A Dual-Bioresponsive Drug-Delivery Depot for Combination of Epigenetic Modulation and Immune Checkpoint Blockade

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Patients with advanced melanoma that is of low tumor-associated antigen (TAA) expression often respond poorly to PD-1/PD-L1 blockade therapy. Epigenetic modulators, such as hypomethylation agents (HMAs), can enhance the antitumor immune response by inducing TAA expression. Here, a dual bioresponsive gel depot that can respond to the acidic pH and reactive oxygen species (ROS) within the tumor microenvironment (TME) for codelivery of anti-PD1 antibody (aPD1) and Zebularine (Zeb), an HMA, is engineered. aPD1 is first loaded into pH-sensitive calcium carbonate nanoparticles (CaCO3 NPs), which are then encapsulated in the ROS-responsive hydrogel together with Zeb (Zeb-aPD1-NPs-Gel). It is demonstrated that this combination therapy increases the immunogenicity of cancer cells, and also plays roles in reversing immunosuppressive TME, which contributes to inhibiting the tumor growth and prolonging the survival time of B16F10-melanoma-bearing mice.

Programmed death-1 (PD-1) receptor is expressed on various immune cells, including activated CD8+ T cells. The interaction between PD-1 and its ligand PD-L1/PD-L2 on tumor cells such as melanoma cells can lead to T cell anergy, impeding anticancer immune responses.[1] Therefore, blocking PD-1/PD-L1 pathway by anti-PD-1 (aPD1) or anti-PD-L1 antibodies (aPD-L1) can potentially revert the exhausted T cells and enhance antitumor immune responses in patients with melanoma or other cancers.[2] However, despite the considerable success of PD-1/PD-L1 blockade therapy, utilization of these antibodies as a single therapeutic is often limited to a subset of patients.[3] Several immune evasion mechanisms account for it, including the absence of tumor-associated antigens (TAAs), and infiltration of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs).[4] Epigenetic alteration like DNA hypermethylation plays a pivotal role in immune evasion of cancer cells during tumorigenesis.[5] It is a common feature of heterogeneous cancer phenotypes for the reason that the TAA promoter regions are hypermethylated in various types of cancers.[6] It has been reported that hypomethylating agents (HMAs), also known as DNA methyltransferase inhibitors, could contribute to enhancing the expression of TAAs, which increased tumor immunogenicity and enhanced infiltration of CD8+ T cells.[7] Additionally, DNA methylation is involved in regulating immune responses as well, and HMAs have been demonstrated to regulate immunosuppressive tumor microenvironment (TME) by reducing MDSCs.[8] Previous studies also showed that HMAs also induced expression of immunosuppressive ligands, such as PD-L1/PD-L2, sensitizing tumors to PD-1/PD-L1 checkpoint blockade therapy.[9]
Herein, we described an in situ formed dual-bioresponsive depot to locally codeliver Zebularine (Zeb), a demethylation agent, and aPD1 antibody (Figure 1A). aPD1 was first loaded in the pH-sensitive CaCO\(_3\) nanoparticles (aPD1-NPs) for locally sustained release, and then the aPD1-NPs and Zeb were encapsulated together into the ROS-responsive hydrogel (Zeb-aPD1-NPs-Gel) crosslinked by mixing polyvinyl alcohol (PVA) and N\(^1\)-(4-boronobenzyl)-N\(^3\)-(4-boronophenyl)-N\(^\circ\),N\(^\circ\),N\(^3\),N\(^3\)-tetramethylpropane-1,3-diaminium (TSPBA) linker. This ROS/H\(^+\) dual-sensitive scaffold was engineered to utilize the acidic TME and ROS within tumors for the controlled release and increasing retention time of therapeutics. We expected that Zeb-loaded hydrogel could regulate the expression of TAAs, reverse the immunosuppressive TME by reducing suppressive immune cells, and upregulate PD-L1 expression. Together with the controlled release of aPD1 that blocks PD-L1 interaction with PD-1, the dual responsive depot would elicit strong antitumor immune response.

![Figure 1. Schematic and characterization of injectable in situ formed ROS/H\(^+\) dual bioresponsive gel depots. A) Schematic illustration of the combination strategy of epigenetic modulation and ICB therapy using ROS/H\(^+\) responsive scaffolds. B) Size distribution of aPD1-loaded CaCO\(_3\) NPs measured by dynamic light scattering (DLS) and morphology observation by transmission electronic microscopy (TEM). Scale bar: 100 nm. C) Representative Cyro-SEM image of hydrogel loaded with aPD1-NPs. Scale bar: 500 nm. D) Release profiles of Zeb from hydrogel incubated in PBS buffer (pH 7.4) with or without 1 \(\times 10^{-3}\) M H\(_2\)O\(_2\). E) Release profiles of aPD1 from NPs-loaded gel depot incubated in PB buffer (pH 7.4 or pH 6.5) with/without 1 \(\times 10^{-3}\) M H\(_2\)O\(_2\). The data are presented as mean \(\pm\) standard deviation (SD), n = 3. F) In vivo retention of Cy5.5-labeled aPD1 in different formulations at different days (day 0 (D0), day 2 (D2), day 4 (D4), day 6 (D6)), injected at peritumoral sites in the B16F10-melanoma-bearing mice (G1: free Cy5.5-aPD1; G2: Cy5.5-aPD1-NPs; G3: Cy5.5-aPD1-NPs-Gel).]
To select the optimal HMA for the combination therapy, four agents including 5-azacytidine (AC), 5-aza-2'-deoxycytidine (DAC), Zeb, and 5-fluoro-2'-deoxycytidine (5-F) have been tested. AC and DAC are two of the most studied HMAS and have been approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia.\textsuperscript{10} As a recently studied agent, Zeb, with better stability and lower toxicity, has shown potential for targeting cancer cells in vitro/vivo.\textsuperscript{11} Another pyrimidine nucleoside analog, 5-F, which has a fluorine atom instead of the proton at C5 position, has been studied to reduce the proliferation of brain tumors.\textsuperscript{12} First, MTT assays were conducted to compare the cytotoxicity among these agents and determine the appropriate concentration to induce the expression of TAAs (Figure S2, Supporting Information). The IC\textsubscript{50} values of DAC and 5-F against B16F10 melanoma cells were $2.55 \times 10^{-6}$ and $1.05 \times 10^{-6}$ m, respectively, which were much lower than that of AC ($48.98 \times 10^{-6}$ m) and Zeb ($69.18 \times 10^{-6}$ m). This high cytotoxicity may significantly impede the application of DAC and 5-F since they would directly cause the death of tumor cells rather than inducing TAAs. Thus AC and Zeb with suitable IC\textsubscript{50} values were selected for the following study. We next compared the induction of TAAs between AC and Zeb. Three of TAAs, including melanoma antigen family E1 (MAGE-E1),\textsuperscript{13} tyrosinase-related protein-1 (TRP1),\textsuperscript{14} and melanoma cell adhesion molecule (CD146) were detected.\textsuperscript{15} It was shown that Zeb induced similar or slightly more tumor antigen expression than AC (Figure S2, Supporting Information). Furthermore, AC is unstable in aqueous solutions while Zeb is stable in both neutral and acidic solutions, making the potential clinical application of the latter more flexible.\textsuperscript{16} Therefore, Zeb was finally chosen as a TAA-inducing agent for further study.

Next, the aPD1-loaded CaCO\textsubscript{3} nanoparticles were prepared using poly(ethylene glycol)–poly(glutamic acid) (PEG-P(Glu)) block copolymers to interact with Ca\textsuperscript{2+} and CO\textsubscript{3}\textsuperscript{2-} in the aqueous solution. Glu provided carboxyl to interact with Ca\textsuperscript{2+} preventing the mineralization of large CaCO\textsubscript{3} blocks, and PEG shell acted to avoid agglomeration and aggregation.\textsuperscript{17} Monodisperse aPD1-NPs were achieved with an average size of about 100 nm (Figure 1B) and encapsulation efficiency of about 50%. The ROS-responsive TSPBA linker was synthesized and then characterized by 1H-NMR (Figure S3, Supporting Information).\textsuperscript{18} The hydrogel was immediately formed when mixing the linker with PVA due to the conjugation between the phenylboronic acid and the cis-1,3-diol in PVA. TSPBA linker solution containing Zeb was added into the PVA solution containing aPD1-NPs, leading to the immediate formation of hydrogel, Zeb-aPD1-NPs-Gel. The cryo-scanning electron microscopy (Cryo-SEM) images of this scaffold showed that the spherical NPs were loaded in the hydrogel with network structure (Figure 1C). Additionally, TSPBA linker could be oxidized and hydrolyzed when exposed to H\textsubscript{2}O\textsubscript{2}, leading to the degradation of the hydrogel and release of Zeb (Figure S4, Supporting Information; Figure 1D). aPD1-NPs could dissolve and release aPD1 in slightly acidic buffer by reacting with H\textsuperscript{+} (Figure S5, Supporting Information), with almost 95% released within 72 h. Furthermore, encapsulation of aPD1-NPs in the gel depot allowed a more sustained release of aPD1. As shown in Figure 1E, $\approx$75% of aPD1 was released in the stimuli of H\textsubscript{2}O\textsubscript{2} and low pH at 72 h. To investigate the retention ability of aPD1 in vivo, the B16F10 melanoma-bearing mice model was established. aPD1 labeled with Cyanine5.5 (Cy5.5) was loaded in the gel depot and then peritumorally injected. The fluorescence signal remained detectable six days after gel injection, while there was nearly no signal for the groups of free Cy5.5-aPD1 and Cy5.5-aPD1-NPs, indicating that encapsulation of aPD1-NPs in gel increased its retention in the tumor sites (Figure 1F).

Then, TAAs expression of the Zeb-loaded gel (Zeb-Gel)-treated group was investigated by western blotting assay. The result showed enhanced expression of MAGE-E1, TRP1, and CD146, indicating that Zeb could facilitate exposure of TAAs and thereby increase the immunogenicity of melanoma, which could potentiate the capture of tumor antigen by antigen presenting cells (Figure 2A; Figure S6, Supporting Information). Treatment with Zeb-Gel also promoted mature dendritic cells (mDCs) with expression of CD80 and CD86 (Figure 2D,E). Furthermore, a significant reduction of MDSCs was detected in the Zeb-Gel group, which was $\approx$30% of untreated group (UnTx) and half of gel-treated group, suggesting that Zeb can potentially reverse immunosuppressive TME (Figure 2F,G). Additionally, the presence of Zeb upregulated the expression of PD-L1 on B16F10 tumors (Figure 2B,C), making the subsequent use of immune checkpoint blockade essential.

To validate whether the combination strategy of epigenetic modulation and ICB therapy could enhance the inhibition of tumor growth, different formulations were injected at the peri-tumoral site separately, including blank Gel, aPD1-NPs-Gel (aPD1, 40 µg per mouse), aPD1-NPs-Gel + Zeb (aPD1, 40 µg per mouse; Zeb, 5 mg kg\textsuperscript{-1}), Zeb-NPs-Gel (Zeb, 5 mg kg\textsuperscript{-1}), and Zeb-aPD1-NPs-Gel (aPD1, 40 µg per mouse; Zeb, 5 mg kg\textsuperscript{-1}). In the in vivo tumor growth was monitored by capturing bioluminescence images of luciferase-tagged B16F10 cells (Figure 3A). The Zeb-aPD1-NPs-Gel-treated group showed the most notable tumor inhibition effect, while blank Gel treated group had shown negligible treatment efficacy, and the single agent treated groups displayed a limited inhibitory effect on tumor growth (Figure 3B). Furthermore, the average tumor volume of Zeb-aPD1-NPs-Gel group at day 12 was 4.27-fold smaller than that of aPD1-NPs-Gel + Zeb treated group, which was attributed to the controlled release of Zeb from the gel depot. Furthermore, the median survival time of mice treated with Zeb-aPD1-NPs-Gel was effectively prolonged to 39.5 days, significantly longer than those of other groups ($p < 0.001$), including the untreated group (16 days), blank Gel (15 days), aPD1-NPs-Gel (18 days), Zeb-NPs-Gel (16 days), and aPD1-NPs-Gel + Zeb (23 days) (Figure 3C). Even one-third of mice treated with Zeb-aPD1-NPs-Gel survived for more than 60 days.

Besides, tumors were harvested for analysis by flow cytometry and immunofluorescence analysis five days after different treatments. As shown in Figure 3D,E, the group treated with Zeb-aPD1-NPs-Gel displayed a significantly higher rate of CD8\textsuperscript{T} cell infiltration in tumors with 4.46 $\pm$ 0.53% of total tumor cells, which was 2.73-fold higher than that of Zeb-NPs-Gel group ($p < 0.001$), and 1.90-fold of aPD1-NPs-Gel + Zeb ($p < 0.01$). In addition, noticeable staining of CD8\textsuperscript{T} cells was observed in the Zeb-aPD1-NPs-Gel treated group by confocal laser scanning microscopy (Figure S7, Supporting Information). As shown in Figure S8, Supporting Information,
treatment with Zeb-aPD1-NPs-Gel further effectively promote the amount of activated CD8+ T cells (CD8+CD44+ T cells), potentiating CD8+ T cell. Additionally, a slight reduction of Tregs has been detected in the group treated with Zeb-aPD1-NPs-Gel (Figure S9, Supporting Information). Furthermore, the results of body-weight change and hematoxylin and eosin staining showed that Zeb-aPD1-NPs-Gel did not cause obvious toxicity to major organs (heart, liver, spleen, lung, and spleen) when compared with the control group (Figures S10 and S11, Supporting Information).

To assess the systemic antitumor immune effect of Zeb-aPD1-NPs-Gel, the mice bearing B16F10 tumors on both sides were constructed. A Zeb-aPD1-NPs-Gel was injected just next to the left tumor. The tumor growth on both sides had a similar tendency and was obviously inhibited (p < 0.001) compared with the control group (Figure 4A, B). The average tumor volumes of the treated group at the left and right sides ten days after treatment were 14.3-fold and 5.5-fold smaller than that of untreated group on the left side, respectively. In addition, increased infiltration of CD8+ T cells and CD4+ T cells of tumors (left and right) was detected by flow cytometry compared to the untreated group (p < 0.05) (Figure 4C–E). Furthermore, a significant increase of activated CD8+ T cells has been detected on both sides in the treated group, while Tregs have been evaluated to decrease (Figures S12 and S13, Supporting Information). These results indicated that local delivery of Zeb-aPD1-NPs-Gel could effectively induce the systemic antitumor immune responses.

In summary, we have engineered a bioreponsive depot loaded with Zeb and aPD1 to combine epigenetic modulation and immunotherapy, which have been proved to effectively enhance antitumor immune responses. This dual-responsive scaffold, composed of pH-sensitive CaCO3 NPs and ROS-responsive hydrogel, enabled to achieve controlled release of payloads by responding to the acidic pH and ROS condition associated with TME. Additionally, local release of Zeb increased the immunogenicity of tumors via enhancing TAAs expression, decreasing immunosuppression. Based on these functions, its combination with aPD1 inhibitors effectively boosted the T cell-mediated antitumor immune response. This delivery strategy integrated with both epigenetic modulators and immune checkpoint blockade treatments may be translated for enhancing objective response rates in clinic.
Experimental Section

Western Blotting Assay: The western blotting analysis was performed as previous reports to investigate the various TAAs expression levels of B16F10 melanoma.[19] For the in vitro study, the B16F10 cells were treated with different demethylation agents at a predetermined concentration for 72 h, and then the drug-loaded medium was removed and replaced by normal Dulbecco’s modified Eagle medium and cells were incubated for another four days. For the in vivo study, the mice bearing melanoma were implanted with Gel or Zeb-Gel for five days. Proteins were collected from cells or tumor tissues using RIPA buffer and the total protein concentrations were quantified using BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of proteins were mixed with 2× laemmli sample buffer, then loaded and separated by Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) and transferred to a membrane (Bio-Rad), blocked in 3% fat-free milk for 1 h at room temperature, and then incubated with the following primary antibodies diluted in 1.5% bovine serum albumin overnight at 4 °C: anti-beta actin antibody, anti-TRP1 antibody, anti-CD146 antibody, and anti-MAGE-E1 antibody. Then, the goat anti-rabbit/mouse HRP-conjugated secondary antibodies were diluted and incubated for 1 h. Images were acquired by chemiluminescence.

Synthesis of ROS-Responsive TSPBA Linker and PEG-P(Glu) Block Copolymers: TSPBA was synthesized from the quaternization reaction between N,N,N′,N′-tetramethyl-1,3-propanediamine (TMPA) and 4-(bromomethyl) phenylboronic acid as previously reported.[18a] Briefly, 4-(bromomethyl) phenylboronic acid and TMPA were mixed (3:1, mmol/mmol) in N,N-dimethylformamide (DMF) and stirred at 60 °C for 24 h. Then, the reaction solution was precipitated in tetrahydrofuran (THF) and filtrated, and further washed with THF three times. Placing the product under vacuum condition overnight to obtain pure TSPBA, which was then characterized by 1H-NMR.

The PEG-P(Glu) block copolymers were synthesized as reported previously.[20] Briefly, N-carboxyanhydride of γ-benzyl l-glutamate (NCA-BLG) was first synthesized by Fuchs–Farthing method using triphosgene and l-glutamic acid γ-benzyl ester. Then,
the PEG-poly(γ-benzyl L-glutamate) (PEG-PBLG) was obtained by following ring-opening procedures in DMF to initiate NCA-BLG by utilizing the primary amino group of CH₃O-PEG-NH₂. Finally, benzyl groups of PEG-PBLG were removed by mixing with 0.5 N NaOH at room temperature to obtain PEG-P(Glu). The repeat unit of the P(Glu) segment of PEG-P(Glu) was calculated to be 50 using 1H-NMR spectroscopy (300 MHz; solvent: D₂O).

Preparation and Characterization of Zeb-aPD1-NPs-Gel: First, aPD1-NPs were prepared via chemical precipitation. Briefly, 5 mg PEG-P(Glu) was dissolved in DI water and then 80 µg aPD1 was added, followed by the addition of 10 mg CaCl₂ aqueous solution. Then, 1 × 10⁻³ M Tris-HCl buffer (pH 8.0) was slowly added to adjust the pH value to pH 7.8 to form Ca²⁺ chelate compounds, and 3 mg NaCO₃ was added dropwise to the mixture until opalescence was observed indicating the formation of aPD1-NPs. The mixture was stirred at 4 °C overnight and then centrifuged to remove the excess ions, copolymers, and antibodies (14800 rpm, 15 min). The size distribution was characterized by DLS and the morphology was observed by TEM (JEOL 2000FX). The encapsulation efficiency of aPD1 in CaCO₃ NPs was measured by ELISA (rat IgG total ELISA kit, Abcam, cat. no. ab189578).

Then a predetermined amount of Zeb was dissolved in 10 wt% TSPBA solution and then added into the 5 wt% PVA containing aPD1-NPs to form the Zeb-aPD1-NPs-Gel. The morphology of this scaffold was characterized by Cyro-SEM (JEOL 7600F with Gatan Alto).

In Vitro Release of Zeb and aPD1 from Hydrogel: For the release of Zeb, 1 × 10⁻³ M H₂O₂ was added into PBS buffer to investigate the release profile of Zeb from ROS-responsive hydrogels, the amount of Zeb was analyzed by HPLC. For aPD1 release, it was studied in PB buffer with different pH values (pH 6.5 or pH 7.4) with or without H₂O₂. Released aPD1 was measured by a Rat IgG total ELISA kit. All the study was conducted at room temperature.

In Vivo Antitumor Effect: The C57BL/6 male mice model bearing subcutaneous B16F10 melanoma was established by implanting about 1 × 10⁶ of luc-B16F10 cells into the right flank of mice. Six days later, the mice were randomly divided into six groups (n = 6) and peritumorally implanted with different formulations, including blank Gel, aPD1-NPs-Gel, aPD1-NPs-Gel + Zeb, Zeb-NPs-Gel, and Zeb-aPD1-NPs-Gel at a dose of 40 µg aPD1 and/or 5 mg kg⁻¹ Zeb per mouse. For bioluminescence imaging, 100 µL d-luciferin substrate solution (30 mg mL⁻¹) was intraperitoneally injected for 5 min and then the mice were photographed via the IVIS imaging system (Perkin Elmer Ltd). The tumor volumes were measured every two days and calculated according to the equation: (long diameter × short diameter²)/2. Survival time of model mice was recorded starting from the day implanting tumor cells, and Kaplan–Meier survival curves were plotted. Animals were euthanized when showing signs of imperfect health or when the size of tumors exceeded 1.5 cm³.

Flow Cytometry: Mice model was built and treated as mentioned above. Five days later, mice were euthanized and tumors were collected and homogenized in cold cell staining buffer to obtain single cell suspensions after filtration. Cells were stained with different fluorescence-labeled antibodies following the instructions. The stained cells were measured...
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on a CytoFLEX flow cytometer (Beckman) and analyzed by the FlowJo software.

In Vivo Systemic Immune Effect on Treating Distant Tumor: 1 × 10^6 of luc-B16F10 cells were implanted on both sides of mice. Seven days later, Zeb-pDO1-NPs-Gel was injected on the left tumor site, while no treatment was performed on the right tumor site. The in vivo bioluminescence images and tumor volumes on both sides were imaged as aforementioned. Ten days later, the mice were sacrificed and tumors were collected to conduct the flow cytometry experiments aforementioned.

All animal studies were strictly in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at UNC-CH and North Carolina State University (NCSU). All mice were kept in accordance with federal and state policies on animal research at UNC-CH and NCSU.

Statistical Analysis: All results are presented as mean ± SD. as ±

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

Z.G. and H.T.R. have applied for patents related to this study.

Keywords

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