

ZINC-BINDER BASED EBNA1-SPECIFIC COMPOUNDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application Number 62/619,093, filed on January 18, 2018, U.S. Provisional Patent Application Number 62/652,304 filed on April 3, 2018, U.S. Provisional Patent Application Number 62/682,161, filed on June 7, 2018, and U.S. Patent Application Number 16/249,987, filed on January 17, 2019 and the disclosures of which are hereby incorporated by reference in their entirety.

FIELD OF INVENTION

[0001] The present disclosure generally relates to compounds useful as therapeutic, imaging, and/or diagnostic agents. More particularly, the present disclosure relates to compounds of binding zinc and EBNA1 with responsive imaging properties and robust inhibition of EBNA1 for the treatment of EBV-positive cells, such as cancers.

BACKGROUND

[0002] Epstein-Barr virus (EBV) infects the majority of the human population worldwide and can establish lifelong latent infection in hosts. The association between latent EBV infection and the development of certain lymphomas and epithelial malignancies is well known. EBV encoded Nuclear Antigen 1 (EBNA1) is a highly restricted gene that is expressed in the latent phase of EBV infection and is the only protein that expressed in all EBV latently-infected cells, EBV encoded Nuclear Antigen 1 (EBNA1), making it a promising and effective target for the treatment of EBV-positive cancers.

[0003] Despite its importance, neither the precise role(s) of EBNA1 in EBV latent infection is well understood, nor have any therapies targeting EBNA1 for the

treatment of EBV-positive cancers have been approved. This emphasizes that continuous efforts are needed to develop potent EBNA1 inhibitors. In the past few years, several strategies have been used to inhibit EBNA1 or EBNA1-dependent cellular functions, including the inhibiting the expression of EBNA1, EBNA1-DNA binding activities, EBNA1-RNA interactions, and EBNA1 homo-dimerization. Although these inhibitors were shown to inhibit their targets *in vitro* and *in vivo*, there were problems with them, such as lack of specificity, poor cellular permeability and poorly understood mechanisms of action. These problems stemmed, in part, from the fact that the inhibitors were identified using screening-based approaches and failed to show the direct binding to EBNA1, which largely limited their application.

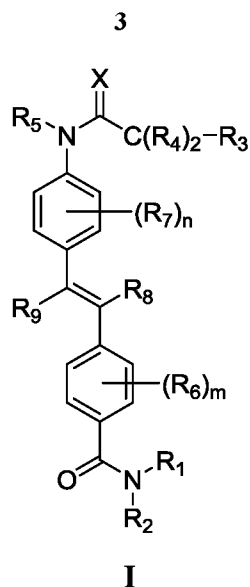
[0004] Furthermore, due to the non-fluorescent nature of conventional EBNA1 inhibitors, this presents obstacles to track and study inhibitor distribution and their cellular functions. Fluorescence imaging is useful for sensing biomolecules in living systems due to its unique potential advantages, such as excellent sensitivity and selectivity, high spatial and temporal resolution, simplicity and implementation, facile visualization, and real-time analysis of intracellular dynamics and biomolecular localization at subcellular levels.

[0005] There thus exists a need for improved EBNA1 inhibitors that address one or more of the aforementioned needs.

SUMMARY

[0006] The present disclosure relates to zinc-binding EBNA1-specific probes for the detection and regulation of EBNA1 and which are useful in the treatment of EBV-positive cancer.

[0007] In a first aspect, provided herein is a compound of Formula I:



[0008] or a pharmaceutically acceptable salt thereof, wherein

[0009] m is a whole number selected from 0-4;

[0010] n is a whole number selected from 0-4;

[0011] p is a whole number selected from 0-4;

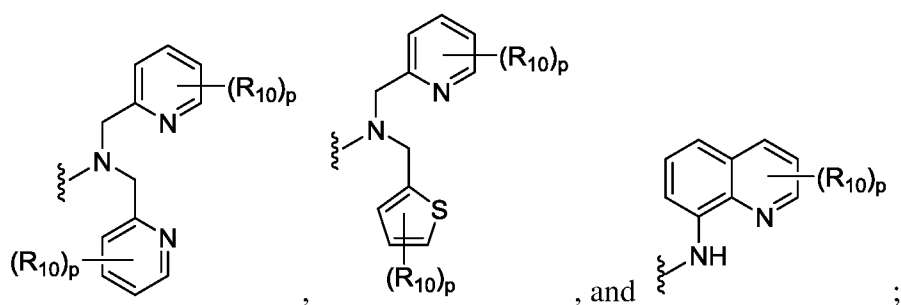
[0012] X is H₂, S, or O;

[0013] each instance of R is independently H or alkyl;

[0014] R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

[0015] R₂ is H or alkyl;

[0016] R₃ is selected from the group consisting of:



[0017] each instance of R₄ is independently H or alkyl;

[0018] R₅ is H or alkyl;

[0019] each instance of R₆ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂;

[0020] each instance of R_7 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[0021] each of R_8 and R_9 is independently H or alkyl; and

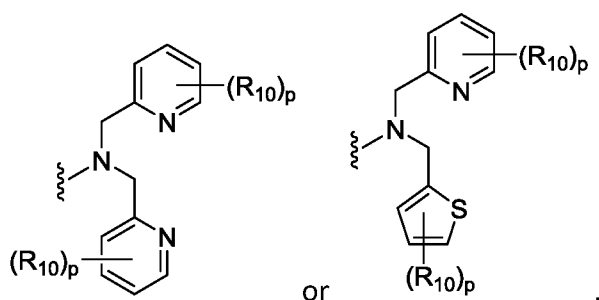
[0022] each instance of R_{10} is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 .

[0023] In a first embodiment of the first aspect, provided herein is the compound of the first aspect, wherein each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.

[0024] In a second embodiment of the first aspect, provided herein is the compound of the first aspect, wherein each of R_2 and R_5 is H.

[0025] In a third embodiment of the first aspect, provided herein is the compound of the first aspect, wherein each of R_4 , R_8 , and R_9 is H.

[0026] In a fourth embodiment of the first aspect, provided herein is the compound of the first aspect, wherein X is O and R_3 is:

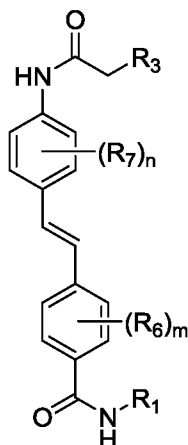


[0027] In a fifth embodiment of the first aspect, provided herein is the compound of the fourth embodiment of the first aspect, wherein R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.

[0028] In a sixth embodiment of the first aspect, provided herein is the compound of the fifth embodiment of the first aspect, wherein each of R_4 , R_8 , and R_9 is H.

[0029] In a seventh embodiment of the first aspect, provided herein is the compound of the first aspect, wherein the compound has Formula II:

5



II

[0030] or a pharmaceutically acceptable salt thereof, wherein

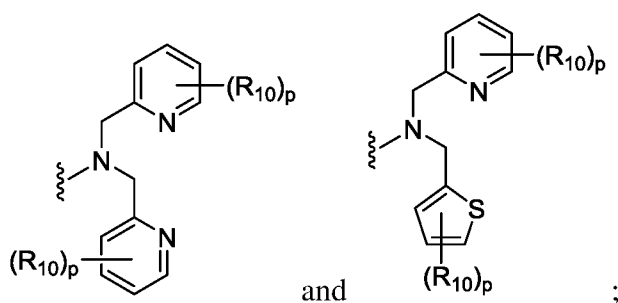
[0031] m is a whole number selected from 0-4;

[0032] n is a whole number selected from 0-4;

[0033] p is a whole number selected from 0-4;

[0034] R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

[0035] R₃ is selected from the group consisting of:



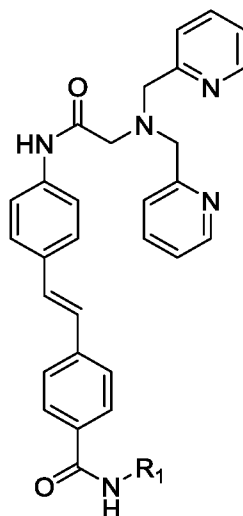
[0036] each instance of R₆ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂;

[0037] each instance of R₇ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂;

[0038] each of R₈ and R₉ is independently H or alkyl; and

[0039] each instance of R₁₀ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂.

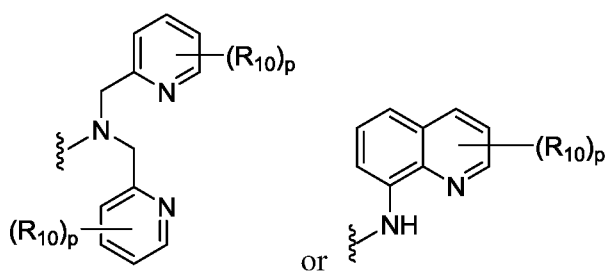
[0040] In an eighth embodiment of the first aspect, provided herein is the compound of the first aspect, wherein the compound has the Formula **III**:



III

[0041] or a pharmaceutically acceptable salt thereof, wherein R_1 is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

[0042] In a ninth embodiment of the first aspect, provided herein is the compound of the first aspect, wherein X is H_2 and R_3 is:



[0043] In a tenth embodiment of the first aspect, provided herein is the compound of the ninth embodiment of the first aspect, wherein each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.

[0044] In an eleventh embodiment of the first aspect, provided herein is the compound of the tenth embodiment of the first aspect, wherein each of R_2 and R_5 is H.

[0045] In a twelfth embodiment of the first aspect, provided herein is the

compound of the eleventh embodiment of the first aspect, wherein each of R₄, R₈, and R₉ is H.

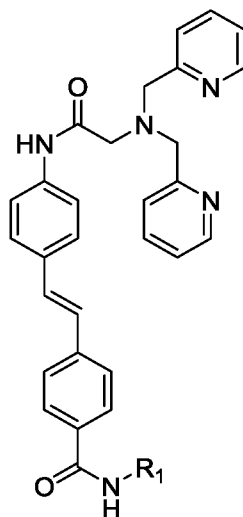
[0046] In a thirteenth embodiment of the first aspect, provided herein is the compound of the twelfth embodiment of the first aspect, wherein each of R₆, R₇, and R₁₀ is H.

[0047] In a second aspect, provided herein is a method of treating an Epstein-Barr virus (EBV)-positive cell in a patient in need thereof comprising administering a therapeutically effective amount of a compound of the first aspect to the patient.

[0048] In a first embodiment of the second aspect, provided herein is the method of the second aspect, wherein the EBV-positive cell is a cancerous cell.

[0049] In a second embodiment of the second aspect, provided herein is the method of the first embodiment of the second aspect, wherein the cancerous cell is Burkitt's lymphoma, non-Hodgkin's lymphoma, Hodgkin's disease, T-cell lymphoma, B-cell lymphoma, transplant-associated lymphoproliferative disorders, nasopharyngeal carcinoma, gastric adenocarcinoma, parotid carcinoma, or leiomyosarcoma.

[0050] In a third embodiment of the second aspect, provided herein is the method of the second aspect, wherein the compound has the Formula **III**:



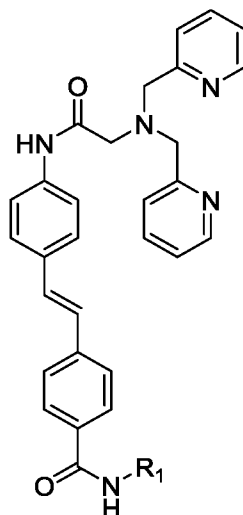
III

[0051] or a pharmaceutically acceptable salt thereof, wherein

[0052] R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

[0053] In a third aspect, provided herein is a method of imaging an EBV-positive cell comprising the step of contacting the EBV-positive cell with a compound of the first aspect and measuring the fluorescence of the compound.

[0054] In a first embodiment of the third aspect, provided herein is the method of the third aspect, wherein the compound has the Formula **III**:



III

[0055] or a pharmaceutically acceptable salt thereof, wherein R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

BRIEF DESCRIPTION OF DRAWINGS

[0056] The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, in which:

[0057] **Figure 1** shows the schematic illustration of the dual-responsive emission for the binding to Zn²⁺/EBNA1 and the blockage on EBNA1 dimerization of a compound as described herein.

[0058] **Figure 2A** shows fluorescence spectral changes of **ZRL₅P₄** (2 μM; excitation at 337 nm) in the presence of Zn²⁺ in aqueous solution (CH₃CN/0.05 M HEPES, pH 7.4 = 50:50).

[0059] **Figure 2B** shows fluorescence spectral changes of **ZRL₅P₄** (2 μM; excitation at 337 nm) upon addition of Zn²⁺ in aqueous solution (CH₃CN/0.05 M

HEPES, pH 7.4 = 50:50).

[0060] **Figure 2C** shows solvation study of **ZRL₅P₄** (2 μ M; excitation at 337 nm) to check whether the ICT-state emission was presented by the probe.

[0061] **Figure 2D** shows fluorescence spectral changes of **ZRL₅P₄** (2 μ M; excitation at 337 nm) upon addition of EBNA1.

[0062] **Figure 2E** shows fluorescence spectral changes of **ZRL₅P₄** (2 μ M; excitation at 337 nm) upon addition of human serum albumin (HSA). The selectivity of **ZRL₅P₄** to EBNA1 over HSA as shown by the fluorescence intensity ratio before and after the addition of the protein.

[0063] **Figure 2F** shows fluorescence spectral changes of **ZRL₅P₄** upon addition of EBNA1 in the presence of 1 equiv. Zn^{2+} . Inset figure shows fluorescence intensity performance in the presence and absence of Zn^{2+} upon the addition of EBNA1.

[0064] **Figure 3A** shows fluorescence spectral changes of **ZRL₅P₂** (2 μ M; excitation at 337 nm) in the presence of Zn^{2+} and Cd^{2+} in aqueous solution ($\text{CH}_3\text{CN}/0.05$ M HEPES, pH 7.4 = 50:50).

[0065] **Figure 3B** shows fluorescence spectral changes of **ZRL₅P₆** (2 μ M; excitation at 337 nm) in the presence of Zn^{2+} and Cd^{2+} in aqueous solution ($\text{CH}_3\text{CN}/0.05$ M HEPES, pH 7.4 = 50:50).

[0066] **Figure 4A** shows fluorescence spectral changes of **ZRL₅P₄** (2 μ M; excitation at 337 nm) in the presence of Cd^{2+} , Cu^{2+} , Co^{2+} , Ni^{2+} in aqueous solution ($\text{CH}_3\text{CN}/0.05$ M HEPES, pH 7.4 = 50:50).

[0067] **Figure 4B** shows fluorescence spectral changes of **ZRL₅P₄** (2 μ M; excitation at 337 nm) in the presence of Hg^{2+} and Mg^{2+} in aqueous solution ($\text{CH}_3\text{CN}/0.05$ M HEPES, pH 7.4 = 50:50).

[0068] **Figure 5A** shows solvation study of **ZRL₅P₂** with a decrease of solvent polarity.

[0069] **Figure 5B** shows solvation study of **ZRL₅P₆** with a decrease of solvent polarity.

[0070] **Figure 6A** shows chemical structures of **L₂P₄** (left) and **ZRL₅P₄** (right).

[0071] **Figure 6B** shows the binding energy of **ZRL₅P₄** and **Zn²⁺-ZRL₅P₄** to the putative EBNA1 DBD monomer calculated via MMPBSA.

[0072] **Figure 7(A-F)** shows 500 ns NPT simulation of P₄-EBNA1 complex model (#9), the complex model is chosen from flexible peptide docking. (A) Root-mean-square fluctuation (RMSF) of the putative EBNA1 DBD monomer. (B) RMSF of **P₄**. (C) RMSD of main-chain atoms from EBNA1 DBD with regard to (w.r.t) the initial and end structure. (D) RMSD of main-chain atoms from **P₄** with regard to the initial and end structure. (E) The salt-bridging between RrRK motif and the acid residues in the C-terminus EBNA1 DBD. (F) The hydrophobic packing between YFMVF (SEQ ID NO:2) motif and the dimeric interface of EBNA1 DBD.

[0073] **Figure 8(A-E)** shows the putative 3D structures of **ZRL₅-NME** and **Zn²⁺-ZRL₅-NME** resulted from molecular mechanics calculation. (A) 2D structure of **ZRL₅-NME**. (B) The calculated 3D structure of **ZRL₅-NME**. (C) 2D structure of **Zn²⁺-ZRL₅-NME**. (D) The calculated 3D structure of **Zn²⁺-ZRL₅-NME**. (E) The superposition of the calculated **Zn²⁺-ZRL₅-NME** 3D structure onto the crystal structure of **ZTF-Zn²⁺**, a highly similar compound reported previously.

[0074] **Figure 9(A-B)** shows the putative structures of **Zn²⁺-ZRL₅-NME** complex with 5-coordinated **Zn²⁺** where solvent provides 1 coordination site. (A) The calculated 3D structure of **DMSO-Zn²⁺-ZRL₅-NME**. (B) The calculated 3D structure of **H₂O-Zn²⁺-ZRL₅-NME**.

[0075] **Figure 10(A-F)** shows 200 ns NPT simulation of **ZRL₅P₄-EBNA1** complex model (#1). (A) RMSF of the putative EBNA1 DBD monomer. (B) RMSF of **ZRL₅P₄**. (C) RMSD of main-chain atoms from EBNA1 DBD with regard to (w.r.t) the initial

and end structure. **(D)** RMSD of main-chain atoms from **ZRL₅P₄** ligand with regard to the initial (red) and end (blue) structure. **(E)** The salt-bridging between RrRK motif and acid residues in the C-terminus of EBNA1 DBD. **(F)** The hydrophobic interactions between YFMVF (SEQ ID NO:2) motif and the dimeric interface of EBNA1 DBD.

[0076] **Figure 11(A-F)** shows 200 ns NPT simulation of **ZRL₅P₄**-EBNA1 complex model (#2). **(A)** RMSF of the putative EBNA1 DBD monomer. **(B)** RMSF of **ZRL₅P₄**. **(C)** RMSD of main-chain atoms from EBNA1 DBD with regard to (w.r.t) the initial and end structure. **(D)** RMSD of main-chain atoms from **ZRL₅P₄** with regard to the initial and end structure. **(E)** The salt-bridging between RrRK motif and acid residues in the C-terminus of EBNA1 DBD. **(F)** The hydrophobic interactions between YFMVF (SEQ ID NO:2) motif and the dimeric interface of EBNA1 DBD.

[0077] **Figure 12(A-F)** shows 200 ns NPT simulation of Zn²⁺-**ZRL₅P₄**-EBNA1 complex model (#1). **(A)** RMSF of the putative EBNA1 DBD monomer. **(B)** RMSF of Zn²⁺-**ZRL₅P₄**. **(C)** RMSD of main-chain atoms from EBNA1 protein with regard to (w.r.t) the initial and end structure. **(D)** RMSD of main-chain atoms from Zn²⁺-**ZRL₅P₄** ligand with regard to the initial and end structure. **(E)** The salt-bridging between RrRK motif and acid residues in the C-terminus of EBNA1 DBD. **(F)** The hydrophobic interactions between YFMVF (SEQ ID NO:2) motif and the dimeric interface of EBNA1 DBD.

[0078] **Figure 13(A-F)** shows 200 ns NPT simulation of Zn²⁺-**ZRL₅P₄**-EBNA1 complex model (#2). **(A)** RMSF of the putative EBNA1 DBD monomer. **(B)** RMSF of Zn²⁺-**ZRL₅P₄**. **(C)** RMSD of main-chain atoms from EBNA1 DBD with regard to (w.r.t) the initial and end structure. **(D)** RMSD of main-chain atoms from Zn²⁺-**ZRL₅P₄** with regard to the initial and end structure. **(E)** The salt-bridging

between RrRK motif and acid residues in the C-terminus of EBNA1 DBD. (F) The hydrophobic interactions between YFMVF (SEQ ID NO:2) motif and the dimeric interface of EBNA1 DBD.

[0079] **Figure 14(A-D)** shows the putative structure of **ZRL₅P₄**-EBNA1 and **Zn²⁺-ZRL₅P₄**-EBNA1 complex. (A, B) Representative conformation of **ZRL₅P₄**-EBNA1 complex resulted from two independent MD simulations. The calculated GB and PB values represented their binding free energy. (C, D) Representative conformation of **Zn²⁺-ZRL₅P₄**-EBNA1 complex resulted from two independent MD simulations. The calculated GB and PB values represented their binding free energy.

[0080] **Figure 15(A-B)** shows ¹H NMR spectra of compound 5 in the presence of **Zn²⁺** in CD₃CN (A) and DMSO-*d*₆ (B). The binding of the carbonyl oxygen to **Zn²⁺** in CD₃CN blocks the resonance structure of the amide group and shifts the proton 8 upfield; and the binding of the amide nitrogen to **Zn²⁺** in DMSO-*d*₆ functions as the electron-withdrawing group to shift the proton 8 downfield.

[0081] **Figure 16** shows the representative **P₄**-EBNA1 complex structure after 500 ns MD simulations. **P₄**-EBNA1 DBD binding was mediated by both hydrophobic interactions from YFMVF (SEQ ID NO:2) to dimeric interface of EBNA1 DBD and salt bridges from RrRK motif to C-terminus of EBNA1 DBD.

[0082] **Figure 17(A-D)** shows the partial charge (A, C) and AMBER atom types (B, D) of **ZRL₅** (A, B) and **Zn²⁺-ZRL₅** (C, D) for MD simulations.

[0083] **Figure 18(A-I)** shows **ZRL₅P₄** inhibited EBNA1 dimerization (A-B) and transactivation by EBNA1 (C-D). MBS-cross-linked, full-length EBNA1 dimerization in the absence/presence of **Zn²⁺** by **L₂P₄/ZRL₅P₄** (control in buffer) (A)

and their calculated EBNA1 monomer/dimer band intensity (**B**). EDTA, TPEN, **ZRL₅P₄** and **L₂P₄** differentially affect the ability of EBNA1 to transactivate oriP-Cp-luciferase in EBV-positive C666-1 (**C**) and NPC43 (**D**) cells. Dashed line in (**C**) and (**D**) was plotted for a better comparison of transactivation between different compounds. Cytotoxicity assays of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** in EBV-negative HK-1 cells (**E**) and HONE-1 cells (**F**) (conc. 1, 5, 10, 20, 50, 100 μ M) and EBV-positive NPC43 cells (**G**), C666-1 cells (**H**), and Raji cells (**I**) (conc. 1, 3, 5, 10, 15, 20 μ M) were measured. Cells were treated with different probes and then incubated for 5 days to test their cytotoxicity (half of the medium was replaced every 4 days with fresh medium containing the appropriate concentration of the probes).

[0084] **Figure 19(A-C)** shows two-photon fluorescence imaging of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** in living EBV-positive (**A**) NPC43 cells and (**B**) C666-1 cells, and EBV-negative (**C**) HONE-1 cells. **ZRL₅P_n**, signal emitted from the respective EBNA1 probe. DRAQ5 is a fluorescent dye used to label the cell nuclei of the living cells as indicated.

[0085] **Figure 20(A-F)** shows *in vivo* anti-tumor activity of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** in EBV-positive C666-1 mouse xenograft model. Mice were treated twice weekly with 4 μ g/injection of the probes for 18 days. Throughout the treatment period, (**A**) tumor volumes and (**B**) body weights were measured. At the experimental endpoint, tumors were excised. (**C**) Tumor weights and (**D**) representative photographs of tumors. Data are expressed as the means $\pm$ SEM. * P < 0.05; ** P < 0.005; *** P < 0.005 vs. vehicle. Scale bars: 10 mm. (**E**) Representative images of tumor sections derived from the above *in vivo* animal study. Cell necrosis (acellular areas indicated by *) was observed in the tumor nodules treated with **ZRL₅P₄** and **ZRL₅P₆**. H&E, hematoxylin and eosin. T, adjacent area with tumor cells.

Magnification, 40 ×. Scale bar, 20 μm. (F) *In vitro* emission spectra (from confocal microscopy) of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** in the nucleus of EBV -positive NPC43 and -negative HONE-1 cells. Emission intensity was much greater for **ZRL₅P₄** and **ZRL₅P₆** in EBV-positive cells.

[0086] **Figure 21(A-D)** shows expression analyses of EBV lytic genes and proteins in response to the EBNA1 probes. (A) IHC analysis of lytic proteins BZLF1 (Zta) and BMRF1 in the transplanted C666-1-derived tumor tissues as described in Figure 20. Representative results are shown. Nuclear staining of Zta and BMRF1 are observed in the necrotic tumor regions in response to **ZRL₅P₂**, **ZRL₅P₄** and **ZRL₅P₆**. Magnification, 400 ×. Scale bar, 2 μm. (B, C) Gene expression analysis of EBV lytic genes, BRLF1 (Rta) and BMRF1, in C666-1 and NPC43 cells cultured with or without 10 μM **ZRL₅P₂** and **ZRL₅P₄** for 3 (C666-1 cells) or 7 (NPC43 cells) days. The gene expression was analyzed by qRT-PCR. Fold change of the relative gene expression after each treatment was compared with the solvent control (DMSO) and compared between the two treatments. (D) Protein expression analysis of early (Rta and BMRF1) and late (gp350/220) EBV lytic proteins in EBV-positive NPC43 cells cultured with or without 10 μM **ZRL₅P₂** and **ZRL₅P₄** for 7 (NPC43 cells) days. The protein expression was detected with Western blot, and β-actin serves as the loading control.

[0087] **Figure 22(A-B)** shows production of EBV particle in response to **ZRL₅P₄**. The HONE-1-EBV cell line, which expresses GFP to indicate the presence of the EBV genome, was used. This cell line was treated with 10 μM **ZRL₅P₂** or **ZRL₅P₄** for 4 days, and the viral particles released in the culture medium was detected by the Raji cell assay. The culture medium was added to Raji cells for 3 days, and GFP expression reflects the re-infection by the HONE-1-released EBV particles. (A)

Relative average viral titer in response to **ZRL₅P₂** or **ZRL₅P₄** was compared with the solvent control (DMSO). The Raji cell assay was performed in triplicates for each treatment. **, $P < 0.01$, statistically significant difference. **(B)** Representative results are shown. The GFP signal was detected by UV light exposure, and the cell morphology was captured by phase-contrast light microscopy and the bright field image was merged with the GFP image. Magnification, 40 ×. Scale bar, 20 μm.

[0088] **Figure 23(A-E)** shows effect of **ZRL₅P₂**/**ZRL₅P₄**/**ZRL₅P₆** on the weights of vital organs. Weights of the **(A)** heart, **(B)** lung, **(C)** liver, **(D)** kidney and **(E)** spleen were normalized to mouse body weights at the experimental endpoint. Data are expressed as the mean ± SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.005$ vs. control. Treatment with **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** did not cause any significant changes in organ weights when compared to the control.

[0089] **Figure 24(A-D)** shows in vivo anti-tumor activity of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** in EBV-negative HeLa xenografts. Mice were treated every three days with 4 μg/ injection of the probes for 19 days. Throughout the treatment period, **(A)** tumor volumes and **(B)** body weights were measured. At the experimental endpoint, tumors were excised and **(C)** tumor weights and **(D)** representative photographs of tumors. Data are expressed as means ± SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.005$ vs. control. Scale bar: 10 mm. There was no significant difference in both the tumor volume and tumor weight between the control mice and those treated with probes. Also, treatment with **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** did not cause any significant changes in body weights compared to the control.

DETAILED DESCRIPTION

[0090] The present disclosure is not to be limited in scope by any of the specific embodiments described herein. The following embodiments are presented for

exemplification only.

[0091] Those skilled in the art will appreciate that the methods and compositions described herein are susceptible to variations and modifications other than those specifically described.

[0092] The present disclosure includes all such variations and modifications. The present disclosure also includes all of the steps and features referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0093] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the present invention.

[0094] Furthermore, throughout the specification and claims, unless the context requires otherwise, the word "include" or variations such as "includes" or "including", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0095] As used herein, "halo", "halogen", or halide refer to fluorine, chlorine, bromine, and iodine.

[0096] As used herein, "alkyl" refers to a straight-chain or branched saturated hydrocarbon group. Examples of alkyl groups include methyl (Me), ethyl (Et), propyl (e.g., n-propyl and z'-propyl), butyl (e.g., n-butyl, z'-butyl, sec-butyl, *tert-butyl*), pentyl groups (e.g., n-pentyl, z'-pentyl, -pentyl), hexyl groups, and the like. In various embodiments, an alkyl group can have 1 to 40 carbon atoms (i.e., C1-40 alkyl group), for example, 1-30 carbon atoms (i.e., C1-30 alkyl group). In some embodiments, an alkyl group can have 1 to 6 carbon atoms, and can be referred to as a "lower alkyl group." Examples of lower alkyl groups include methyl, ethyl, propyl (e.g., n-propyl and z'-propyl), and butyl groups (e.g., n-butyl, z'-butyl, *sec-butyl*, *tert-butyl*). In some embodiments, alkyl groups can be substituted as described herein. An alkyl group is generally not substituted with another alkyl group, an alkenyl group, or an alkynyl group.

[0097] As used herein, "cycloalkyl" by itself or as part of another substituent means, unless otherwise stated, a monocyclic hydrocarbon having between 3-12 carbon atoms in the ring system and includes hydrogen, straight chain, branched chain, and/or cyclic substituents. Exemplary cycloalkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and the like.

[0098] As used herein, "alkenyl" refers to a straight-chain or branched alkyl group having one or more carbon-carbon double bonds. Examples of alkenyl groups include ethenyl, propenyl, butenyl, pentenyl, hexenyl, butadienyl, pentadienyl, hexadienyl groups, and the like. The one or more carbon-carbon double bonds can be internal (such as in 2-butene) or terminal (such as in 1-butene). In various embodiments, an alkenyl group can have 2 to 40 carbon atoms (i.e., C2-40 alkenyl group), for example,

2 to 20 carbon atoms (i.e., C2-20 alkenyl group). In some embodiments, alkenyl groups can be substituted as described herein. An alkenyl group is generally not substituted with another alkenyl group, an alkyl group, or an alkynyl group.

[0099] As used herein, a "fused ring" or a "fused ring moiety" refers to a polycyclic ring system having at least two rings where at least one of the rings is aromatic and such aromatic ring (carbocyclic or heterocyclic) has a bond in common with at least one other ring that can be aromatic or non-aromatic, and carbocyclic or heterocyclic. These polycyclic ring systems can be highly p-conjugated and optionally substituted as described herein.

[00100] As used herein, "heteroatom" refers to an atom of any element other than carbon or hydrogen and includes, for example, nitrogen, oxygen, silicon, sulfur, phosphorus, and selenium.

[00101] As used herein, "aryl" refers to an aromatic monocyclic hydrocarbon ring system or a polycyclic ring system in which two or more aromatic hydrocarbon rings are fused (i.e., having a bond in common with) together or at least one aromatic monocyclic hydrocarbon ring is fused to one or more cycloalkyl and/or cycloheteroalkyl rings. An aryl group can have 6 to 24 carbon atoms in its ring system (e.g., C6-24 aryl group), which can include multiple fused rings. In some embodiments, a polycyclic aryl group can have 8 to 24 carbon atoms. Any suitable ring position of the aryl group can be covalently linked to the defined chemical structure. Examples of aryl groups having only aromatic carbocyclic ring(s) include phenyl, 1-naphthyl (bicyclic), 2-naphthyl (bicyclic), anthracenyl (tricyclic), phenanthrenyl (tricyclic), pentacenyl (pentacyclic), and like groups. Examples of polycyclic ring systems in which at least one aromatic carbocyclic ring is fused to one or more cycloalkyl and/or cycloheteroalkyl rings include, among others, benzo

derivatives of cyclopentane (i.e., an indanyl group, which is a 5,6-bicyclic cycloalkyl/aromatic ring system), cyclohexane (i.e., a tetrahydronaphthyl group, which is a 6,6-bicyclic cycloalkyl/aromatic ring system), imidazoline (i.e., a benzimidazolyl group, which is a 5,6-bicyclic cycloheteroalkyl/aromatic ring system), and pyran (i.e., a chromenyl group, which is a 6,6-bicyclic cycloheteroalkyl/aromatic ring system). Other examples of aryl groups include benzodioxanyl, benzodioxolyl, chromanyl, indolyl groups, and the like. In some embodiments, aryl groups can be substituted as described herein. In some embodiments, an aryl group can have one or more halogen substituents, and can be referred to as a "haloaryl" group. Perhaloaryl groups, i.e., aryl groups where all of the hydrogen atoms are replaced with halogen atoms (*e.g.*, -C₆F₅), are included within the definition of "haloaryl." In certain embodiments, an aryl group is substituted with another aryl group and can be referred to as a biaryl group. Each of the aryl groups in the biaryl group can be substituted as disclosed herein.

[00102] As used herein, "heteroaryl" refers to an aromatic monocyclic ring system containing at least one ring heteroatom selected from oxygen (O), nitrogen (N), sulfur (S), silicon (Si), and selenium (Se) or a polycyclic ring system where at least one of the rings present in the ring system is aromatic and contains at least one ring heteroatom. Polycyclic heteroaryl groups include those having two or more heteroaryl rings fused together, as well as those having at least one monocyclic heteroaryl ring fused to one or more aromatic carbocyclic rings, non-aromatic carbocyclic rings, and/or non-aromatic cycloheteroalkyl rings. A heteroaryl group, as a whole, can have, for example, 5 to 24 ring atoms and contain 1-5 ring heteroatoms (i.e., 5-20 membered heteroaryl group). The heteroaryl group can be attached to the defined chemical structure at any heteroatom or carbon atom that results in a stable structure.

Generally, heteroaryl rings do not contain O-O, S-S, or S-O bonds. However, one or more N or S atoms in a heteroaryl group can be oxidized (e.g., pyridine N-oxide, thiophene S-oxide, thiophene S,S-dioxide). Examples of heteroaryl groups include, for example, the 5- or 6-membered monocyclic and 5-6 bicyclic ring systems shown below: where T is O, S, NH, N-alkyl, N-aryl, N-(arylalkyl) (e.g., N-benzyl), SiH₂, SiH(alkyl), Si(alkyl)₂, SiH(arylalkyl), Si(arylalkyl)₂, or Si(alkyl)(arylalkyl). Examples of such heteroaryl rings include pyrrolyl, furyl, thienyl, pyridyl, pyrimidyl, pyridazinyl, pyrazinyl, triazolyl, tetrazolyl, pyrazolyl, imidazolyl, isothiazolyl, thiazolyl, thiadiazolyl, isoxazolyl, oxazolyl, oxadiazolyl, indolyl, isoindolyl, benzofuryl, benzothienyl, quinolyl, 2-methylquinolyl, isoquinolyl, quinoxalyl, quinazolyl, benzotriazolyl, benzimidazolyl, benzothiazolyl, benzisothiazolyl, benzisoxazolyl, benzoxadiazolyl, benzoxazolyl, cinnolinyl, 1H-indazolyl, 2H-indazolyl, indoliziny, isobenzofuryl, naphthyridinyl, phthalazinyl, pteridinyl, purinyl, oxazolopyridinyl, thiazolopyridinyl, imidazopyridinyl, furopyridinyl, thienopyridinyl, pyridopyrimidinyl, pyridopyrazinyl, pyridopyridazinyl, thienothiazolyl, thienoxazolyl, thienoimidazolyl groups, and the like. Further examples of heteroaryl groups include 4,5,6,7-tetrahydroindolyl, tetrahydroquinolyl, benzothienopyridinyl, benzofuropyridinyl groups, and the like. In some embodiments, heteroaryl groups can be substituted as described herein.

[00103] The compounds described herein may include one or more groups that can exist as stereoisomers. All such stereoisomer isomers are contemplated by the present disclosure. In instances in which stereochemistry is indicated (for example E/Z double bond isomers), it is understood that for the sake of simplicity that only one stereoisomer is depicted. However, all stereoisomers and mixtures thereof are contemplated by the present disclosure.

[00104] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

[00105] The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[00106] The term "EBV-positive cancer cells" refers to those cancers and cancer cells which express, e.g., in a latent form, EBV. Examples of such cancers include, but are not limited to, nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's Disease, T-cell lymphoma, B-cell lymphoma, transplant-associated lymphoproliferative disorders, gastric carcinoma, parotid carcinoma, breast carcinoma, leiomyosarcoma. In certain embodiments EBV-positive cancers are cancers wherein greater than about 30%, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, or greater than about 80% contain the EBV virus. The most preferred cancer and/or cancer cell for treatment with the method of the invention is nasopharyngeal carcinoma (NPC).

[00107] The term a "therapeutically effective amount" of an agent as used herein is that amount sufficient to effect beneficial or desired results, including clinical results, and, as such, a "therapeutically effective amount" depends upon the context in which it is being applied. For example, in the context of administering a compound that

inhibits EBV-positive cancer cell proliferation, an effective amount of a compound is, for example, an amount sufficient to achieve such an inhibition as compared to the response obtained without administration of the agent.

[00108] The term "pharmaceutically acceptable salt" or "salt" refers to a salt of one or more compounds. Suitable pharmaceutically acceptable salts of compounds include acid addition salts which may, for example, be formed by mixing a solution of the compound with a solution of a pharmaceutically acceptable acid, such as hydrochloric acid, hydrobromic acid, sulfuric acid, fumaric acid, maleic acid, succinic acid, benzoic acid, acetic acid, citric acid, tartaric acid, phosphoric acid, carbonic acid, or the like. Where the compounds carry one or more acidic moieties, pharmaceutically acceptable salts may be formed by treatment of a solution of the compound with a solution of a pharmaceutically acceptable base, such as lithium hydroxide, sodium hydroxide, potassium hydroxide, tetraalkylammonium hydroxide, lithium carbonate, sodium carbonate, potassium carbonate, ammonia, alkylamines, or the like. Pharmaceutically acceptable salts can also include zwitterions.

[00109] The term "composition" is intended to encompass a product comprising the specified ingredients in the specified amounts, as well as any product that results, directly or indirectly, from combinations of the specified ingredients in the specified amounts.

[00110] The term "pharmaceutically acceptable carrier" refers to a medium that is used to prepare a desired dosage form of a compound. A pharmaceutically acceptable carrier can include one or more solvents, diluents, or other liquid vehicles; dispersion or suspension aids; surface active agents; isotonic agents; thickening or emulsifying agents; preservatives; solid binders; lubricants; and the like. Remington's Pharmaceutical Sciences, Fifteenth Edition, E. W. Martin (Mack Publishing Co.,

Easton, Pa., 1975) and Handbook of Pharmaceutical Excipients, Third Edition, A. H. Kibbe ed. (American Pharmaceutical Assoc. 2000), disclose various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof.

[00111] The term "subject" as used herein, refers to an animal, typically a mammal or a human, that will be or has been the object of treatment, observation, and/or experiment. When the term is used in conjunction with administration of a compound or drug, then the subject has been the object of treatment, observation, and/or administration of the compound or drug.

[00112] As used herein, and as well understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

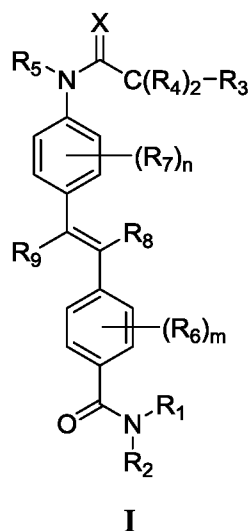
[00113] To "inhibit" or "suppress" or "reduce" a function or activity, such as EBV-positive cancer cell proliferation, is to reduce the function or activity when compared to otherwise same conditions except for a condition or parameter of interest, or alternatively, as compared to another condition(s).

[00114] Other definitions for selected terms used herein may be found within the detailed description of the present disclosure and apply throughout. Unless otherwise defined, all other technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the present disclosure belongs.

[00115] Other aspects and advantages of the present invention will be apparent to those skilled in the art from a review of the ensuing description.

[00116] The compounds provided herein can comprise an intramolecular charge transfer (ICT)-characterized fluorophore, a peptide comprising a peptide inhibitor sequence and optionally a nucleus localization sequence (NLS), and a zinc binding moiety to increase the inhibitory effect against EBV-positive cancers and maintain the responsive signal of the compound. The resulting compound has improved inhibition on EBNA1, as indicated by MTT assay. Luminescence titration showed that compounds provided herein are highly selective towards EBNA1 over HSA, in addition, the strong binding to EBNA1 was also demonstrated in the presence of Zn^{2+} . The compounds provided herein not only provide a powerful and promising probe for use in the treatment, imaging, and diagnosis of EBV-positive cells (e.g., cancer cells).

[00117] Provided herein is a compound of Formula I:



[00118] or a pharmaceutically acceptable salt thereof, wherein

[00119] m is a whole number selected from 0-4;

[00120] n is a whole number selected from 0-4;

[00121] p is a whole number selected from 0-4;

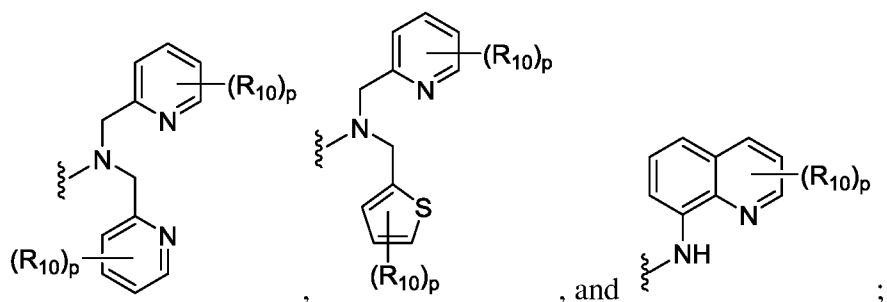
[00122] X is H_2 , S, or O;

[00123] each instance of R is independently H or alkyl;

[00124] R_1 is selected from the group consisting of SEQ ID NO:1 (CYFMVF), SEQ ID NO:2 (YFMVF), SEQ ID NO:3 (CAhxRrRKGGYFMVF), SEQ ID NO:4 (CAhxYFMVFGGRrRK), SEQ ID NO:5 (AhxYFIVFGGRrRK), and SEQ ID NO:6 (YFIVF);

[00125] R_2 is H or alkyl;

[00126] R_3 is selected from the group consisting of:



[00127] each instance of R_4 is independently H or alkyl;

[00128] R_4 is H or alkyl;

[00129] R_5 is H or alkyl;

[00130] each instance of R_6 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[00131] each instance of R_7 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

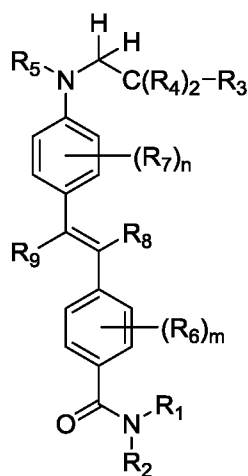
[00132] each of R_8 and R_9 is independently H or alkyl; and

[00133] each instance of R_{10} is independently H, alkyl, alkene, alkyne, cycloalkyl,

aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂.

[00134] The compounds described herein include an olefin. The olefin can exist as an (E)-isomer, a (Z)-isomer, or a combination thereof. In certain embodiments, the olefin is an (E)-isomer.

[00135] In instances in which X is H₂, the compound of Formula I can be represented by the following structure:

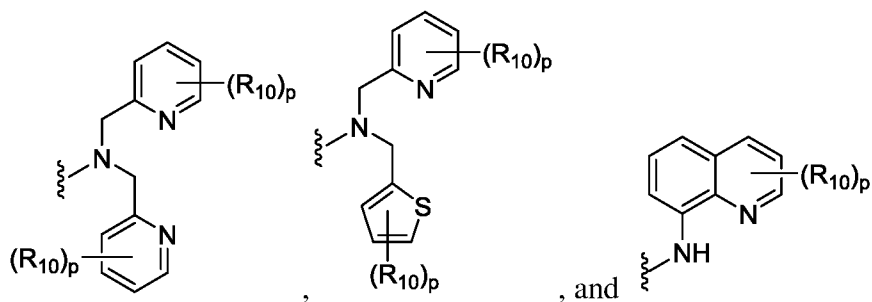


[00136] In certain embodiments, R₁ is selected from the group consisting of SEQ ID NO:2 (YFMVF), SEQ ID NO:4 (CAhxYFMVFGGRrRK), and SEQ ID NO:6 (YFIVF).

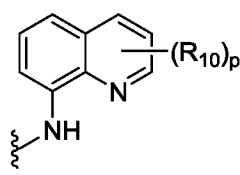
[00137] In certain embodiments, each of m, n, and p is independently a whole number selected from 0, 1, and 2. In certain embodiments, each of m, n, and p is independently a whole number selected from 0 and 1.

[00138] In certain embodiments, R₂ is H or C₁-C₆ alkyl. In certain embodiments, R₂ is H.

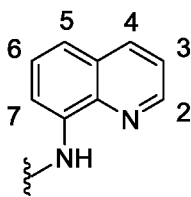
[00139] In certain embodiments, R₃ is selected from the group consisting of:



[00140] In instances in which R_3 is



between 1-4 instances of R_{10} can be bound to positions 2, 3, 4, 5, 6, or 7 as shown below:



[00141] In certain embodiments, R_4 is H or C_1 - C_6 alkyl. In certain embodiments, R_4 is H.

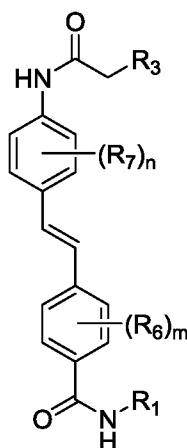
[00142] In certain embodiments, R_5 is H or C_1 - C_6 alkyl. In certain embodiments, R_5 is H.

[00143] In certain embodiments, each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, C_1 - C_6 alkyl, C_2 - C_6 alkene, C_2 - C_6 alkyne, C_3 - C_6 cycloalkyl, C_6 - C_{10} aryl, heteroaryl, halide, nitrile, nitro, -OR, and NR_2 , wherein R for each occurrence is H or C_1 - C_6 alkyl. In certain embodiments, each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, C_1 - C_6 alkyl, halide, nitrile, and nitro. In certain embodiments, each of

R₆, R₇, and R₁₀ is H.

[00144] In certain embodiments, each of R₈ and R₉ is H or C₁-C₆ alkyl. In certain embodiments, R₈ and R₉ are each H.

[00145] In certain embodiments, the compound has Formula II:



II

[00146] or a pharmaceutically acceptable salt thereof, wherein

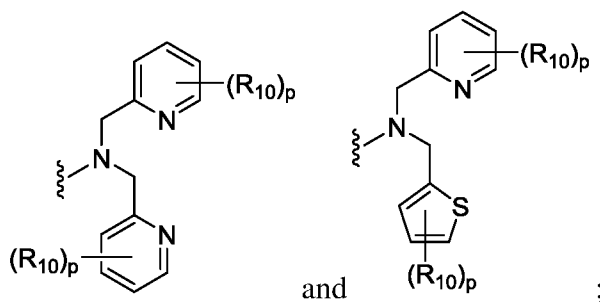
[00147] m is a whole number selected from 0-4;

[00148] n is a whole number selected from 0-4;

[00149] p is a whole number selected from 0-4;

[00150] R₁ is selected from the group consisting of SEQ ID NO:1 (CYFMVF), SEQ ID NO:2 (YFMVF), SEQ ID NO:3 (CAhxRrRKGGYFMVF), SEQ ID NO:4 (CAhxYFMVFGGRrRK), SEQ ID NO:5 (AhxYFIVFGGRrRK), and SEQ ID NO:6 (YFIVF);

[00151] R₃ is selected from the group consisting of:



[00152] each instance of R_6 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[00153] each instance of R_7 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[00154] each of R_8 and R_9 is independently H or alkyl; and

[00155] each instance of R_{10} is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 .

[00156] In certain embodiments of the compound of Formula **II**, each of m, n, and p is independently selected from 0, 1, and 2.

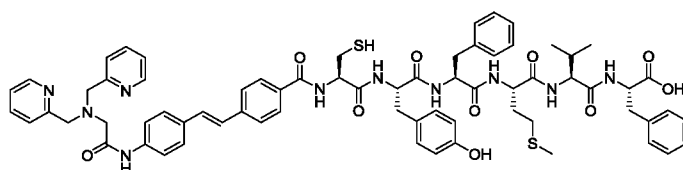
[00157] In certain embodiments of the compound of Formula **II**, each instance of R_6 and R_7 is independently H, C_1 - C_6 alkyl, C_2 - C_6 alkene, C_2 - C_6 alkyne, C_3 - C_6 cycloalkyl, C_6 - C_{10} aryl, heteroaryl, halide, nitrile, nitro, -OR, or NR_2 , wherein R for each occurrence is H or C_1 - C_6 alkyl. In certain embodiments of the compound of Formula **II**, each instance of R_6 and R_7 is independently H, C_1 - C_6 alkyl, halide, nitrile, or nitro. In certain embodiments of the compound of Formula **II**, each R_6 and R_7 is H.

[00158] In certain embodiments of the compound of Formula **II**, each of R_8 and R_9 is independently H or C_1 - C_6 alkyl. In certain embodiments of the compound of Formula **II**, R_8 and R_9 are each H.

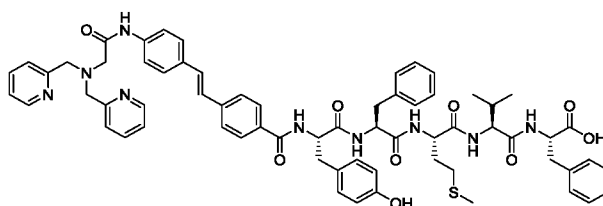
[00159] In certain embodiments of the compound of Formula **II**, each instance of

R₁₀ is independently H, C₁-C₆ alkyl, C₂-C₆ alkene, C₂-C₆ alkyne, C₃-C₆ cycloalkyl, C₆-C₁₀ aryl, heteroaryl, halide, nitrile, nitro, -OR, or NR₂, wherein R for each occurrence is H or C₁-C₆ alkyl. In certain embodiments of the compound of Formula **II**, each instance of R₁₀ is independently H, C₁-C₆ alkyl, halide, nitrile, or nitro. In certain embodiments of the compound of Formula **II**, R₁₀ is H.

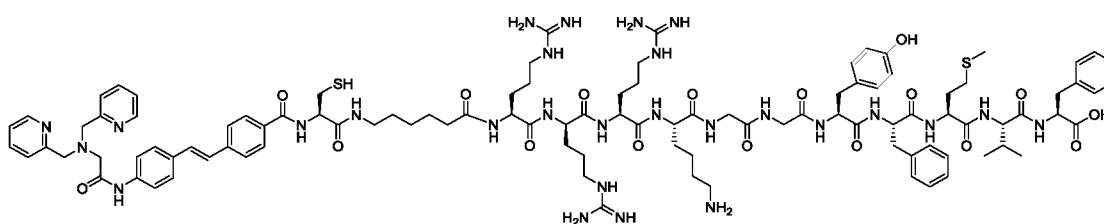
[00160] In certain embodiments of the compound is selected from the group consisting of:



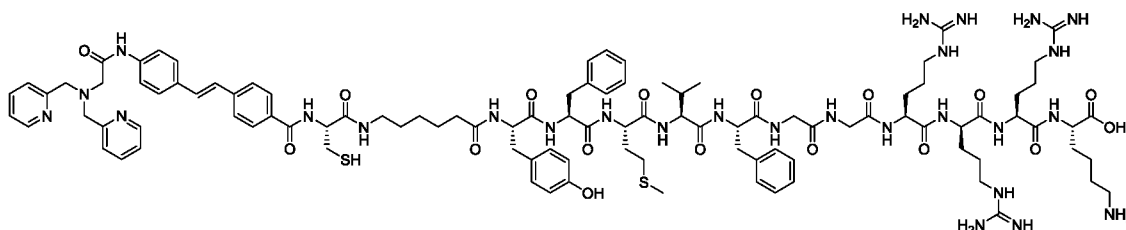
ZRL₅P₁ [SEQ ID NO:1 (CYFMVF)];



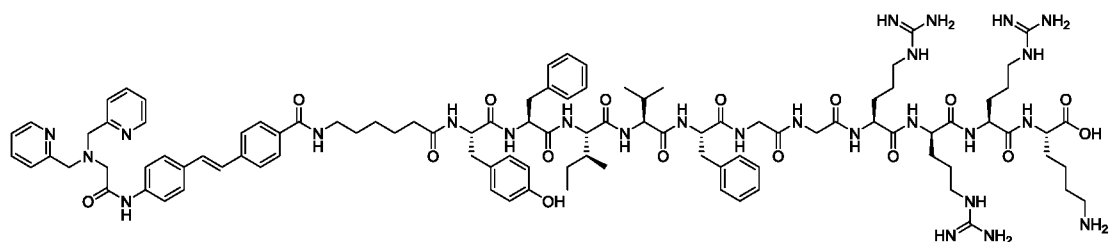
ZRL₅P₂[SEQ ID NO:2 (YFMVF)];



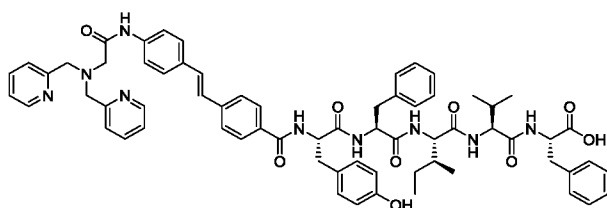
ZRL₅P₃[SEQ ID NO:3 (CAh_xRrRKGGYFMVF)];



ZRL₅P₄[SEQ ID NO:4 (CA_hxYFMVFGGRrRK)];



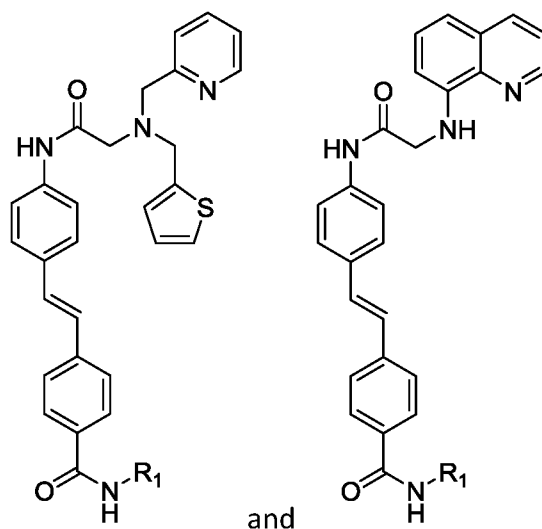
ZRL₅P₆ [SEQ ID NO:5 (AhxYFIVFGGRrRK)]; and



ZRL₅P₅ [SEQ ID NO:6 (YFIVF)]

or a pharmaceutically acceptable salt thereof.

[00161] In certain embodiments, the compound is selected from the group consisting of:

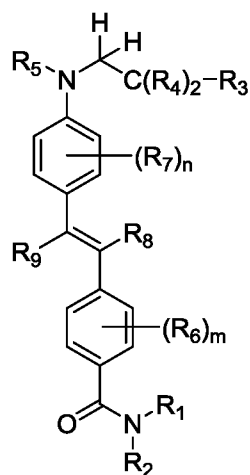


and

[00162] or a pharmaceutically acceptable salt thereof, wherein R₁ is selected from the group consisting of SEQ ID NO:1 (CYFMVF), SEQ ID NO:2 (YFMVF), SEQ ID NO:3 (CAhxRrRKGGYFMVF), SEQ ID NO:4 (CAhxYFMVFGGRrRK), SEQ ID NO:5 (AhxYFIVFGGRrRK), and SEQ ID NO:6 (YFIVF).

[00163] In certain embodiments, the compound has the Formula **IV**:

32



IV

[00164] or a pharmaceutically acceptable salt thereof, wherein

[00165] m is a whole number selected from 0-4;

[00166] n is a whole number selected from 0-4;

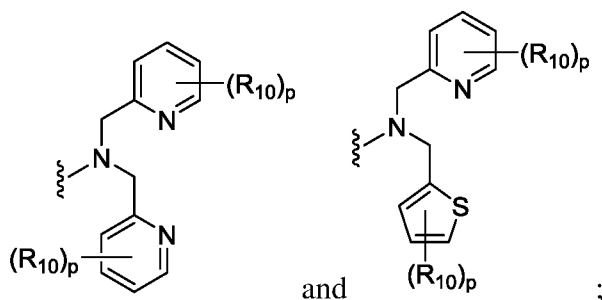
[00167] p is a whole number selected from 0-4;

[00168] each instance of R is independently H or alkyl;

[00169] R_1 is selected from the group consisting of SEQ ID NO:1 (CYFMVF), SEQ ID NO:2 (YFMVF), SEQ ID NO:3 (CAhxRrRKGGYFMVF), SEQ ID NO:4 (CAhxYFMVFGGGrRK), SEQ ID NO:5 (AhxYFIVFGGGrRK), and SEQ ID NO:6 (YFIVF);

[00170] R_2 is H or alkyl;

[00171] R_3 is selected from the group consisting of:



[00172] each instance of R_4 is independently H or alkyl;

[00173] R_4 is H or alkyl;

[00174] R_5 is H or alkyl;

[00175] each instance of R_6 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[00176] each instance of R_7 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

[00177] each of R_8 and R_9 is independently H or alkyl; and

[00178] each instance of R_{10} is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 .

[00179] In certain embodiments of the compound of Formula **IV**, each of m, n, and p is independently selected from 0, 1, or 2.

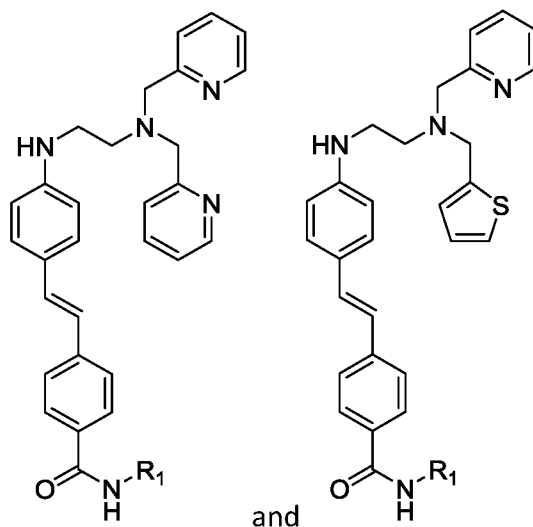
[00180] In certain embodiments of the compound of Formula **IV**, R_2 and R_5 are each H.

[00181] In certain embodiments of the compound of Formula **IV**, R_6 , R_7 , and R_{10} for each instance is independently selected from the group consisting of H, C_1 - C_6 alkyl, C_2 - C_6 alkene, C_2 - C_6 alkyne, C_3 - C_6 cycloalkyl, C_6 - C_{10} aryl, heteroaryl, halide, nitrile, nitro, -OR, and NR_2 , wherein R for each occurrence is H or C_1 - C_6 alkyl. In certain embodiments, R_6 , R_7 , and R_{10} for each instance is independently H, C_1 - C_6 alkyl, halide, nitrile, or nitro. In certain embodiments, R_6 , R_7 , and R_{10} are each H.

[00182] In certain embodiments of the compound of Formula **IV**, R_8 and R_9 are each H.

[00183] In certain embodiments of the compound of Formula **IV**, the compound is

selected from the group consisting of:



[00184] or a pharmaceutically acceptable salt thereof, wherein R_1 is selected from the group consisting of SEQ ID NO:1 (CYFMVF), SEQ ID NO:2 (YFMVF), SEQ ID NO:3 (CAhxRrRKGGYFMVF), SEQ ID NO:4 (CAhxYFMVFGGRrRK), SEQ ID NO:5 (AhxYFIVFGGRrRK), and SEQ ID NO:6 (YFIVF).

[00185] The present disclosure also provides a pharmaceutical composition comprising any one of the compounds described herein and at least one pharmaceutically acceptable excipient.

[00186] The compounds described herein and their pharmaceutically acceptable salts can be administered to a subject either alone or in combination with pharmaceutically acceptable carriers or diluents in a pharmaceutical composition according to standard pharmaceutical practice. The compounds can be administered orally or parenterally. Parenteral administration includes intravenous, intramuscular, intraperitoneal, subcutaneous and topical, the preferred method being intravenous administration.

[00187] Accordingly, the present disclosure provides pharmaceutically acceptable compositions, which comprise a therapeutically-effective amount of one or more of the compounds described herein, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. The pharmaceutical compositions of the present disclosure may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; and (2) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, e.g., those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue. The preferred method of administration of compounds of the present invention is parental administration (intravenous).

[00188] As set out herein, certain embodiments of the compounds described herein may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. The term "pharmaceutically-acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* in the administration vehicle or the dosage form manufacturing process, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed during subsequent purification. Representative salts include the bromide, chloride, sulfate, bisulfate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate,

phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like.

[00189] The pharmaceutically acceptable salts of the compounds of the present disclosure include the conventional nontoxic salts or quaternary ammonium salts of the compounds, e.g., from nontoxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloride, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

[00190] In other cases, the compounds described herein may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared in situ in the administration vehicle or the dosage form manufacturing process, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like.

[00191] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives, solubilizing agents, buffers and antioxidants can also be present in the compositions.

[00192] Methods of preparing these formulations or compositions include the step of bringing into association a compound described herein with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers (liquid formulation), liquid carriers followed by lyophilization (powder formulation for reconstitution with sterile water or the like), or finely divided solid carriers, or both, and then, if necessary, shaping or packaging the product.

[00193] Pharmaceutical compositions of the present disclosure suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, chelating agents, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. In the examples, the active ingredients are brought together with the pharmaceutically acceptable carriers in solution and then lyophilized to yield a dry powder. The dry powder is packaged in unit dosage form and then reconstituted for parental administration by adding a sterile solution, such as water or normal saline, to the powder.

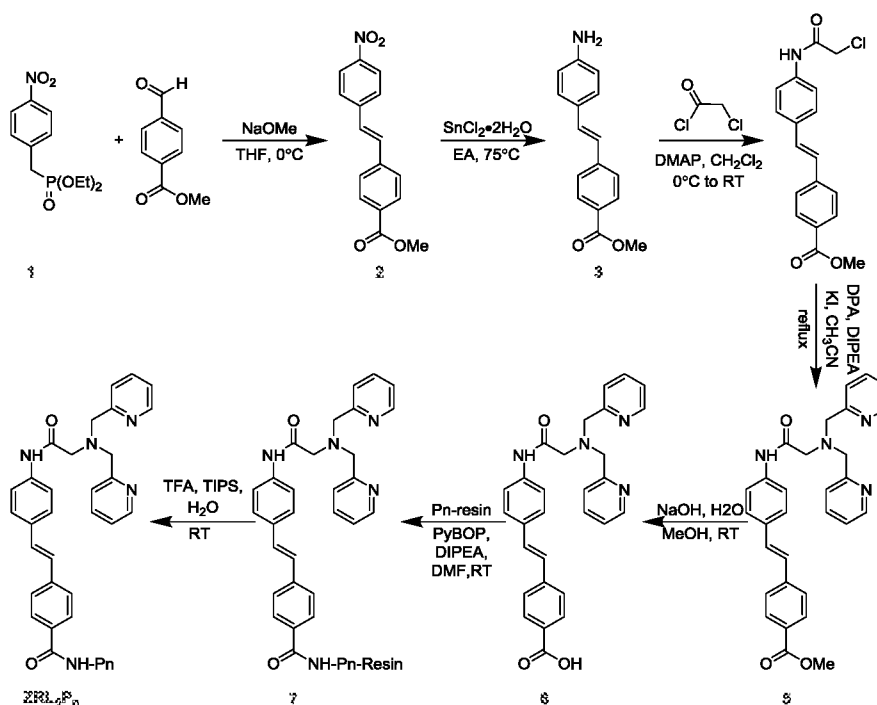
[00194] Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[00195] These compositions may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms upon the compounds of the present invention may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[00196] In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

[00197] **Synthesis.** An exemplary synthesis of ZRL_5P_n is shown in Scheme 1. The synthetic route started from a Horner-Wadsworth-Emmons olefination to prepare **2**.

Reduction of the nitro group in **2** using stannous chloride gave **3**, which was then reacted with chloroacetyl chloride to produce **4**. Reaction of **4** with di-2-picolyamine (DPA) using N,N-diisopropylethylamine (DIPEA) as base followed by ester saponification and peptide conjugation gave **7**. Cleavage of the resin on **7** produced the target probe **ZRL₅P_n**.



Scheme 1 Synthetic route of **ZRL₅P₂**/**ZRL₅P₄**/**ZRL₅P₆** (**P₂**, SEQ ID NO:2 (YFMVF); **P₄**, SEQ ID NO:4 (CAhxYFMVFGGRrRK); **P₆**, SEQ ID NO:5 (AhxYFIVFGGRrRK)).

[00198] The compound described herein can bind to and inhibit the dimerization of EBNA1. Consequently, the compounds described herein can be used to treat conditions or disease states in which inhibition of the dimerization of EBNA1 has a therapeutic effect. Provided herein is a method of treating an Epstein-Barr virus (EBV)-positive cell in a patient in need thereof comprising administering a therapeutically effective amount of a compound as described herein to the patient. In certain embodiments, the EBV-positive cell is a cancerous cell. In certain

embodiments, the cancerous cell is Burkitt's lymphoma, non-Hodgkin's lymphoma, Hodgkin's disease, T-cell lymphoma, B-cell lymphoma, transplant-associated lymphoproliferative disorders, nasopharyngeal carcinoma, gastric adenocarcinoma, parotid carcinoma, or leiomyosarcoma.

[00199] Also provided is a method of imaging an EBV-positive cell comprising the step of contacting the EBV-positive cell with a compound as described herein and measuring the fluorescence of the compound. In certain embodiments, the step of measuring the fluorescence comprises two-photon excitation microscopy. In certain embodiments, the step of measuring the fluorescence comprises comprising irradiating the EBV-positive cell in the presence of the compound as described herein with light at a wavelength between 650 to 750 nm and detecting emitted light.

[00200] EBNA1 has several functional domains, among them, there is a small domain, termed unique region 1 ("UR1", a.a. 64-89), was reported to coordinate zinc through a pair of cysteines contained within it. Point mutations of the cysteines in UR1 eliminates the ability of EBNA1 to coordinate zinc, self-associate, and activate transcription. Thus, the coordination of zinc is critical for the self-association, transactivation and transactivation-related cellular functions of EBNA1.

[00201] The compounds provided herein not only localized to the nucleus where EBNA1 is primarily located, but also showed responsive emission towards the binding of EBNA1.

[00202] In order to determine whether the overall efficacy of a probe could be enhanced by taking advantage of the necessity of zinc coordination by EBNA1, an all-in-one probe, **ZRL₅P₄**, was designed and synthesized (Figure 6A).

[00203] In this scaffold, an amide group was used to link two moieties-the peptide

conjugated fluorophore and a DPA zinc chelator. The amide linked DPA served as a responsive zinc chelator, and amide tautomerization with red-shifted emission can be triggered when it coordinates zinc. The N atom in the amide linker can also act as donor, with the carbonyl group in the other side of the probe serving as the acceptor, thus the ICT (intramolecular charge transfer) characterized emission with enhanced blue-shifted emission can be triggered if the probe binds EBNA1. Thereby, the dual-responsive emission can be reflected for the probe when binding Zn^{2+} and/or EBNA1. Advantageously, as described in detail below, the zinc binding moiety and the peptide motif in the compound of Formula I can function synergistically to inhibit EBNA1, such as to inhibit in self-association of EBNA1 and transactivation by EBNA1, and the blockage on EBNA1 dimerization, and thus achieved better inhibitory effect as shown in Figure 1.

[00204] A critical constraint for the development of overexpressed protein targeting cancer therapy is the genetic variants in different human species. For EBV associated cancers, as the only viral protein expressed in all EBV-associated tumors, EBNA1 plays a vital role in regulating viral genome in the infected cells. Thus, EBNA1 protein not only serves as a perfect marker for clinical imaging, but also a molecular target for treatment. Yet, current studies present that the gene varies frequently on EBNA1 that exists in Chinese and Indonesian nasopharyngeal carcinoma (NPC) samples. Under this circumstance, the inhibition of EBV associated cancer growth has also been weakened by the reported EBNA1 targeting agents.

[00205] It is known that zinc coordination by EBNA1 contributes to EBNA1 self-association and activates transcription by EBNA1. It is show that the blockage of zinc coordination by EBNA1 can increase the inhibitory effect. From preliminary data, the inhibition effect of EBV cancer development can be 4 times stronger than EBNA1

dimerization inhibitors using only a “single” approach (i.e., without a zinc binding site). The “twin” inhibition effect can be “directly” monitored by the dual-responsive emission change (Zn-21nm red shift of the emission and EBNA1-39nm blue shift of the emission; kinetic) in aqueous, *in vitro/in vivo* and the heat change by isothermal calorimetry titration (thermodynamic) in aqueous upon binding to EBNA1 (K_d , 19.1 μ M). The compounds described herein provide a new cancer inhibition approach – interference in both cellular cofactor and physical binding between the protein dimerization, which can be used in the treatment of EBV latently-infected tumors and puts forward the design strategy by incorporating other co-factors to enhance the overall treatment effect.

[00206] **Rational probe design and molecular dynamic simulations of the new zinc chelating probe ZRL₅P₄.** To determine the interactions between ZRL₅P₄ and EBNA1 DNA-binding domain (DBD), as well as to determine whether the Zn²⁺-bound ZRL₅P₄ might affect its interaction with the DBD, molecular dynamics (MD) simulations were conducted using the previous resolved structure of the EBNA1 DBD. The X-ray crystal structures have been solved only for EBNA1 DBD in its apo and DNA-bound forms, whereas the crystal structures of the rest of an EBNA1 monomer are not available at present. The X-ray crystal structure of EBNA1 DBD (protein database ID, 1B3T; chain A; residue 461 to 607) is an α/β -mixed fold comprised of four α -helix and four β -sheet motifs that are linked by several loops (Figure 1). The beta sheets, $\beta 1$ - $\beta 4$, drive formation of the dimer via hydrophobic interaction which represents the first EBNA1 dimerization interface which is located at the carboxyl-terminus. The ligand P₄ was first docked into the dimeric interface of the EBNA1 DBD monomer using the flexible peptide docking tool CABS. The previously defined P₄--EBNA1 complex model was chosen and subjected to a further

500 ns of simulation (Figure 7). The penta-peptide P_2 (YFMVF (SEQ ID NO:2)) can occupy the dimerization interface through a hydrophobic interaction, and the NLS tetrapeptide-RrRK can form salt bridges with the adjacent negatively charged residues (D_{601} , D_{602} , D_{605}) which further enhances the interaction, confirming that P_4 is a physical blocker of the EBNA1 dimerization.

[00207] The 3D models of the zinc-chelator and responsive fluorophore ZRL_5 in both Zn^{2+} -free and Zn^{2+} -coordinated scenarios were built and optimized with molecular mechanics and a conformational searching tool LowModeMD. The Zn^{2+} -coordinated DPA chelator fragment from the final optimized Zn^{2+} -- ZRL_5 superposed very well with the crystal structure of a highly similar compound Zn^{2+} -- ZTF^{11} (Figure 8), suggesting that the two configurations converged at the global energy minimum. Considering the five-coordinate complex formed in the Zn^{2+} -- ZTF crystal structure (one coordination site is occupied by the solvent molecule) and the high structural similarity between ZRL_5 and ZTF , models of $DMSO$ -- Zn^{2+} -- ZRL_5 and H_2O -- Zn^{2+} -- ZRL_5 were constructed, with the solvent O atom acting as an electron donor (Figure 9).

[00208] The tertiary interaction models of the Zn^{2+} -bound and -unbound ZRL_5P_4 with EBNA1 (ZRL_5P_4 --EBNA1 and Zn^{2+} -- ZRL_5P_4 --EBNA1 complexes) were constructed based on the aforementioned P_4 --EBNA1 DBD and ZRL_5/Zn^{2+} -- ZRL_5 models. The two interaction models were then subjected to 200 ns MD simulations for structure optimization and binding energy calculations (Figure 10-13). Both interaction models have demonstrated stable probe-protein interactions, and the final optimized models are shown in Figure 7B 6B (simulation #1, both complexes were simulated twice, and the second simulation is shown in Figure 11, 13 and 14). The featured interaction model of the P_4 --EBNA1 complex with the hydrophobic

interaction mediated by YFMVF (SEQ ID NO:2) and the salt bridge formed by RrRK, are well-conserved. The results indicate that the presence of Zn^{2+} did not affect **ZRL₅P₄** to interact with the EBNA1 DBD, this observation is further supported by the calculated binding energies using MMPBSA (Figure6B, Figure 14 and Table 1).

[00209] **Table 1. The calculated binding affinity between **ZRL₅P₄/Zn²⁺**-**ZRL₅P₄** and EBNA1 DBD.**

System	Simulation	GB (kcal/mol)	PB (kcal/mol)
ZRL₅P₄ -EBNA1 DBD	#1	-90.1365±8.5697	-88.8280±8.7654
	#2	-79.9369±11.5724	-78.9043±13.2484
Zn²⁺ - ZRL₅P₄ -EBNA1 DBD	#1	-71.6652±9.1082	-74.9505±10.8770
	#2	-69.1021±12.3023	-74.3209±13.8954

[00210] **Dual-responsive emission by the three new EBNA1 probes when binding to **Zn²⁺** and EBNA1.** The fluorescence spectral changes of the three new EBNA1 probes (**ZRL₅P₂**, **ZRL₅P₄**, **ZRL₅P₆**) in the presence of 2 equiv. of Zn^{2+} in aqueous solution [$\text{CH}_3\text{CN}/0.05\text{ M HEPES}$ (pH 7.4), 50:50] were firstly measured (Figure 2A and Figure 3A-B; all of the metal salts used in this work were perchlorates, which should be used with caution). Only **ZRL₅P₄** demonstrated a 21 nm redshifted emission (451 to 472nm) upon addition of Zn^{2+} , while the other two probes either showed a slightly blue shifted emission (**ZRL₅P₂**, 448 to 445 nm) or their maximum emission wavelength was unchanged (**ZRL₅P₆**, 438nm). So, the detailed kinetics of the redshifted emission by **ZRL₅P₄** was focused on. The luminescence of **ZRL₅P₄** upon titration with various concentrations of Zn^{2+} to check the binding stoichiometry was investigated. A gradually redshifted emission with a concomitant decrease in the emission intensity was observed, and this trend ceased if 1 equiv. of Zn^{2+} was added,

indicating that the **ZRL₅P₄**-Zn²⁺ complex had 1:1 stoichiometry (Figure 2B). Additionally, the selectivity of **ZRL₅P₄** towards Zn²⁺ over various heavy and transition metal ions was also measured (Figure 4A-B). The results show that Cd²⁺, Cu²⁺, Co²⁺, and Ni²⁺ (Figure 4A) differentially decreased the emission intensity with Cd²⁺ displayed a 10 nm blue shifted emission, and Hg²⁺ and Mg²⁺ (Figure 4B) barely affected the **ZRL₅P₄** emission; none of these results indicate that competitive responsive emission was induced by Zn²⁺.

[00211] The EBNA1-binding activity of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** were next investigated. Before performing the assay, a solvation study was performed to determine the intramolecular charge transfer (ICT) characteristics of each probe. The responsive signal emitted by the fluorophore of the EBNA1 probes when binding to EBNA1 was based on the principle of the ICT mechanism. It has been well-documented that ICT-mediated emission is strongly solvent-dependent; that is, emission is enhanced and blue shifted with the decrease of solvent polarity (Figure 2C). As shown in Figure 2A and Figure 5A-B, **ZRL₅P₄** demonstrated the best ICT characteristics, whereas **ZRL₅P₆** also showed enhancement of emission intensity but no blue shifted emission. Thus, **ZRL₅P₄** represented the best candidate probe and was assayed for its activity towards the EBNA1 DBD protein. It was found that the addition of 1.2 μM EBNA1 to **ZRL₅P₄** caused a 2.7-fold emission enhancement, which was blue shifted by 39 nm (466 to 427 nm) (Figure 2D). The irrelevant human serum albumin (HSA) was used as a negative control target protein, and the addition of HSA to **ZRL₅P₄** showed only a 1.2-fold increase of its emission intensity (Figure 2E). Figure 2E (inset) clearly shows that the binding of **ZRL₅P₄** was highly selective for EBNA1 over HSA. In addition, the fluorescence response of **ZRL₅P₄** to EBNA1 was also measured in the presence of Zn²⁺ (Figure 2F). A 2.4-fold emission

enhancement with a 51 nm blueshift (477 nm to 426 nm) was observed, demonstrating that the presence of Zn^{2+} did not affect the **ZRL₅P₄**-EBNA1 DBD binding, as reflected by the same order of enhanced emission without Zn^{2+} (Figure 2D). This observation was supported by the MD simulation results (Figure 6B). Moreover, this observation also indicates that the spectral response in the presence of Zn^{2+} will result in a larger blueshift with a slight emission enhancement of **ZRL₅P₄**.

[00212] **Confirmation of the interaction of ZRL₅P₄ with zinc(II) by nuclear magnetic resonance (NMR) study.** To characterize the mechanism of how **ZRL₅P₄** interacts with zinc(II), ¹H NMR titration experiments of **ZRL₅P₄** with and without 1 equiv. zinc(II) were conducted. As reported by a previous study, an amide-imidic acid tautomer binding mode was adopted by this zinc chelator in different solvents; in detail, the zinc(II) chelate complex is an amide tautomer in CH₃CN whereas it is an imidic tautomer in DMSO. To determine whether **ZRL₅P₄** can interact with zinc(II) in a similar way, the ¹H NMR spectroscopy of compound 5 (the precursor of **ZRL₅**) with/without zinc(II) in CD₃CN and DMSO-d₆ was conducted. Compound 5 was used as a substitute of **ZRL₅P₄** in this study because the presence of the peptide moiety will make it hard to assign peaks in the region of interest. Consistent with the previous work, proton 8 showed a large upfield shift from 10.93 to 9.43 ppm in CD₃CN, but was shifted downfield from 10.72 to 10.84 ppm in DMSO-d₆ (Figure 15A-B). This finding confirmed the two binding modes of **ZRL₅P₄** in MeCN and DMSO, thereby it can be inferred that the zinc(II)-triggered amide-tautomerization, which has been described in the previous study, will also occur in **ZRL₅P₄**. **ZRL₅P₄ diminishes EBNA1 homodimerization and transcription activated by EBNA1.** The ability of **ZRL₅P₄** to prevent EBNA1 dimerization in the absence and presence of Zn^{2+} was assayed and compared with **L₂P₄**. Since Zn^{2+} mediates zinc finger formation at

EBNA1 UR1 (a.a. 64-89) and **ZRL₅P₄** strongly binds Zn²⁺, the full-length EBNA1, rather than EBNA1 DBD, was employed in this assay. Without probe treatments, the presence of Zn²⁺ slightly increased EBNA1 dimerization (buffer, - or + Zn²⁺), confirming the role of Zn²⁺ in EBNA1 dimerization. **ZRL₅P₄** decreased EBNA1 dimer to a similar level as shown by **L₂P₄** since in the absence of Zn²⁺ dimerization inhibition comes only from the peptide moiety (left part in Figure 18A-B). However, the two probes behaved distinctly in the presence of Zn²⁺, **ZRL₅P₄** demonstrated a much stronger dimerization inhibition than **L₂P₄**. This observation indicates that the exhausting of Zn²⁺ by **ZRL₅P₄** resulted in a greater inhibition, and encouraged further exploration of the *in vitro* cytotoxicity of **ZRL₅P₄** in EBV-positive cells.

[00213] Before assaying the cytotoxicity of **ZRL₅P₄**, its ability to bind cellular zinc and affect EBNA1 transactivation was measured as shown in Figure 18C-D. EBV-positive C666-1 and NPC43 cells were exposed to **ZRL₅P₄**, two chelators known to have high specificity for zinc – ethylenediaminetetraacetic acid (EDTA) and N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), as well as **L₂P₄**. Both **ZRL₅P₄** and **L₂P₄** diminished transactivation by EBNA1 in dose-dependent manner, and the effects of **ZRL₅P₄** was more potent than **L₂P₄** in both C666-1 (p value = 0.00616) and NPC43 cell lines (p value = 0.00007).

[00214] **ZRL₅P₄ reduces the viability of EBV-positive cells and localizes to their nuclei.** The cell viability of a panel of EBV-negative (HK-1 and HONE-1 NPC cells) and EBV-positive (NPC43, C666-1, Raji lymphoma cells) cells treated with **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** (Figure 18E-I) was next measured. **ZRL₅P₂** was included to show that the nucleus permeability is critical for the cytotoxicity initiated by the zinc-chelator and the EBNA1 binding peptide, while **ZRL₅P₆** was included to examine if the variation of the EBNA1 binding sequence is essential for the tumor cell

growth. Cytotoxicity was measured by using the MTT assay. Treatment in the EBV-negative cells showed negligible inhibition over the range of 1-100 μM for all three probes (Figure 18E-F), whereas obvious cytotoxicity was observed in the EBV-positive cells (both EBV-positive nasopharyngeal carcinoma cells and EBV-positive Burkitt's lymphoma cells), even when treated with low dosages of 1-20 μM probes (Figure 18G-I). **ZRL₅P₄** and **ZRL₅P₆** displayed very similar growth-inhibitory effects, with **ZRL₅P₂** being the least efficient. When compared to the cytotoxicity study of **L₂P₄** (IC₅₀; 23 μM in NPC43, 27 μM in C666-1 and 27 μM in Raji cells), **ZRL₅P₄** is likely to be more potent (IC₅₀; 5 μM in NPC43, 13 μM in C666-1 and 12 μM in Raji cells), indicating that the exploitation of the zinc chelator will enhance the cytotoxic activity of the EBNA1 probes in EBV latently infected cells, regardless of epithelial or lymphoid origin. On the other hand, the results of **ZRL₅P₆** suggest that the variation in the amino acid sequence in the EBNA1 binding penta-peptide is not essential in inhibiting the *in vitro* biological activities.

[00215] Given that nuclear localization is a prerequisite for EBNA1 to maintain and replicate the EBV viral genome, a probe with nuclear permeability is preferred. The above MTT assay results also show that the entry into the nuclei is essential for the EBNA1 probes to inhibit the tumor cell viability. Therefore, the sub-cellular localization of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** was assessed in both EBV-positive (C666-1 and NPC43) and EBV-negative cell lines (HONE-1) under two-photon excitation microscopy (λ_{ex} : 700 nm). Two-photon excitation microscopy is superior for deeper tissue penetration and reduced photobleaching. After incubation for 3 h, the fluorescence signals of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** were collected in the blue channel. As shown in Figure 19A-C, **ZRL₅P₄** and **ZRL₅P₆** demonstrated clear nuclear localization in the EBV-positive cells with the assistance of the incorporated

NLS(RrRK) and hence achieved better growth inhibition (Figure 18E-18I), whereas **ZRL₅P₂** was only found in the cytoplasm (Figure 19A-B, 20F). Moreover, all three probes showed no nuclear permeability in EBV-negative cells (Figure 19C, 20F). Furthermore, majority of **ZRL₅P₄** and **ZRL₅P₆** was found in the nuclei of the EBV-infected cells, and this is in contrast to the imaging results of **L₂P₄** we previously reported, which show that majority of **L₂P₄** was localized to the cytoplasm, only a small portion was found in the nuclei.

[00216] **ZRL₅P₄ inhibits the growth of EBV-positive tumors in BALB/c mice.**

After the investigation of the *in vitro* cytotoxicity, the *in vivo* effects of the three new EBNA1 probes (**ZRL₅P₂**, **ZRL₅P₄**, **ZRL₅P₆**) were then examined. BALB/c nude mice transplanted with the EBV-positive C666-1 xenografts were treated with these probes (4 µg/injection), or with a vehicle control. Intra-tumoral injection was performed to deliver the agents to the animal, and injection was carried out biweekly. Body weight and tumor volumes were measured twice weekly. After an 18-day treatment period, mice were sacrificed and the tumors and organs were excised and weighed.

[00217] Tumor growth was significantly inhibited by treatment with **ZRL₅P₄** and **ZRL₅P₆**. At the end of the treatment period (day 18), the average tumor volume of mice treated with **ZRL₅P₄** was decreased by 41.9% ($p < 0.01$), compared to the control. Treatment with **ZRL₅P₆** led to a 29.6% decrease in tumor volume compared to the control ($p < 0.05$) (Figure 20A and 20D). There was no significant difference in both tumor volume and tumor weight when comparing the effects of **ZRL₅P₄** and **ZRL₅P₆**. **ZRL₅P₂** without NLS, however, did not significantly affect tumor growth at either dose. Tumors were also weighed at the experimental endpoint. Compared to the control, the average tumor weight was decreased by 49.2% ($p < 0.05$) and 31.4% ($p <$

0.05) after treatment with **ZRL₅P₄** and **ZRL₅P₆**, respectively (Figure 20C). As shown in Figure 20B and Figure 23A-E, treatment with **ZRL₅P₂**, **ZRL₅P₄**, or **ZRL₅P₆** did not cause significant changes in body or organ weights compared to the vehicle control, indicating that neither probe exhibited a toxic effect *in vivo*. Together, these results indicate that **ZRL₅P₄** and **ZRL₅P₆** are potential anti-EBV agents having no appreciable *in vivo* toxicity. Also, **ZRL₅P₄** performed better as a reporter probe in binding with EBNA1 and Zn²⁺. When the tumor sections were stained with H & E to examine the tissue morphology, cell necrosis was much more frequently observed in the tumor tissues with the treatment of **ZRL₅P₄** or **ZRL₅P₆** than the **ZRL₅P₂** and the solvent control (Figure 20E). The cell death could be due to the cytotoxic activities of **ZRL₅P₄** and **ZRL₅P₆** observed in the MTT assay for C666-1 cells (Figure 19B), and that can also explain why the tumors shrank after treatments with the new EBNA1 probes. For the HeLa EBV-negative xenografts (Figure 24A-D), there was no significant difference in both tumour volume and tumour weight between the control mice and those treated with probes.

[00218] **Reactivation of EBV lytic cycle by ZRL₅P₄**. To investigate the mechanism by which **ZRL₅P₄** and **ZRL₅P₆** resulted in the necrotic tumors *in vivo* (Figure 20E), it was next determined whether this phenomenon could be due to the induction of the EBV lytic gene products, as the full lytic induction will cause tumor cell death and release of EBV particles. The immediate early and early lytic proteins, BZLF1 (Zta) and BMRF1, were examined in the C666-1 derived tumor tissues in the above animal study (Figure 20A-E). The tumor sections were analyzed with immunohistochemistry (IHC), the EBV lytic proteins, Zta and BMRF1, were mainly detected in the tumors injected with **ZRL₅P₄** or **ZRL₅P₆**. Nuclear staining of these two lytic proteins was observed in tumor cells adjacent to the necrotic tumor tissues (Figure 21A). After

treatment with **ZRL₅P₄**, about 12.5 and 18.8% tumor areas were positive for Zta and BMRF1, respectively. On the other hand, only negative staining was detected in the solvent control for the two proteins, and 1.15 % was observed in **ZRL₅P₂** for BMRF1. The IHC results of Zta and BMRF1 in response to **ZRL₅P₆** were similar to the **ZRL₅P₄** (18.8% for Zta and 15.6% for BMRF1). Thus, these IHC results indicate that the necrotic tumor tissues could be the consequence of the induced lytic cycle by **ZRL₅P₄** and **ZRL₅P₆**.

[00219] The inventor also included the other EBV-infected cell line NPC43 for the lytic phase analysis, the gene expression of the immediate early and early lytic genes, *BRLF1* (*Rta*) and *BMLF1*, was studied in both NPC43 and C666-1 cell lines in response to 10 μ M **ZRL₅P₄**. The gene expression was detected by qRT-PCR. The gene expression of both *Rta* and *BMRF1* was strikingly induced by **ZRL₅P₄**, whereas their expression in response to **ZRL₅P₂** was similar to that of the solvent control (Figure 21B-C). The change was ~26- to ~59-fold in response to **ZRL₅P₄** in NPC43 cells, and ~6 to ~11-fold induction for C666-1 cells. The gene expression results of *Rta* and *BMRF1* were supported by the Western blot analysis which shows that the protein levels were specifically induced by **ZRL₅P₄** in the two NPC cell lines (Figure 21D). Besides these two early lytic proteins, the late protein gp350/220 for virion assembly was also induced by **ZRL₅P₄**. Although the expression of *Rta* and *BMRF1* was also induced by **ZRL₅P₂**, the increased levels of expression were much weaker than that of **ZRL₅P₄** and the gp350/220 levels were similar to the solvent control. It is likely that the entry of **ZRL₅P₄** to the nuclei is critical for the induction of late lytic protein expression.

[00220] In order to verify if the EBV particles could be actually generated by **ZRL₅P₄**, the recombinant EBV-containing HONE-1 (HONE-1-EBV) cell line was

used. The presence of the virions was detected by infecting Raji cells (an established B-cell line), because the recombinant EBV genome encodes a green fluorescence protein (GFP), the GFP-expressing Raji cells reflect the production of virions by the HONE-1-EBV cells. As can be seen, 10 μ M **ZRL₅P₄** could lead to the production of virions and which is 10.4-fold more than the DMSO control ($p = 0.009$), and is ~4-fold more than the NLS-null version **ZRL₅P₂** ($p = 0.006$) (Figure 22A-B). Although the viral titer of **ZRL₅P₄** was 2.6-fold higher than the solvent control but the difference is not significant ($p = 0.06$). Taken together, the entry of **ZRL₅P₄** into EBV-infected cells can induce the reactivation of EBV and release of the virions, and which can mediate shrinkage of the transplanted C666-1 tumors (Figure 20A-E) by causing the cell death of these tumor cells when exposed to **ZRL₅P₄**.

Discussion

[00221] Despite the effort spent on identifying and testing various well-known and novel EBV lytic inducing agents over recent decades, no specific agent against EBV genes or proteins has been established. The **ZRL₅P₄** probe might represent the first of its type to specifically disrupt the EBNA1 protein and to potently reactivate EBV from latency, leading to tumor cell lysis and/or induction of viral proteins that can be targeted by immune cells and anti-viral agents to eliminate EBV-infected tumor cells. The strategy of targeted reactivation of the latent viral genome and induction of cytotoxic effects in virus-driven tumors is known as cytolyticvirus activation (CLVA) therapy, and some EBV lytic inducers have recently entered phase I/II clinical trials. In addition, the *in vivo* administration of **ZRL₅P₄** was demonstrated to shrink the subcutaneous tumors, and the IHC staining results indicated that the necrotic tumor tissues might be due to an induction of EBV lytic cycle. The EBV early and late lytic gene/protein expression analyses and the *in vitro* RAJI cell infection assay have

shown that **ZRL₅P₄** could specifically induce the viral reactivation, which might result in tumor cell viability (as reflected by the MTT assay) and shrinkage of the EBV-associated tumors (derived from C666-1 cells) by disruption of the functional EBNA1 dimer. In concordance to this finding, it was reported that the depletion of EBNA1 gene expression by small interfering RNA (siRNA) in the EBV-infected epithelial cell lines could activate spontaneous lytic cycle induction, indicating that EBNA1 has a functional role in suppressing reactivation of EBV. The association of PML proteins and EBNA1 might provide an explanation of how **ZRL₅P₄** can induce the EBV lytic cycle. As can be seen from the current and other studies, it has become clear that EBNA1 is not limited to maintain the stable persistence of EBV episomes and it is also involved in other cellular functions including regulation of the viral reactivation which contribute to the cell proliferation and survival. The present report represents one of the few studies to demonstrate the functional role of EBNA1 and EBV in the *in vivo* tumor formation and lytic induction.

[00222] As mentioned earlier, previous studies show that Zn^{2+} is essential for EBNA1 to cooperatively activate the *oriP*-associated transcription and dimerize through the amino-terminal domain. The current results of EBNA1 dimerization and *oriP* reporter assays are in concordance with their results, as the zinc chelator in **ZRL₅P₄** could further disrupt both the self-association of the EBNA1 monomers and the closely related transactivation activity, as well as the cell viability and the EBV lytic gene induction. For the EBNA1 self-association analysis, in the absence of Zn^{2+} there was no significant difference in the inhibition between the **ZRL₅P₄** and **L₂P₄** (lack of zinc chelator) treatments; but when Zn^{2+} was present, **ZRL₅P₄** was more efficient to inhibit the dimer formation (Figure 18A and 18B). As aforementioned, the UR1 region in EBNA1 coordinates Zn^{2+} through a pair of essential cysteine and this

coordination of Zn^{2+} is essential for UR1 to form a second EBNA1 dimerization site in addition to the first dimerization site at the DBD. The presence of Zn^{2+} likely contributes to the increased affinity of the full length of two adjacent EBNA1 monomers. Thus, **ZRL₅P₄** may interrupt both dimerization interfaces at UR1 and the DBD, whereas **L₂P₄** can only interfere with the dimerization site at the DBD. As shown in the current results of MD simulations and fluorescence titration, the presence of Zn^{2+} did not affect **ZRL₅P₄** to bind with the EBNA1 DBD. The DBD region is supposed to have no binding residues of Zn^{2+} , so this binding of **ZRL₅P₄** with the DBD is solely due to the **P₄** peptide present in the probe. It implies that the additional inhibitory effects contributed by the zinc chelator is not directly related with the DBD.

[00223] The increased potency of **ZRL₅P₄** on the various EBNA1 and cellular activities upon **L₂P₄** is also reflected by the cellular imaging for the EBV-positive cell lines. The imaging results can explain that the stronger inhibition attributes to the nuclear localization of the probe, where EBNA1 is primarily located, and that nuclear EBNA1 was of critical importance for its dependent function. In contrast, a major proportion of **L₂P₄** is located in the cytoplasm, the remaining probe is located in the nucleus. As can be seen, the new bioprobe **ZRL₅P₄** can selectively enter the nuclei of EBV-positive cells and can disrupt the function of EBNA1 more effectively than **L₂P₄**. Both the NLS RrRK sequence and the zinc chelator in **ZRL₅P₄** play a critical role in enhancing the more specific binding to the nuclear EBNA1 protein and/or facilitating the entry to the nucleus.

[00224] On the other hand, the inventor initially thought that the genetic variation at the EBNA1 dimerization sequence (the amino acid 563) could be an important factor to design the EBNA1 probes. **ZRL₅P₆** was constructed to target the EBNA1 protein

with an "I" residue at the variation position. However, the experimental results of various biological assays show that both **ZRL₅P₄** and **ZRL₅P₆** were equally effective in all these assays, indicating that this amino acid residue is not critical for disrupting the self-association of the EBNA1 monomer. These findings suggest that **ZRL₅P₄** and **ZRL₅P₆** should be equally potent for suppressing the EBNA1 proteins in various EBV stains.

[00225] In conclusion, Zn²⁺ has been made use of as an important co-factor for EBNA1 to function and have constructed a new series of EBNA1 probes with a zinc chelator and an EBNA1 binding peptide. The zinc chelator can further enhance the inhibitory activities of the functional self-associated EBNA1 dimer and the associated *oriP*-associated transactivation. Interestingly, the new **ZRL₅P₄** probe could reactivate the EBV lytic induction which is associated with the shrinkage of EBV-positive tumors in the animal model. This is the first study to successfully target a single protein (EBNA1) to induce the EBV lytic cycle. As EBNA1 is a foreign protein to the host, in theory, this strategy of elimination of EBV-associated tumor cells warrants absolute specificity over all other lytic induction therapies. The current lytic analysis results also suggest that the function of EBNA1 as well as the role of EBV is associated with maintenance of tumor cell survival through suppression of the lytic cycle reactivation.

[00226] **Experimental**

[00227] **General.** Unless otherwise stated, all chemicals were used as purchased without further purification. Full-length EBNA1 with an N-terminal His tag was purchased from Abcam (ab138345). Solvents were dried using standard procedures. Purification of **ZRL₅P₂**, **ZRL₅P₄**, and **ZRL₅P₆** was performed on a Waters semi-preparative HPLC system. NMR spectra were recorded on a Bruker 400 MHz

NMR spectrometer, and the chemical shifts were referenced internally to tetramethylsilane or the corresponding solvent residues in parts per million (ppm); coupling constants are reported in hertz (Hz). High-resolution mass spectra, reported as m/z , were obtained on either a Bruker Autoflex MALDI-TOF or an Agilent 6450 UHD Accurate-Mass Q-TOF spectrometer. UV-visible absorption spectra were recorded on a Cary 8454 spectrometer.

[00228] **Syntheses.** All new compounds were characterized by ^1H and ^{13}C NMR spectroscopy and HR MALDI-TOF MS.

[00229] **Molecular Dynamics Simulation.** All-atom unbiased MD simulations in AMBER 14 with aff99SBildn force field were used. The system preparation and simulation procedures were the same as those reported previously. In brief, a periodic boundary, cubic, TIP3 explicit water box with a 20 Å buffer was used with charge neutralized by adding Cl^- ions. The system was then minimized and equilibrated by sander using three stages: (1) heating from 100 to 300 K in 20 ps; (2) adjusting the solvent density to 1 g/mL in 20 ps; and (3) equilibrating in 200 ps with NPT ensemble. A subsequent 100 ns NPT simulation was performed with CUDA-accelerated PMEMD. A 2 fs time step and SHAKE-enabled settings were used for all of the equilibration and production stages. A Berendsen thermostat was adopted for temperature control at all stages.

[00230] **Luminescence Measurements.** Luminescence, excitation and emission spectra were recorded on a Horiba Fluorolog-3 spectrofluorometer equipped with a xenon lamp. Specifically, a luminescence titration assay of the selectivity of **ZRL₅P₄** for metal ions was performed in HEPES buffer (0.05 M, pH 7.4)/CH₃CN(50:50) using perchlorate salts as the metal source [$\text{Zn}(\text{ClO}_4)_2$, $\text{Cd}(\text{ClO}_4)_2$, $\text{Cu}(\text{ClO}_4)_2$, $\text{Co}(\text{ClO}_4)_2$, $\text{Ni}(\text{ClO}_4)_2$, $\text{Hg}(\text{ClO}_4)_2$ and $\text{Mg}(\text{ClO}_4)_2$]. The luminescence titration assay of the

selective binding of **ZRL₅P₄** with proteins was conducted in phosphate-buffered saline (PBS). Luminescence titration experiments were performed by gradually increasing the concentration of the analytes in the aqueous solution of **ZRL₅P₄**, such as Zn²⁺, EBNA1 and HSA; the experiment was stopped when the change in **ZRL₅P₄** luminescence ceased.

[00231] **Expression and Purification of EBNA1 DBD.** Plasmids for the expression of EBNA1 DBDs of amino acids 379-641 fused with glutathione S-transferase were expressed in *Escherichia coli* and purified with glutathione sepharose 4B rinse (GE Dharmacon).

[00232] **MBS-Cross-Linked Dimerization Assay.** Full-length EBNA1 (residues 1-641) with an N-terminal His tag (ab138345, Abcam) was used in this assay. Each 5 µg of EBNA1 was incubated without or with 10 µM Zn²⁺ at room temperature in the presence of the cross-linking reagent 3-maleimidobenzoylN-hydroxysuccinimide ester (MBS) and probe (buffer/**L₂P₄**/**ZRL₅P₄**) for 2 h to allow the pre-existing dimer to be covalently cross-linked. After incubation, SDS loading buffer was added to each system, which were then separated using denaturing SDS-PAGE, transferred onto a nitrocellulose membrane, which was blotted with antibody; the obtained protein bands provided information of dimerization inhibition.

[00233] **Luciferase Reporter Assay for EBNA1-oriPI Dependent Transactivation.** To study EBNA1-dependent transactivation, the luciferase vector J988F containing the EBV C promoter and *oriPI* (family of repeats, FR) was constructed. The EBV C promoter and *oriPI* (nucleotides 7447-11412) regions were subcloned from the previously described plasmid pgCp(-3889)CAT as a HindIII fragment into the pGL3Basic luciferase vector (Promega). Correct sequences were ascertained by Sanger sequencing using the ABI PRISM Big Dye terminator cycle

sequencing kit (Applied Biosystems). EBV-positive C666-1 and NPC43 cells were then transiently transfected with the J988F reporter plasmid. Cells were seeded in 12-well plates and co-transfected with the J988F plasmid (2 µg/well) and a pRL*Renilla* luciferase control reporter (500 ng/well) (Promega) using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were treated with **ZRL₅P₄**, **L₂P₄**, EDTA or TPEN (10 µM) for another 8 h. Cells were lysed with Passive Lysis Buffer (Promega), and the lysate was then transferred onto a white, opaque, 96-well plate. The luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) with the GloMax 96 Microplate Luminometer (Promega). The pRL *Renilla* luciferase reporter was used as an internal control to normalize the transfection efficiency among the samples.

[00234] **Cell Culture.** Five cell lines were used in this work: the EBV-negative HK-1 and HONE-1 lines and the EBV-positive NPC43, C666-1 and Raji lines. HK-1, HONE-1, C666-1 and Raji cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37 °C and 5% CO₂. NPC43 cells were maintained in RPMI 1640 with 10% FBS and 4 µM Y27362 (inhibitor of Rho-associated, coiled-coil-containing protein kinase; Enzo Life Sciences).

[00235] **MTT Assay.** All cells were sub-cultured in 96-well plates at the optimal growth density (HK-1, 1×10⁴ cells/100 µl per well; HONE-1, 8×10³ cells/100 µl per well; NPC43, 8×10³ cells/100 µl per well; C666-1, 3×10⁴ cells/100 µl per well; Raji, 1×10⁴ cells/100 µl per well) for 24 h. The growth medium was then replaced by solutions of **ZRL₅P₂**, **ZRL₅P₄**, or **ZRL₅P₆** at concentrations of 1, 5, 10, 20, 50 and 100 µM for EBV-negative cells, or at concentrations of 1, 3, 5, 10, 15 and 20 µM for EBV-positive cells (for the suspensions of C666-1 and Raji cells, the 96-well plate

was centrifuged at 1000 rpm for 3 min before each replacement/withdrawal of medium and operated with care during the assay). After culturing for a further 5 days (half of the volume of medium was replaced every 4 days with fresh medium containing the appropriate drug concentration), the cells were rinsed with PBS and then incubated with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml, 50 μ l) in PBS at 37 °C for 3 h. Then, 70% of the medium was carefully removed, DMSO (100 μ l) was added, and the plate was shaken for 30 min to solubilize the formazan produced by the living cells. The optical densities were measured with a dual-wavelength Labsystem Multiskan microplate reader (Merck Eurolab) at wavelengths of 540 and 690 nm and expressed as a percentage relative to control cells (cells without drug treatment served as the control). Measurements were performed in triplicate and repeated twice. Cell viability (%) was calculated according to Eq. (2):

$$\text{Viability (\%)} = (\text{OD}_i / \text{OD}_c) \times 100\% \quad (2)$$

[00236] Where OD_i and OD_c are the optical densities of the surviving cells treated with or without drug, respectively.

[00237] **Two-Photon Imaging and Co-staining.** Cells were incubated with **ZRL₅P₂/ZRL₅P₄/ZRL₅P₆** (10 μ M) for 3 h and then co-stained with DRAQ5 (5 μ M) for 30 min. Images were acquired using a Leica TCS SP8 confocal laser-scanning microscope equipped with a coherent femtosecond laser (680-1050 nm), argon laser (432, 457 and 488 nm), He-Ne laser (632 nm), ultraviolet lamp and a controlled CO₂-content stage-top tissue culture chamber (37 °C, 2-7% CO₂). *In vitro* images of **ZRL₅P₂/ZRL₅P₄/ZRL₅P₆** were obtained under two-photon excitation ($\mu_{\text{ex}} = 700\text{nm}$), whereas images of DRAQ5 were acquired under single-photon excitation ($\mu_{\text{ex}} = 638\text{nm}$).

[00238] **Nude Mice Xenograft and Intratumoral Injection.** C666-1 cells (8×10^6) suspended in 100 μ l of serum-free RPMI medium were injected into the right flanks of 6- to 8-week-old male BALB/c nude mice. After 21 days of inoculation, when tumor had grown to an average volume of approximately 220 mm³, mice were assigned to treatment groups ($n=5$ per group) such that the average tumor volumes varied between groups by no more than 10%. Twice weekly, mice received 4 μ g/tumor intra-tumoral injections of **ZRL₅P₂**, **ZRL₅P₄**, or **ZRL₅P₆** in 0.1% using a 29-gauge syringe. Mice that received an equivalent volume of 0.1% DMSO alone served as controls. The treatment period lasted for 18 days. Body weight and tumor volumes were measured twice weekly, and tumor volumes were calculated as $(\text{length} \times \text{width}^2)/2$. At the end of the treatment period, mice were sacrificed, and their tumors and major organs were harvested and weighed. The investigators were blind to treatment grouping during the experiments and analysis of data. All animal experiments were approved by the Department of Health of the Hong Kong Government and the Animal Subjects Ethics Sub-Committee of Hong Kong Polytechnic University.

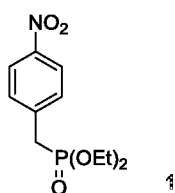
[00239] **Quantitative Polymerase Chain Reaction gene expression analysis.** qPCR was performed as reported.

[00240] **Western Blot analysis.** Western blot analysis of BRLF1 (Rta), BMRF1, and gp350/220 was performed as reported. The Rta antibody was supplied from Argene (Verniolle, France) and the β -actin antibody for loading control was supplied from Cell Signaling Technology (Danvers, MA, US). Antibodies against BMRF1 and gp350/220 were generously given by Prof. Jaap M. Middeldorp (VU University Medical Center, The Netherlands). A 1:400 dilution of the primary antibodies was used.

[00241] **Preparation of formalin-fixed, paraffin-embedded (FFPE) tumor tissues, hematoxylin and eosin (H & E) stain, and immunohistochemistry (IHC)** The transplanted tumor tissues were fixed with formalin, and embedded in paraffin accordingly to the general practice. The histologic sections were prepared and stained with H & E. The lytic protein markers were stained with IHC antibodies against Zta and dBMF1. The BZLF1 (Zta) antibody was supplied from Argene and the BMRF1 antibody was the one used for Western blot analysis. The slides were incubated with the primary antibodies (1:100 dilution) for IHC as previously described.

EBV infection assay The HONE-1-EBV cell line was used to produce the infectious EBV particles for the lytic analysis, this cell line was generated by introducing a green fluorescent protein (GFP) open reading frame in the recombinant Akata EBV genome into the EBV-negative NPC cell line HONE-1. The procedures of production of viral particles and quantitation of virus titers were followed as previously described. In brief, after incubation with various EBNA1 probes for 96 hours, the supernatants were filtered through 0.45 μ M pore filters and the viral particles were enriched by centrifugation at 20,000g for 2 hours. The relative virus titers were determined by the Raji cell assay and were quantified with the GFP expression of Raji cells infected with the virus stocks to be analyzed. The Raji cells (1×10^5) were incubated in 96-well plates and cultivated for 3 days at 37°C to allow expression of GFP. The number of GFP-positive cells was counted by ultraviolet microscopy.

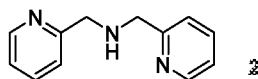
[00242] Synthesis of diethyl 4-nitrobenzylphosphonate (**1**)



[00243] Synthesis of diethyl 4-nitrobenzylphosphonate (**1**). Compound **1** was

prepared according to a previous report. A mixture of 4-nitrobenzyl bromide (200 mg, 0.93 mmol) and triethylphosphite (216 mg, 1.30 mmol) was heated at 160 °C under a nitrogen atmosphere for 2 h. Excess triethylphosphite was removed under reduced pressure to provide **1** as a brown oil (241mg, 95%). ¹H NMR (400 MHz, CDCl₃): δ 8.19 (dd, J = 8.8, 0.8 Hz, 2H), 7.48 (dd, J = 8.8, 2.4 Hz, 2H), 4.07 (dq, J = 7.6, 0.4 Hz, 2H), 4.05 (dq, J = 7.6, 0.4 Hz, 2H), 3.25 (d, J = 22.4 Hz, 2H), 1.27 ppm (t, J = 6.8 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃): δ 139.7 (d, ²J_(C,P) = 8.9 Hz), 130.6 (d, ³J_(C,P) = 6.4 Hz), 123.7 (d, ⁴J_(C,P) = 2.9 Hz), 62.4 (d, ²J_(C,P) = 6.7 Hz), 34.0 (d, ¹J_(C,P) = 136.7 Hz), 16.4 ppm (d, ³J_(C,P) = 5.9 Hz); HRMS (MALDI-TOF): *m/z*: calcd for C₁₁H₁₆NO₅P 273.0766 [*M*]⁺; found: 273.0755.

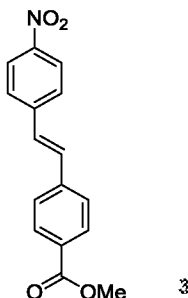
[00244] Synthesis of di-(2-picolyl)amine (**2**)



[00245] A solution of 2-picolylamine (5.0 g, 46.24 mmol) in absolute EtOH (100 ml) was added dropwise to a suspension of 2-pyridinecarboxaldehyde (4.9 g, 46.05 mmol) in absolute EtOH (100 ml) at 0 °C. After the addition, the reaction solution was stirred for 4 h and then cooled to 0 °C. NaBH₄ (3.5 g, 92.10 mmol) was added into the cooled solution in small portions. The reaction was stirred for another 12 h at room temperature. Then, HCl (5 N, 120 ml) was added slowly and the mixture was stirred for 1 h. Aqueous NaOH (2 N) was then added until the pH reached 11. The mixture was extracted with DCM (6 × 50 ml), and the combined organic phases were dried over anhydrous MgSO₄ and filtered. Removal of the solvent afforded **2** in analytical purity as a brown oil (7.8 g, 85%). ¹H NMR (400 MHz, CD₃OD): δ 8.49 (qd, J = 4.8, 0.8 Hz, 2H), 7.81 (dt, J₁ = 7.6, 1.6 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.31 (m, 2H), 3.93 ppm (s, 4H); ¹³C NMR (100 MHz, CD₃OD): δ 160.3, 149.8, 138.7, 124.1, 123.8, 54.8 ppm; HRMS (MALDI-TOF): *m/z*: calcd for C₁₂H₁₄N₃ 200.1188 [*M*+H]⁺; found:

200.1326.

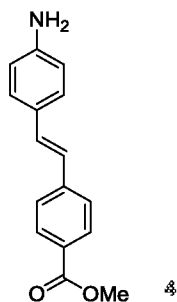
[00246] Synthesis of (*E*)-methyl 4-(4-nitrostyryl)benzoate (**3**)



[00247] A solution of diethyl 4-nitrobenzylphosphonate (200 mg, 0.73 mmol) and methyl 4-formylbenzoate (120 mg, 0.73 mmol) in THF (3 ml) was added to a suspension of sodium methoxide (79 mg, 1.46 mmol) in THF (6 ml). The mixed solution was stirred for 20 min at 0 °C. The solvent was removed under reduced pressure. H₂O (100 ml) was added, and the resulting aqueous solution was neutralized with HCl (0.1 N), followed by extraction with DCM (3 × 50 mL). The combined organic phases were dried over anhydrous MgSO₄ and filtered. Then, the solvents were removed under reduced pressure. The pure product **3** (yellow solid) was obtained by filtering the crude material through silica gel with DCM as the eluent (182 mg, 88%). ¹H NMR (400 MHz, CDCl₃): δ 8.25 (dd, J₁ = 6.8, 2 Hz, 2H), 8.07 (dd, J₁ = 6.8, 1.6 Hz, 2H), 7.67 (dd, J₁ = 7.2, 2 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.28-7.26 (2H, coincided with residual CHCl₃), 3.94 ppm (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 166.7, 147.2, 143.2, 140.5, 132.1, 130.2, 130.0, 128.7, 127.2, 126.9, 124.2, 52.2 ppm; HRMS (MALDI-TOF): *m/z*: calcd for C₁₆H₁₄NO₄ 284.0923 [*M*+H]⁺; found: 284.0895.

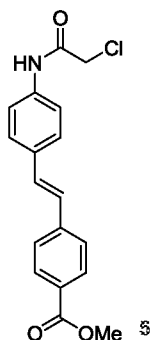
[00248] Synthesis of (*E*)-methyl 4-(4-aminostyryl)benzoate (**4**)

64



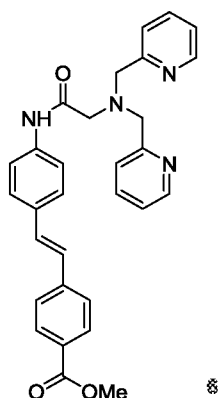
[00249] A solution of **3** (200 mg, 0.71 mmol) in EtOAc (10 ml) was stirred under a nitrogen atmosphere at 75 °C. Stannous chloride dihydrate (801 mg, 3.55 mmol) was added and the mixture was heated at 75 °C and stirred overnight. After the reaction was complete (determined by TLC, thin-layer chromatography), the mixture was allowed to cool to room temperature, followed by the addition of aqueous NaHCO₃ until the pH reached 8. The suspension was filtered through a pad of celite and washed with EtOAc. The filtrate was washed with H₂O (twice) and brine, then dried over anhydrous MgSO₄ and filtered and concentrated under reduced pressure to afford **4** as an orange-yellow solid (144 mg, 80%). ¹H NMR (400 MHz, CDCl₃): δ 7.99 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.13 (d, J = 16.4 Hz, 1H), 6.93 (d, J = 16.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 2H), 3.92 ppm (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 167.0, 146.7, 142.6, 131.3, 130.0, 128.2, 128.1, 127.4, 125.8, 123.8, 115.2, 52.0 ppm. HRMS (MALDI-TOF): *m/z*: calcd for C₁₆H₁₅NO₂ 253.1103 [*M*]⁺; found: 253.1084.

[00250] Synthesis of (*E*)-methyl 4-[4-(2-chloroacetamido)styryl]benzoate (**5**)



[00251] A solution of 2-chloroacetyl chloride (53 mg, 0.47 mmol) in DCM (3 ml) was added dropwise to a solution of **4** (100 mg, 0.39 mmol) and 4-(dimethylamino)pyridine (81 mg, 0.66 mmol) in DCM (15 ml) at 0 °C and stirred under a nitrogen atmosphere. After stirring for 2 h at room temperature, the solvent was removed under reduced pressure. The obtained pale-yellow solid was purified by silica gel with DCM as the eluent to give **5** as an orange solid (109mg, 85%). ¹H NMR (400 MHz, CDCl₃): δ 8.28 (s, 1H), 8.03 (d, J = 8.4 Hz, 2H), 7.60-7.53 (m, 6H), 7.19 (d, J = 16.4 Hz, 1H), 7.09 (d, J = 16.4 Hz, 1H), 4.22 (s, 2H), 3.93 ppm (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 167.8, 163.7, 141.7, 136.5, 133.7, 130.2, 130.0, 128.9, 127.5, 127.3, 126.2, 120.1, 52.1, 42.9 ppm; HRMS (MALDI-TOF): *m/z*: calcd for C₁₈H₁₆ClNO₃ 329.0819 [*M*]⁺; found: 329.0807.

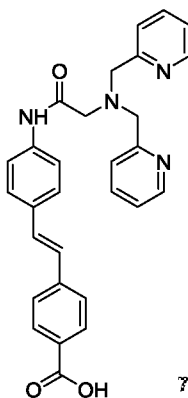
[00252] Synthesis of (E)-methyl 4-(4-{2-[bis(pyridine-2-ylmethyl)amino]acetamido}styryl)benzoate (**6**)



[00253] Compound **5** (50 mg, 0.15 mmol), di(2-picolyl)amine (DPA, 36 mg, 0.18 mmol), *N,N*-diisopropylethylamine (DIPEA, 33 mg, 0.26 mmol), and potassium iodide (16 mg, 0.67 mmol) were added to MeCN (25 ml). After stirring and heating at reflux overnight under a nitrogen atmosphere, the mixture was allowed to cool to room temperature, and the solvent was removed under reduced pressure to give a yellow-brown solid. The crude material was then purified by silica gel column

chromatography using DCM/MeOH (100:1; the DCM was extracted with aqueous NH_3 before use, DCM/ NH_3 200:1 v/v) to obtain **6** (59mg, 80%). ^1H NMR (400 MHz, CDCl_3): δ 11.04 (s, 1H), 8.64 (qd, $J_1 = 4.8, 0.8$ Hz, 2H), 8.02 (d, $J = 8.4$ Hz, 2H), 7.82 (d, $J = 8.4$ Hz, 2H), 7.64 (dt, $J = 7.6, 2.0$ Hz, 2H), 7.56 (d, $J = 8$ Hz, 2H), 7.53 (d, $J = 8.4$ Hz, 2H), 7.29 (d, $J = 7.6$ Hz, 2H), 7.22-7.18 (m, 3H), 7.07 (d, $J = 16.4$ Hz, 1H), 3.95 (s, 4H), 3.93 (s, 3H), 3.49 ppm (s, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 169.8, 166.9, 158.0, 149.4, 142.0, 138.7, 136.7, 132.2, 130.8, 130.0, 127.4, 126.2, 126.1, 123.3, 122.6, 119.8, 60.4, 58.7, 52.1 ppm; HRMS (MALDI-TOF): m/z : calcd for $\text{C}_{30}\text{H}_{28}\text{N}_4\text{NaO}_3$ 515.2059 $[M+\text{Na}]^+$; found: 515.2654.

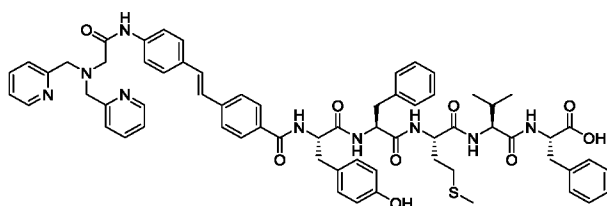
[00254] Synthesis of **ZRL₅** (**7**)



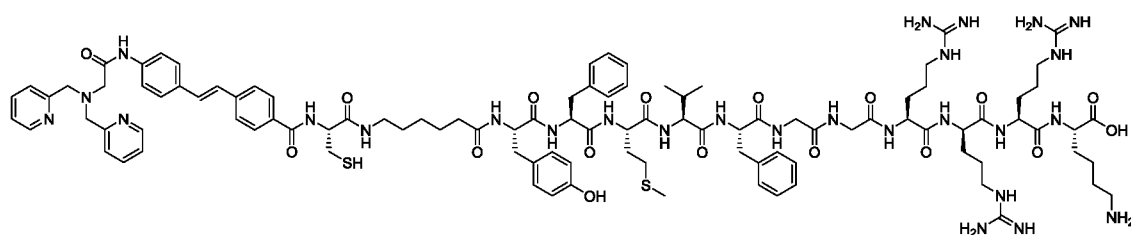
[00255] Synthesis of **ZRL₅** (**7**). KOH (114 mg, 2.03 mmol) was added to a suspension of **6** (200 mg, 0.41 mmol) in EtOH/ H_2O (1:1, 8 ml). After stirring and heating at reflux overnight, the reaction mixture was allowed to cool to room temperature. The solvent volume was reduced to approximately 50%, and then HCl (1 N) was added until the solution became acidic. The resulting precipitate was filtered and collected to give **7** as a yellow solid (157 mg, 78%). ^1H NMR (400 MHz, CDCl_3): δ 10.74 (s, 1H), 8.68 (d, $J = 4.4$ Hz, 2H), 8.00 (d, $J = 8.4$ Hz, 2H), 7.73 (d, $J = 8.4$ Hz, 2H), 7.67 (dt, $J = 7.6, 1.6$ Hz, 2H), 7.51 (d, $J = 8.4$ Hz, 2H), 7.43 (d, $J = 8.8$ Hz, 2H), 7.33 (d, $J = 7.6$ Hz, 2H), 7.26-7.22 ppm (m, 2H), 7.16 (d, $J = 16.4$ Hz, 1H),

7.02 (d, $J = 16.4$ Hz, 1H), 4.02 (s, 4H), 3.54 ppm (s, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 169.4, 157.4, 149.1, 142.4, 138.5, 137.2, 132.4, 130.9, 130.5, 127.3, 126.3, 126.1, 123.7, 122.9, 120.1, 60.3, 59.0 ppm; HRMS (MALDI-TOF): m/z : calcd for $\text{C}_{29}\text{H}_{26}\text{N}_4\text{NaO}_3$ 501.1903 $[M+\text{Na}]^+$; found: 501.1902.

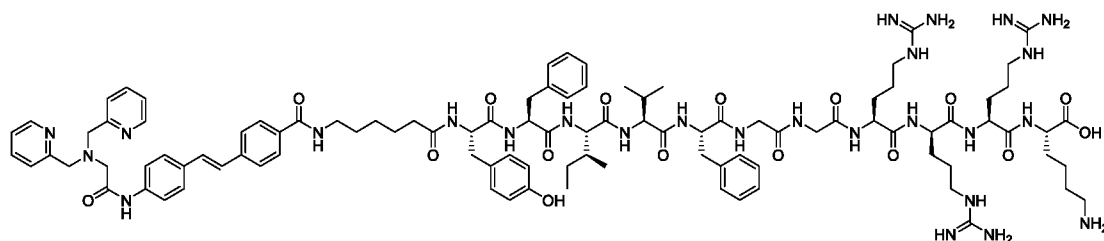
[00256] Synthesis of $\text{ZRL}_5\text{P}_2/\text{ZRL}_5\text{P}_4/\text{ZRL}_5\text{P}_6$



ZRL_5P_2



ZRL_5P_4



ZRL_5P_6

[00257] **Conjugation.** A solution of **7** (148 mg, 0.30 mmol) and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 156 mg, 0.3 mmol) was dissolved in DMF (3 ml). DIPEA (90 mg, 0.70 mmol) was added and the solution was stirred for 1 min. The solution was added to the appropriate peptide ($\text{P}_2/\text{P}_4/\text{P}_6$, 0.1 mmol) loaded onto Wang resin on an 8 ml solid-phase extraction (SPE) cartridge with PE frits. The peptide resin was allowed to swell in

DMF overnight, and the chamber was drained before addition. The resulting mixture was agitated on a shaker for 45 min, and the solution was then filtered and washed with DMF three times. A second round of coupling was performed according to the same procedures, and a blank coupling cycle with PyBOP (156mg, 0.3 mmol) and DIPEA (90 mg, 0.70 mmol) in DMF (3 mL) was performed. After 45 min, the solution was filtered and washed as described above.

[00258] Cleavage of the resin. A mixture of TFA/H₂O/TIPS (0.95:0.25:0.25; TFA, trifluoroacetic acid; TIPS, triisopropylsilane) was added to the crude resin shrunken with diethyl ether. The mixture was agitated on a shaker for 3 h. The resin was then removed by filtration and washed with MeOH three times. The combined filtrate was then concentrated under reduced pressure to give the crude **ZRL₅P₂/ZRL₅P₄/ZRL₅P₆**, which was then purified by preparative HPLC (Table 2).

[00259] The compounds provided herein can be purified using any conventional purification technique. In certain embodiments, the compounds provided herein are purified using preparative HPLC. Table 2 below exemplary preparative HPLC purification conditions.

[00260] **Table 2.** The gradient of the solvent for preparative HPLC. Solvent A is DI H₂O with 0.5 % TFA, and solvent B is HPLC grade MeCN.

Time/min	Flow/ml·min	%A	%B
0	8	80	20
35	8	20	80
37	8	0	100
40	8	80	20

[00261] **ZRL₅P₂**. Retention time, 24.061 min. HRMS(Q-TOF): calcd for $[M+H]^+$ 1166.5174, found: 1166.5195; calcd for $[M+2H]/2$ 583.7626, found: 583.7619.

[00262] **ZRL₅P₄**. Retention time, 16.845 min. HRMS (MALDI-TOF): calcd for $[M+H]$ 2094.0552, found: 2094.2452.

[00263] **ZRL₅P₆**. Retention time, 17.319 min. HRMS (Q-TOF): calcd for $[M+2H]/2$ 987.0487, found: 987.0501; calcd for $[M+3H]/3$ 658.3684, found: 658.3686; calcd for $[M+4H]/4$ 494.0282, found: 494.0280.

[00264] **DSS-Cross-Linked Dimerization Assay**. Full-length EBNA1 (a.a. 1-641) with N-terminal His tag (ab138345, Abcam) was used in this assay. Each 5 μ g of EBNA1 was incubated without (-) or with (+) Zn^{2+} at room temperature in the presence of DSS-cross-linker and analyzed probes (buffer/**L₂P₄**/**ZRL₅P₄**) for 2 hours to allow a preexisting dimer to be covalently cross-linked. After incubation, SDS loading buffer was added to each system, which were separated on SDS-PAGE denaturing gel, transferred onto the nitrocellulose membrane, and blotted with antibody, with the obtained protein bands providing information on dimerization inhibition.

[00265] **Molecular dynamic (MD) simulations**

[00266] **Modeling and simulations of P₄-EBNA1 complex**. **P₄** (CAhxYFMVFGGRrRK, SEQ ID NO:4) was docked into the dimeric interface of the putative EBNA1 DBD monomer from a previous work with the online CABS-dock tool. 10 **P₄-EBNA1** complex models were generated and subjected to constant pressure and temperature (NPT) simulations in AMBER 14 with CUDA acceleration. Through observing the fluctuation of **P₄** in the each complex (data not shown), the most stable complex model was chosen and subjected to an additional 500 ns NPT

simulations. The results showed that **P₄** stably occupied the dimeric interface of the putative EBNA1 DBD monomer, suggesting it is a potent EBNA1 dimerization inhibitor (Figure 7A-F). The representative **P₄**-EBNA1 DBD monomer complex structure was obtained with the conformational clustering analysis. Well consistent with the previous work, the hydrophobic packing effect mediated by YFMVF (SEQ ID NO:2) and the salt bridging formed by RrRK contributed to the **P₄**-EBNA1 DBD monomer binding (Figure 16).

[00267] **Modeling of Zn²⁺-ZRL₅ complex.** The 3D models of **ZRL₅** in both Zn²⁺-free (denoted as L5 in Figure 8 and 17) and Zn²⁺-coordinated (denoted as Zn²⁺-L5 in Figure 8 and 17) scenarios were built by the Molecular Operation Environment (MOE, version 2012.10). MMFF94x force field (FF) was used to minimize the energy, besides, the LowModeMD was employed to identify the global energy minimum conformation (Figure 8A-E). The partial charge of **ZRL₅** and Zn²⁺-**ZRL₅** was calculated by online RED server, and their atom types were manually determined with output from parmchk tool (Figure 17A-D). Besides, the missing FF parameters of **ZRL₅** and Zn²⁺-**ZRL₅** were determined from comparable atoms in GAFF or measurements of RED-derived results (Table 3 and Table 4).

[00268] **Table 3. The modified AMBER force field parameters for zinc-free ZRL₅ residues.**

Non-standard residues	Type	Item	Parameters
ZRL₅	Bonded	CA-CD	$k=361.3 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$, $d_0=1.4763 \text{ \AA}$
		CA-N	$k=384.2 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$, $d_0=1.4121 \text{ \AA}$
	Angle	CA-C -N	$k=67.68 \text{ kcal}/(\text{mol} \cdot \text{rad}^2)$, $\theta_0=115.25$

	CA-C -O	$k=68.72 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=122.60$
	CA-CA-CD	$k=64.50 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=120.82$
	CA-CA-N	$k=67.87 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=120.19$
	CA-CA-NC	$k=68.83 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=122.94$
	CT-CA-NC	$k=67.30 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=116.68$
	H4-CA-NC	$k=51.88 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=116.03$
	CA-CD-CD	$k=63.68 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=127.52$
	CA-CD-HA	$k=46.96 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=115.13$
	C -CT-NT	$k=66.32 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=111.14$
	CA-CT-H1	$k=46.99 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=109.56$
	CA-CT-NT	$k=66.22 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=112.16$
	C -N -CA	$k=63.82 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=123.71$
	CT-C -N	$k=66.79 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=115.18$
	N -C -OH	$k=74.29 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=112.82$
	CA-NC-CA	$k=68.35 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=117.22$
Dihedral		$v=26.6 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
	CA-CA-CD-CD	period=2
		$v=26.6 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
	CA-CA-CD-HA	period=2
		$v=10.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
	CA-N -C -OH	period=2

	$v=10.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
CA-N -C -CT	period=2
	$v=14.5 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
N -CA-CA-HA	period=2
	$v=14.5 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
N -CA-CA-CA	period=2
	$v=4.60 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
N -C -OH-HO	period=2
	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
N -C -CT -H1	period=2
	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
N -C -CT-NT	period=2
	$v=1.80 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=180.0,
CA-CA-N -C	period=2

[00269] **Table 4.** The modified AMBER force field parameters for Zn^{2+} -**ZRL₅** residues.

Non-standard residues	Type	Item	Parameters
Zn^{2+} - ZRL₅	Bonded	NE-ZN	$k=640.0 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$, $d_0=2.048 \text{ \AA}$
		NF-ZN	$k=640.0 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$, $d_0=2.201 \text{ \AA}$
		NG-ZN	$k=640.0 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$, $d_0=2.049 \text{ \AA}$
	Angle	CA-C -N	$k=70.790 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=115.08$

	CA-NE-ZN	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=121.90$
	CM-NE-ZN	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=114.0$
	NE-ZN-NF	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=81.2$
	NE-ZN-NG	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=118.0$
	CT-NF-ZN	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=105.0$
	NF-ZN-NG	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=81.30$
	CA-NG-ZN	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=120.0$
	NG-ZN-NG	$k=50.0 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, $\theta_0=117.40$
		$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
	X -NE-ZN-X	period=2
		$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
	X -ZN-NT-X	period=2
	X -ZN-NC-X	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
		period=2
	X -CA-NE-X	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
		period=2
	X -NE-ZN-X	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
		period=2
	X -NE-CA-X	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
		period=2
	X -CM-NE-X	$v=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0,
		period=2

X -NC-ZN-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -ZN-NF-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -NF-ZN-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -ZN-NG-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -CA-NG-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -CT-NF-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -NG-ZN-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2
X -CT-NF-X	$\nu=0.00 \text{ kcal}/(\text{mol} \cdot \text{rad}^{-2})$, phase=0.0, period=2

[00270] **Modeling and simulations of ZRL₅P₄-EBNA1 and Zn²⁺-ZRL₅P₄-EBNA1 complex.** The initial structure of ZRL₅P₄-EBNA1 (denoted as L5P4-EBNA1 in the Figure 10, 11, 14 and Zn²⁺-ZRL₅P₄-EBNA1 (denoted as Zn²⁺-L5P4-EBNA1 in the Figure 12, 13, 14 complex was built based on the P₄-EBNA1 complex structure and putative Zn²⁺-ZRL₅ structure, with CAhx linking the ZRL₅P₄/Zn²⁺-ZRL₅P₄ to P₄. It was then arranged for the 200 ns, all atoms unbiased MD simulations in AMBER 14 with ff99SBildn force field. System preparation and simulation procedures were the same as the previous work. Briefly,

the periodic boundary, cubic, TIP3 explicit water box with a 20 Å buffer was employed with neutralized charge by adding Cl⁻. The system was then minimized and equilibrated by sander following three stages: (1) heated from 100 K to 300 K in 20 ps; (2) adjusting solvent density to 1 g/mL in 20 ps; and (3) equilibrated in 200 ps with NPT ensemble. A subsequent 100 ns NPT simulation was carried out with CUDA-accelerated PMEMD. A 2 fs time step and SHAKE-enabled setting were used for all equilibration and production stages. Berendsen thermostat was adopted for temperature control for all stages.

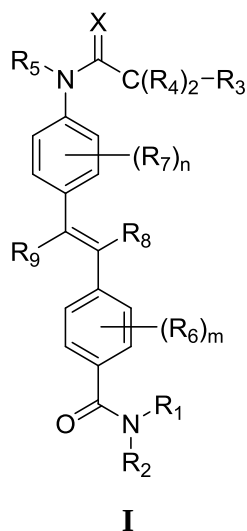
[00271] **Post-simulation analysis.** 10 conformational clusters of **P₄-EBNA1 DBD** complex were calculated via cpptraj by using default settings with distance defined by main chain atoms root-mean-square deviation (RMSD). The representative conformation of most abundant cluster (cluster 0) was saved for **P₄-EBNA1 DBD** interaction analysis. The binding free energies for whole production trajectory were calculated by MMPBSA. The time intervals were adjusted to make sure 200 frames were included in the calculation. The mbondi2 was used and the salt concentration was set to 0.1 M in Generalized Born (GB) calculation; the radii from the prmtop file was used and the ion strength was set to 0.1 mM for Poisson-Boltzman (PB) calculation.

INDUSTRIAL APPLICABILITY

[00272] The present disclosure is in the field of pharmaceuticals and chemical industries. More particularly, the present disclosure relates to zinc binding EBNA1-specific compounds with responsive imaging and inhibition of EBNA1 for the treatment of EBV-positive cells, such as cancer.

What we claim:

1. A compound of Formula I:



or a pharmaceutically acceptable salt thereof, wherein

m is a whole number selected from 0-4;

n is a whole number selected from 0-4;

p is a whole number selected from 0-4;

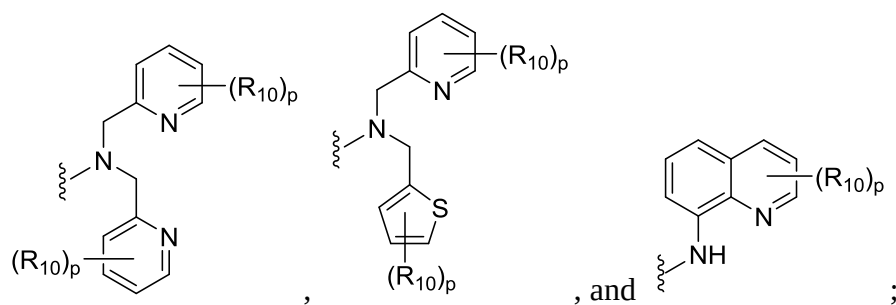
X is H₂, S, or O;

each instance of R is independently H or alkyl;

R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

R₂ is H or alkyl;

R₃ is selected from the group consisting of:



each instance of R_4 is independently H or alkyl;

R_5 is H or alkyl;

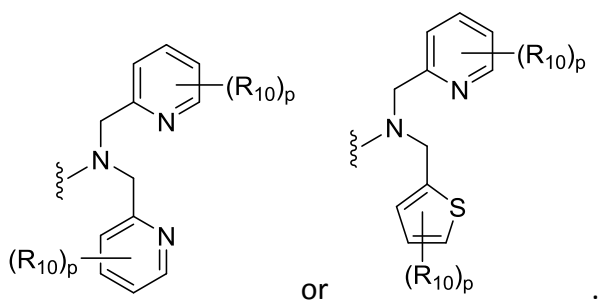
each instance of R_6 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

each instance of R_7 is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 ;

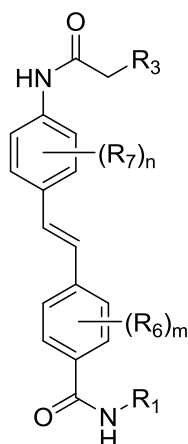
each of R_8 and R_9 is independently H or alkyl; and

each instance of R_{10} is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR_2 .

2. The compound of claim 1, wherein each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.
3. The compound of claim 1, wherein each of R_2 and R_5 is H.
4. The compound of claim 1, wherein each of R_4 , R_8 , and R_9 is H.
5. The compound of claim 1, wherein X is O and R_3 is:



6. The compound of claim 5, wherein R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.
7. The compound of claim 6, wherein each of R_4 , R_8 , and R_9 is H.
8. The compound of claim 1, wherein the compound has Formula **II**:



II

or a pharmaceutically acceptable salt thereof, wherein

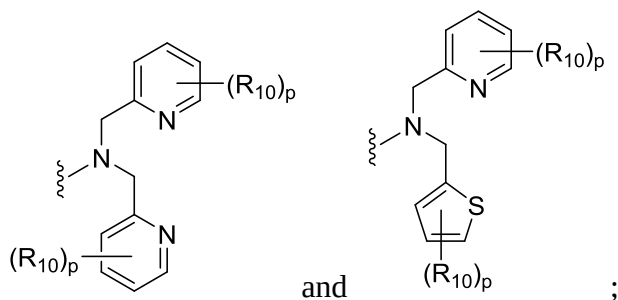
m is a whole number selected from 0-4;

n is a whole number selected from 0-4;

p is a whole number selected from 0-4;

R_1 is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

R_3 is selected from the group consisting of:



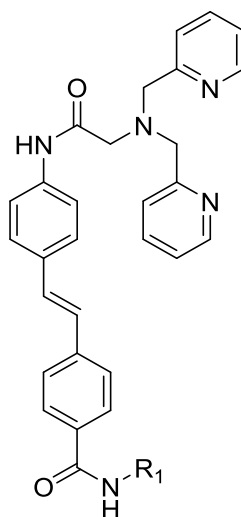
each instance of R₆ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂;

each instance of R₇ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂;

each of R₈ and R₉ is independently H or alkyl; and

each instance of R₁₀ is independently H, alkyl, alkene, alkyne, cycloalkyl, aryl, heteroaryl, halide, nitrile, nitro, OR, or NR₂.

9. The compound of claim 1, wherein the compound has the Formula **III**:

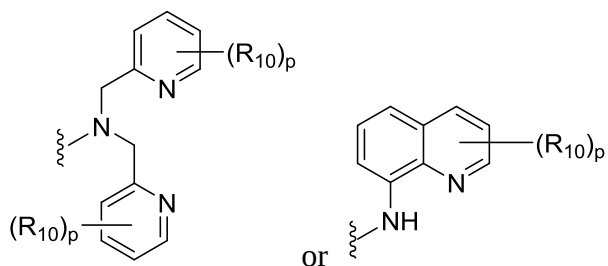


III

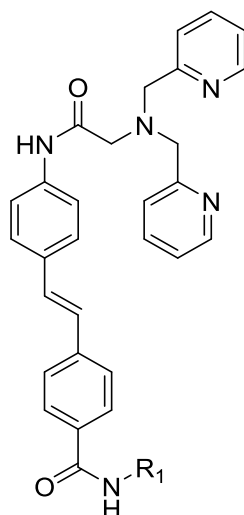
or a pharmaceutically acceptable salt thereof, wherein

R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

10. The compound of claim 1, wherein X is H₂ and R₃ is:



11. The compound of claim 10, wherein each of R_6 , R_7 , and R_{10} for each occurrence is independently selected from the group consisting of H, alkyl, halide, nitrile, and nitro.
12. The compound of claim 11, wherein each of R_2 and R_5 is H.
13. The compound of claim 12, wherein each of R_4 , R_8 , and R_9 is H.
14. The compound of claim 13, wherein each of R_6 , R_7 , and R_{10} is H.
15. Use of a therapeutically effective amount of a compound of claim 1 in the manufacture of a medicament for treating an Epstein-Barr virus (EBV)-positive cell in a patient in need thereof.
16. The use of claim 15, wherein the EBV-positive cell is a cancerous cell.
17. The use of claim 16, wherein the cancerous cell is Burkitt's lymphoma, non-Hodgkin's lymphoma, Hodgkin's disease, T-cell lymphoma, B-cell lymphoma, transplant-associated lymphoproliferative disorders, nasopharyngeal carcinoma, gastric adenocarcinoma, parotid carcinoma, or leiomyosarcoma.
18. The use of claim 15, wherein the compound has the Formula **III**:



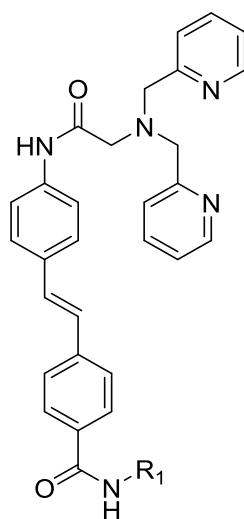
III

or a pharmaceutically acceptable salt thereof, wherein

R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

19. A method of imaging an EBV-positive cell comprising the step of contacting the EBV-positive cell with a compound of claim 1 and measuring the fluorescence of the compound.

20. The method of claim 19, wherein the compound has the Formula **III**:



III

or a pharmaceutically acceptable salt thereof, wherein

R₁ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.