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EBNA1-specific luminescence small molecules for imaging and inhibition of latently EBV-infected tumor cells

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EBNA1-specific small molecule (JLP2) had been syntheised with strong binding and inhibition on the dimerization of EBNA1 in vitro, JLP2 may be used as a selective luminescence agent for the imaging and inhibition of EBV-latently-infected cancer cells.

Epstein-Barr virus (EBV) is etiologically implicated in several lymphoid and epithelial malignancies, substantially contributing to the growth of a diversity of lymphomas and carcinomas. Such presence of EBV in the cells suffering from EBV-associated cancers can, therefore, provide an overarching basis for specific therapy.1 Although current treatments for the EBV-associated carcinoma, such as radiotherapy and chemotherapy, have long been adopted, the former is inadequate either to kill advanced metastatic tumors or to prevent their recurrence, while the latter is still under development.2,3 In fact, a dimeric viral oncoprotein, Epstein-Barr nuclear antigen (EBNA1), is found to be responsible for the development of EBV-related malignancies and the maintenance of the EBV episome. Given that carcinogenesis of EBV-associated carcinomas is symbiotically connected with EBV infection and that EBNA1 can function (e.g. replication, DNA binding and transactivation) only upon dimerization (formation of the active form), we hypothesize that a fluorescent probe consisting of a chromophore and an EBNA1-specific molecule which hampers the dimer formation can be used for the imaging and inhibition of latently EBV-infected cells (Figure 1a); the study will provide a novel strategy to interfere the growth of EBV-associated tumors.4-7 Recently, two groups of novel EBNA1 inhibitors, known as Eik1 and peptide inhibitors, have been introduced. Eik1, developed under a high-throughout screening, is a small molecule which can effectively inhibit the dimerization via association with the EBNA1 amino acid 459-607 of the dimerization domain, whilst the peptidic counterpart functions similarly in the region of 560-574.8-9 On the basis of the in vitro cell line reporter studies, both inhibitors worked well on the EBNA1-dependent OriP-enhanced transcription of SEAP (secreted alkaline phosphatase). However, their activities in the EBV-positive tumor cells have not been reported, and these inhibitors cannot be visualized under confocal microscopy. In particular, the poor solubility of the published peptides (e.g. p85) also obscures their real applications.9,10

Figure 1 (a) Schematic model shows the inhibition process of EBV infection of the host cell and virus reproduction by our compounds; (b) Chemical structures of EBNA1 specific peptides (P1 and P2) conjugated with water soluble luminescence moieties; (c) and (d) the binding fitting via molecular modeling for comparisons of interactions between JLP1/JLP2 and EBNA1 structure.
There are many validated anticancer drug targets, such as Cyclin(s), polo like kinase (Plk) or even EBV; recent research has reported inhibition of these targets can be done by various tailor made peptides. However, the major drawbacks of peptides as anti-wtumor polo like kinase (Plk) or even EBV; recent research has reported binding with EBNA1 specifically in aqueous and bands are very similar at 375 nm (JL NMR and high resolution mass spectroscopy. (Supporting present cationic surfaces can permeate cell membranes of eukaryotic and endogenous cells. The combination of luminescent cargos (i.e. organic molecules/nanomaterial/metal complexes) can provide real time monitoring of these peptide function and improve their cell permeability and solubility. Herein, we report our newly developed EBNA1 specific dual bioprobels (JLP1, Figure 1b) which conjugated with EBNA1 specific peptides. The design rationale is based on molecular computation (vide infra) and synthetic simplicity. Experiment-wise, JLP1 and a well-known mitochondria marker, chromophore JL11 were served as the control. Expectably, JLP2 illustrates selective and responsive emission enhancement upon the binding with EBNA1 specifically in aqueous and in vitro with EBV-infected tumor cells. The IC50 of JLP2 in latently EBV infected tumor cells is much higher than the non-EBV-infected tumor cells. In addition, we had calculated the binding affinity via molecular docking and compared the interactions between Eik1, JLP1, and JLP2 to EBNA1 structures.12 (Figure 1b and lc – JLP1 with EBNA1, -7.6 kCal/mol and JLP2 with EBNA1, -9.0 kCal/mol) The result shows that JLP2 does exert stronger interaction energy than JLP1 and Eik1 binding with EBNA1. (Eik1 = -6.9 kCal/mol) (Figure 1c and 1d). Through comprehensive studies (molecular docking, spectroscopy, MBS cross-linked SDS-PAGE assay and in vitro imaging), JLP2 manifests the potential as a specific dual bioprobe that can trace EBNA1 in vitro for both imaging and inhibition of EBV-infected tumor cells. (Known EBNA1 specific compounds namely Eik1 and the peptides, are not visualized in cells).

Figure 2. The UV absorption, excitation (JLP1 -λem = 545 nm and λex = 560 nm) and emission spectra (λem = 405 nm) of JLP1 and JLP2 in aqueous solution. Inset: The emission enhancement of JLP2 upon the addition of the 200 nM EBNA1.

JLP1 and JLP2 had been purified by HPLC and characterized by NMR and high resolution mass spectroscopy. (Supporting Information) The absorption and emission bands of the JLP1 and JLP2 are 30 nm red shifted compared with JL (peptides P1/P2 conjugated to JL). For JLP1 and JLP2, the absorption and excitation bands are very similar at 375 nm (JLP1) and 390 nm (JLP2) in aqueous solution respectively. (Figure 2) The emission of both JLP1 and JLP2 are green (λem = ~560 nm, λex = 400 nm, Figure 2) In comparison with the EBNA1 small molecules/peptides in the literature, our compounds are fluorescent, meaning that the binding of EBNA1 in situ/in vitro or cellular uptake can be evaluated directly by fluorescent detection, i.e. flow cytometry and fluorescence microscopy. The general photophysical properties of JLP1 and JLP2 are similar with only one amino acid different in their structures; however, the in situ and in vitro behaviours of JLP1 and JLP2 are completely distinct. For instance, after the addition of EBNA1 into the aqueous solution of JLP1 and JLP2 respectively, emission enhancement could be detected only in JLP2, more or less 1.5-fold upon addition of 200 nM EBNA1 (Inset of Figure 2), whereas in

Figure 3. The confocal images and cellular uptake (by flow cytometry) of JLP1 and JLP2 in latently EBV-infected nasopharyngeal carcinoma C666-1 cells (a, b, c - JLP1 ; d, e, f - JLP2) and non-latently EBV-infected human cervical carcinoma HeLa cells (g, h, i - JLP1 ; j, k, l - JLP2).

JLP1, the emission intensity slightly diminished with the same dosed amount of EBNA1 in water (Figure S14). Aside from this, we had also confirmed the specific binding of JLP1 to EBNA1 by MBS (3-maleimidobenzy N-hydroxysuccinimide) cross-linked SDS-PAGE assay. MBS can covalently form cross-linkages with dimerized EBNA1 proteins which can be separated using SDS-PAGE. That is, EBNA1, disrupted by the compound in the assay, can no longer exist in dimmer. In Figure S16 (black box), only JLP2 obviously decreased the dimmer of EBNA1. In addition, the cellular uptake of JLP1 and JLP2 are different. JLP1 and JLP2 (10 µM) had been tested in the latently EBV-infected
nasopharyngeal carcinoma C666-1 cells for 12 hours. Only in JLp2 can the uptake be observed more apparently: the confocal images from the C666-1 cells of JLp1 and JLp2 are co-related in the findings from the flow cytometry uptake studies. (Figure 3) No emission can be obtained from JLp1 in C666-1 cells and only JLp2 displays impressive emission in the latently EBV-infected nasopharyngeal carcinoma C666-1 cell under the same experiment. In the cytotoxicity experiment (JLp1 and JLp2 in EBV/ non-EBV infected tumor cells and Eik1 as the control), JLp2 demonstrates the smaller IC50 values in EBV infected cancer cells (C666-1, ~ IC50 = 20 µM, similar to the Eik1) than non-EBV infected cancer cells (HeLa ~ 1 mM). On the other hand, JLp1, p1 and p2 demonstrated similar IC50 values in HeLa and C666-1 cells under the same experimental conditions. Peptides p1 and p2 are not cell permeable and alone is not effective in growth, these two peptides are served as control experiments in MTT assays. (Figure 4)

Figure 4. Cell viability inhibitory activities of two EBNA1 specific peptides (p1 and p2), two small molecules (JLp1 and JLp2) and Eik1 (control) on (a) non- latently EBV-infected human cervical carcinoma HeLa, (b) latently EBV-infected nasopharyngeal carcinoma C666-1 cells were assayed

Conclusion
A specific and selective agent for both imaging and targeting of the virus-associated tumor has not been available in the literature currently. In this regard, we had synthesised a dual-function fluorescent probe (JLp2) of verified applicability for simultaneously imaging and controlling the growth of EBV latently infected tumor (e.g. nasopharyngeal carcinoma) on account of the underlying mechanism to stymie the dimerization of EBNA1 specifically.

Notes and references
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†Electronic Supplementary Information (ESI) available: Experimental for synthesis, purification of EBNA1, MTT assays and in vitro imaging; High resolution mass spectra of p1, p2, JLp1 and JLp2; NMR spectra of intermediates, ligands of JLp1 and JLp2; emission spectra of JLp with the addition of various amount of EBNA1. See DOI: 10.1039/c000000x/