μM) in the same medium was recorded for comparison (bottom). Spectra was obtained by exciting at 510 nm.
In the fluorescence spectra of enzyme treated BOD2, t0 and t3 correspond to spectrum immediately after and 3 h after enzyme addition, respectively.

**Figure S7.** Structures of kojic acid (tyrosinase inhibitor) and benzil (esterase inhibitor).

![Structures of kojic acid and benzil](image)

**Figure S8.** B16-F10 cell viabilities in the presence of varying concentrations of BOD2. Cells are either incubated in the dark for 24 h (blue) or first irradiated with 506 nm light for 4 h and then incubated for 20 h in the dark (orange). Average of three independent experiments were used. IC\(_{50}\) value was calculated from the equation of linear fit plot. IC\(_{50}\) value was calculated to be 20 \(\mu\)M for irradiated cells. Compound is not toxic in the dark for B16-F10 cells at the application dose, which is less than 8 \(\mu\)M.
**Figure S9.** Viability of B16-F10 cells in the presence of various concentrations of BOD1 (0-32 μM). Cells are either exposed to light for 4 h and then kept in dark for 20 h (orange) or kept in dark for the entire 24 h (blue).

**Figure S10.** B16-F10 cell viabilities in the presence of varying concentrations of BOD1. Cells are either incubated in the dark for 24 h (blue) or first irradiated with 506 nm light for 4 h and then incubated for 20 h in the dark (orange). Average of at least three independent experiments were used. IC50 value was calculated from the equation of linear fit plot. IC50 value was calculated to be 15 μM for irradiated cells and 59 μM for the cells kept in dark.
3. Synthesis and Other Experimental Procedures:

Singlet Oxygen Generation Experiments

Singlet oxygen trap molecule 1,3-diphenyl isobenzofurane (DPBF) is used to compare relative singlet oxygen generations by BOD1 and BOD2. Cary Eclipse 60 Agilent spectrophotometer was used to follow the change in the absorbance of this compound upon $^{1}$O$_{2}$ production. All the measurements were performed in dimethyl sulfoxide (DMSO). To check the stability of DPBF, 50 µM DPBF was prepared and tested under the same experimental conditions. Solution was kept in dark for 8 min and irradiated with 506 nm LED light for 22 min (Bright Light Electronics Corp. BL-BG43V4V, power density 6.4 mW.cm$^{-2}$) from 20 cm distance. Absorbance was recorded for every 2 min. The same experiment was repeated with DPBF solution in the presence of BOD1 or BOD2 (0.5 µM each). Decrease in the absorbance values at 418 nm were compared.

Singlet oxygen quantum yields were separately determined in acetonitrile using the Formula 1 taking Rose Bengal (RB) as reference compound ($\phi_{\Delta}$ is 0.53 in acetonitrile). 50 µM DPBF in the presence of 0.5 µM of RB, BOD1 or BOD2 were irradiated with 506 nm light from 20 cm distance. Absorbance spectra were recorded at 2 min intervals. Change in the absorbance at 411 nm was plotted against time and the slope of the linear fit plot is used in calculations (S).

\[
\phi_{\Delta} = \phi_{\Delta}^{r} \left( \frac{\alpha^{s}}{\alpha^{r}} \right) \left( \frac{S}{S^{r}} \right)
\]

\[
\alpha = 1 - 10^{-A(506 \text{ nm})}
\]

where $\phi_{\Delta}^{s}$ and $\phi_{\Delta}^{r}$ refer to singlet oxygen quantum yields of sample and reference Rose Bengal respectively, $\alpha$ is the absorption correction factor calculated from the absorbances at 506 nm (Formula 2). $S'$ and $S^r$ correspond to slopes of absorbance change vs time graph of reference compound and sample respectively, as shown in Figure S5.

Spectroscopic Analysis with Enzymes

BOD2 (50 µM) solution in high glucose DMEM culture medium was prepared (pH 6.5). UV-Vis Absorbance and Fluorescence spectra are recorded. Fluorescence spectra were obtained by exciting the sample at 510 nm. Tyrosinase (Mushroom, Sigma, 100 U/ml) and esterase (Porcine
Liver, Sigma, 100 U/ml) were added, and fluorescence spectrum was recorded immediately after addition. Then sample was incubated at 37 °C for 3 h, and spectral analysis was done.

**Cell Culture Experiments**

Three different cell lines were used for experiments. B16-F10 (ATCC) mouse melanoma cells are used as target tyrosinase expressing cells. MCF7 mammary cancer cells and HEP3B hepatocellular carcinoma cell line were also used as control cells. HEP3B, B16-F10 cells and MCF7 cells were grown in Eagle's minimal essential medium (EMEM), high glucose Dulbecco's Modified Eagle Medium (DMEM) medium and Roswell Park Memorial Institute 1640 medium (RPMI 1640) medium respectively, supplied with 10% Fetal Bovine Serum (FBS) and 1% gentamycin at 37 °C, under 5 % CO₂ in a humidified incubator. For fluorescence microscopic analysis of cell death, cells (17x10⁴) were seeded in 6-well plate with 3 mL culture medium and incubated for 48 h. 0.5 μM **BOD2** is introduced to cells, and cells were either incubated in the dark for 24 h or first irradiated with 506 nm LED array light having 6.4 mW.cm⁻² power density from approximately 12 cm, then incubated in dark for 20 h. Then, medium is discarded, and cells are washed with Phosphate Buffer Saline (PBS) twice. 3 mL fresh medium was added and propidium iodide solution is added to each well according to suppliers’ recommendation (Acros Organics). Cells were incubated in the dark for 20 min. Medium was removed, cells were washed with PBS twice and cells were fixed with 4% paraformaldehyde. Cells were visualized with Zeiss Fluorescence Microscopy. For experiments with inhibitor, kojic acid was applied to cell medium (2 mg/mL) 1 h prior to **BOD2** addition.

For cell viability analysis, B16-F10 cells (7x10³) were seeded in 96-well plate with 0.2 mL medium and incubated for 48 h at 37 °C under 5% CO₂. **BOD1** or **BOD2** stock solution was prepared in dimethyl sulfoxide, then applied to cells at varying concentrations. Cells were either kept in dark for 24 h, or first irradiated with 506 nm LED light for 4 h and then incubated
for 20 additional hours in the dark. 10 µL MTT solution was added (from the stock solution of 5 mg/mL) and cells were incubated for 4 h in the dark. Medium was removed gently, and formazan crystals were dissolved in 100 µL DMSO. After 15 min incubation in the dark, absorbance at 570 nm was recorded using Quant ELISA plate reader. Relative viabilities were calculated using untreated control cell group as reference. The half maximal inhibitory concentration (IC₅₀) values were calculated using the linear fit equation of the percent viability / concentration graph. For MTT analysis with inhibitors, kojic acid and/or benzil (each 2 mg/mL) were applied to cell medium 2 h prior to BOD2 (10 µM) addition (Figure S7). Three replicates of the experiment were used for the analysis of MTT. For inhibitor analysis two replicates were used.

**Synthesis**

Synthesis was performed using the reactions shown in Scheme S1. Molecule 2 is synthesised using the literature procedure. Details of the synthesis are given below.

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**Scheme S1**

1. **i**
2. **ii**
3. **iii**
4. **iv**
5. **v**

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**Molecule 2**

**Molecule 3**

**BOD1**

**BOD2**
Scheme S1. Synthesis of BOD1 and BOD2. Reaction conditions: i) Et$_3$N, CH$_3$COCl, CH$_2$Cl$_2$; ii) PPh$_3$, NBS, DMF; iii) a. 2,4-dimethyl pyrrole, N$_2$, TFA, CH$_2$Cl$_2$; b) p-chloranil; c) Et$_3$N, BF$_3$OEt$_2$; v) I$_2$, HIO$_3$, EtOH, 60 °C; vi) anhydrous MeCN, 80 °C.

**Synthesis Compound 1:** Solution of 3-hydroxy benzyl alcohol (1.24 g, 10 mmol) in 30 mL dichloromethane was cooled with ice bath. Triethyl amine (Et$_3$N, 1.38 mL, 9 mmol) was added. Then, acetyl chloride (720 μL, 10 mmol) was added slowly to the reaction mixture. Reaction was stirred for 16h at room temperature, extracted with dichloromethane and water. Organic layer was collected, dried over sodium sulphate. Solvent was evaporated under reduced vacuo. Compound was purified with silica column chromatography using 25 % ethyl acetate in hexane as mobile phase (colorless liquid, 81%).

$^1$H NMR (400 MHz, Chloroform-$$d$$) δ 7.33 (t, J = 7.9 Hz, 1H), 7.17 (ddd, J = 7.6, 1.7, 0.9 Hz, 1H), 7.07 (dt, J = 1.7, 1.0 Hz, 1H), 7.01 – 6.93 (m, 1H), 4.61 (s, 2H), 2.50 (b, 1H), 2.28 (s, 3H).

$^{13}$C NMR (101 MHz, Chloroform-$$d$$) δ 169.97, 151.01, 143.09, 129.72, 124.41, 120.75, 120.17, 64.69, 21.32.

**Synthesis Compound 2:** Compound 1 (1.33 g, 8.0 mmol) was dissolved in dimethyl formamide (DMF) and dichloromethane (6 mL, 34 mL) and cooled with an ice bath. N-Bromo succinimide (NBS, 1.85 g, 10.4 mmol) was added. Then, triphenyl phosphine (PPh$_3$, 2.72 g, 10 mmol) was added slowly. Reaction was stirred for 30 min - 1 h and followed by thin layer chromatography (TLC) until all starting material is consumed. Then, the crude sample was extracted with dichloromethane and water. Organic layer was collected, dried over sodium sulfate. Solvent was evaporated under reduced vacuo. Compound was purified with silica column chromatography using 25% ethyl acetate in hexane as mobile phase (colourless liquid, yield 63%).
$^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.34 (t, $J = 7.9$ Hz, 1H), 7.27 – 7.21 (m, 1H), 7.13 (d, $J = 2.2$ Hz, 1H), 7.06 – 6.98 (m, 1H), 4.46 (s, 2H), 2.29 (s, 3H). $^{13}$C NMR (101 MHz, Chloroform-$d$) $\delta$ 169.61, 151.02, 139.24, 129.96, 126.14, 121.99, 121.85, 45.71, 21.36. Theoretical m/z value for (M+Na)$^+$ is 250.9684 and experimental value is 250.18276.

**Synthesis of BOD1.** Compound 3 (250 mg, 0.77 mmol), which was synthesised according to literature, was dissolved in 50 mL ethanol (EtOH). 2.2 equiv of I$_2$ (429 mg, 1.69 mmol) was added and immediately after iodic acid solution was added dropwise (297 mg, 1.69 mmol, in 0.4 mL H$_2$O). Reaction was heated to 60 °C and the progress of reaction was followed with thin layer chromatography using hexane-ethyl acetate mobile phase (2:1, by volume). When all the starting material was consumed, reaction was cooled to room temperature, quenched with 10 mL saturated sodium thiosulfate solution in water. Ethanol is concentrated under reduced vacuo and then extracted with dichloromethane and brine. Red solid product was obtained with 52 % yield.

$^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.82 (d, $J = 5.9$ Hz, 2H), 7.28 (d, $J = 6.0$ Hz, 2H), 2.64 (s, 6H), 1.42 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 158.06, 151.18, 145.06, 143.54, 137.35, 130.44, 123.27, 86.60, 17.49, 16.39. MS (ESI): m/z theoretical 577.9573 (M+H)$^+$; experimental value 577.95765 ($\Delta$: 0.61 ppm).

**Synthesis of BOD2.** BOD2 (50 mg, 90 µmol) was dissolved in 5 mL anhydrous acetonitrile (MeCN). Compound 2 (60 mg, 0.26 mmol) was added and the reaction was stirred at 80 °C for 16 h. Solvent was concentrated under vacuo and a few mL of petroleum ether was added to precipitate the product. Precipitate was filtered and further washed with petroleum ether and dichloromethane solvent mixture (1:1 by volume). Purple solid product was obtained with 43 % yield.
$^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.77 (d, $J = 6.1$ Hz, 2H), 7.96 (d, $J = 5.9$ Hz, 2H), 7.56 (d, $J = 8.0$ Hz, 1H), 7.49 – 7.32 (m, 2H), 7.16 (d, $J = 8.3$ Hz, 1H), 6.54 (s, 2H), 2.64 (s, 6H), 2.29 (s, 3H), 1.41 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 169.42, 159.97, 153.19, 151.67, 146.79, 144.41, 134.24, 132.08, 131.30, 129.38, 128.34, 127.16, 123.91, 123.14, 87.93, 64.46, 29.93, 21.37, 18.70. MS (ESI): m/z theoretical value: 726.0097 (M)$^+$; experimental value 726.00858, ($\Delta$: 1.54 ppm).

4. NMR and HRMS Spectra

![Figure S6. $^1$H NMR spectrum of compound 1 (400 MHz, CDCl$_3$).](image-url)
Figure S7. $^{13}$C NMR spectrum of compound 1 (101 MHz, CDCl$_3$).

Figure S8. $^1$H NMR spectrum of compound 2 (400 MHz, CDCl$_3$).
Figure S9. $^{13}$C NMR spectrum of compound 2 (101 MHz, CDCl$_3$).

Figure S10. High Resolution Mass Spectrum (ESI-MS) of compound 2.
Figure S11. $^1$H NMR spectrum of BOD1 (400 MHz, CDCl$_3$).

Figure S12. $^{13}$C NMR spectrum of BOD1 (101 MHz, CDCl$_3$).

Figure S13. High Resolution Mass Spectrum (ESI-MS) of BOD1.
Figure S14. $^1$H NMR spectrum of BOD2 (400 MHz, CDCl$_3$). An expanded partial spectrum is given at the bottom.
**Figure S15.** $^{13}$C NMR spectrum of BOD2 (101 MHz, CDCl$_3$). An expanded partial spectrum is given at the bottom.

**Figure S16.** High Resolution Mass Spectrum (ESI-MS) of BOD2.
5. References
