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ORIGINAL ARTICLE

Selective killing of Burkitt's lymphoma cells by mBAFF-targeted delivery of PinX1

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Increased expression of BAFF (B cell-activating factor belonging to the TNF family) and its receptors has been identified in numerous B-cell malignancies. A soluble human BAFF mutant (mBAFF), binding to BAFF receptors but failing to activate B-lymphocyte proliferation, may function as a competitive inhibitor of BAFF and may serve as a novel ligand for targeted therapy of BAFF receptor-positive malignancies. Pin2/TRF1-interacting protein X1 (PinX1), a nucleolar protein, potently inhibits telomerase activity and affects tumorigenicity. In this study, we generated novel recombinant proteins containing mBAFF, a polyarginine tract 9R and PinX1 (or its *C/N* terminal), to target lymphoma cells. The fusion proteins PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF specifically bind and internalize into BAFF receptor-positive cells, and subsequently induce growth inhibition and apoptosis. The selective cytotoxicity of the fusion proteins is a BAFF receptor-mediated process and depends on mBAFF, PinX1/C and 9R. Moreover, the fusion proteins specifically kill BAFF receptor-expressing Burkitt's lymphoma (BL) cells by inhibiting telomerase activity and the consequent shortening of telomeres. Therapeutic experiments using PinX1C–G_4S–9R–G_4S–mBAFF in severe combined immunodeficient (SCID) mice implanted with Raji cells showed significantly prolonged survival times, indicating the *in vivo* antitumor activity of the fusion protein. These results suggest the potential of PinX1/C–G_4S–9R–G_4S–mBAFF in targeted therapy of BL.
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Introduction

The traditional therapeutic approach for Burkitt's lymphoma (BL) has been shown to be highly successful; however, relapse typically occurs, and emergence of chemoresistance is common. The agents used for the treatment of BL are non-selective and induce undesired side effects. Ligand-mediated targeting of fusion proteins to cell surface receptors is increasingly being recognized as an effective strategy for improving therapeutic efficacy.[1,2] Fusion proteins are composed of three functional domains: a ligand that binds specifically to target receptors, a translocation domain and the toxic protein.[3,4] Typically, the targeting moiety of the fusion protein recognizes and delivers the entire molecule to specific receptors expressed on the surface of target cells. After binding to the cell surface, the conjugates are internalized via a receptor-mediated endocytosis, followed by cleavage-dependent release of toxic protein from endosomes to the cytosol. Finally, the protein localizes in the cytosol or nucleus and eventually kills the targeted cells.

BAFF (B cell-activating factor belonging to the TNF family), a member of the TNF superfamily of cytokines, is critical for the maintenance of normal B-cell development and homeostasis.[5,6] The biological effects of BAFF are mediated by three cell surface receptors, including BAFF-R (B cell-activating factor receptor), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antibody (BCMA).[7] Increased expression of BAFF and its receptors has been identified in numerous B-cell malignancies.[8–10] The study by Riccobene *et al.*[11] has shown that radiolabeled BAFF specifically and rapidly localizes in B-cell tumors in mice, suggesting the potential for targeted therapy. Indeed, BAFF and its receptors have recently received increasing attention as therapeutic targets for the management of a number of B-cell malignancies.[1,2] Interestingly, a soluble human BAFF mutant (mBAFF), in which amino acids 217–224 are replaced by two glycine residues, can also bind to BAFF receptors but does not activate B-lymphocyte proliferation.[12] Therefore, mBAFF likely competes with BAFF for receptor binding and blocks the biological activity of wild-type BAFF, resulting in suppression of lymphocyte activation and proliferation. Hence, mBAFF may serve as a competitive inhibitor of BAFF in the treatment of relevant malignant hematological diseases. In addition, mBAFF may also be used as a novel ligand for the selective delivery of toxic proteins or toxins to BAFF receptor-positive malignant B cells.

Telomerase, having a crucial role in maintaining telomere length, has been implicated in cancers.[13] Telomerase from all species minimally consists of two essential components: the telomerase reverse transcriptase and the telomerase RNA moiety. In humans, telomerase reverse transcriptase is the key target for telomerase regulation and its expression is the rate-limiting step for telomerase activation.[14,15] Human Pin2/TRF1-interacting protein X1 (PinX1), a 328-aa nucleolar protein, binds telomerase reverse transcriptase directly and potently inhibits its activity.[13] Overexpression of PinX1 or its telomerase inhibitory domain suppresses telomerase activity, causes telomere shortening and induces cells into crisis, whereas depletion of PinX1 increases tumorigenicity in nude mice. However, exogenous PinX1 alone cannot gain cell entry and it must be introduced into the cell as a conjugate. The polyarginine (consisting of nine arginine residues, 9R), shown to function as a protein transduction domain, is important for intracellular processing and meets the most stringent furin specificity requirements, as it contains six furin protease cleavage sites.[16] After furin cleavage, the cleaved 9R aids in translocation of toxic protein from the endosomes to the cytosol. Subsequently, the toxic protein localizes and functions in the cytosol or nucleus.

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In this study, we designed fusion proteins containing the 328-aa full-length human PinX1, 74-aa *C*-terminal fragments (PinX1C) or 142-aa *N*-terminal fragments (PinX1N) (referred to hereafter as PinX1/C/N) linked to mBAFF. A 9R tract, which aids in the translocation of the toxic molecules from endosomes to the cytosol, was inserted between PinX1/C/N and mBAFF. It is suspected that the 5-aa G_4S linker (four glycine residues and one serine residue) may be important to avoid spatial constraints that may interrupt proper folding of the fused fragments. Therefore, we prepared two kinds of fusion proteins including PinX1/C/N–G_4S–9R–G_4S–mBAFF and PinX1/C/N–9R–mBAFF. Subsequently, we characterized the biological activity of these targeted fusion proteins against a variety of lymphoma cells and demonstrated that mBAFF selectively targets the fusion proteins to BAFF receptor-expressing lymphoma cells and internalizes rapidly into target cells. Cytotoxicity assays showed that both PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF (but not PinX1N–G_4S–9R–G_4S–mBAFF and PinX1N–9R–mBAFF) cause selective death of lymphoma cells. In addition, the cytotoxicity of the targeted fusion proteins was associated with a decline in telomerase activity and with the shortening of telomeres. Moreover, therapeutic administration of PinX1C–G_4S–9R–G_4S–mBAFF in severe combined immunodeficient (SCID) mice implanted with Raji cells showed significantly prolonged survival time compared with treatment with free mBAFF, PinX1C or PinX1C-mBAFF, indicating the marked *in vivo* antitumor activity of the fusion protein. These results suggest that the targeted fusion proteins (especially PinX1C–G_4S–9R–G_4S–mBAFF) may be the potential candidates for the treatment of BL and/or other B-cell malignancies expressing high levels of BAFF receptor(s) and exhibiting high telomerase activity.

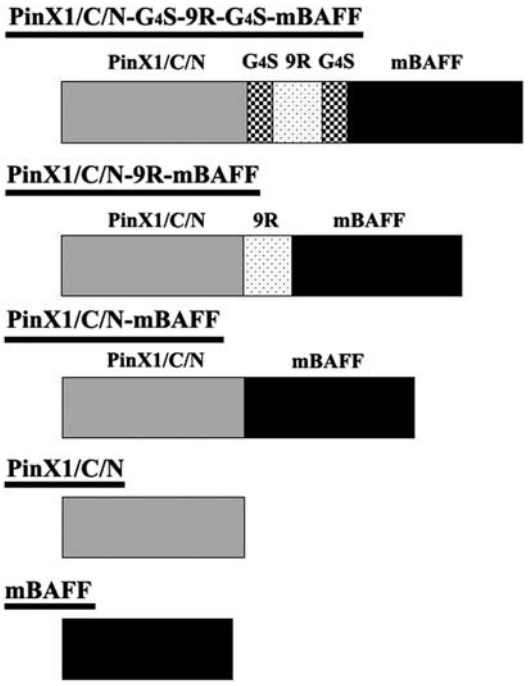

Figure 1 Schematic representation of the recombinant fusion proteins used in this work. PinX1, the 328-aa full-length PinX1; PinX1C, the 74-aa *C*-terminal fragment of PinX1; PinX1N, the 142-aa *N*-terminal fragment of PinX1; PinX1/C/N, PinX1 or PinX1C, or PinX1N; 9R, nine arginine residues; G_4S, four glycine residues and one serine residue; mBAFF, a soluble human BAFF mutant, in which amino acids 217–224 are replaced by two glycine residues.

Materials and methods

Tumor cell lines

The human Burkitt's lymphoma (BL) cell lines (Raji, Namalwa and Daudi), the mantle cell lymphoma (MCL) cell line (JeKo-1), the acute monocytic leukemia cell line (THP-1) and the human acute T-cell leukemia cell line (Jurkat) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate at 37 °C in a humidified atmosphere of 5% CO_2.

Generation and purification of the fusion proteins

The schematics of the fusion proteins generated in this study are shown in Figure 1. The assembly of the fusion genes (from the 5′ to 3′ ends) encoding PinX1/C/N–G_4S–9R–G_4S–mBAFF (refered to as PinX1–G_4S–9R–G_4S–mBAFF, PinX1C–G_4S–9R–G_4S–mBAFF or PinX1N–G_4S–9R–G_4S–mBAFF) was created using overlap extension PCR. The cloned genes were separately ligated into the pET28a expression vector and confirmed by DNA sequence. Similarly, the genes encoding PinX1/C/N-9R-mBAFF, PinX1/C/N-mBAFF and PinX1/C/N were created using the same techniques. Thereafter, the fusion proteins were expressed in *E. coli* BL21(DE3) after being induced by isopropyl-h-D-thiogalactopyranoside (IPTG). In addition, mBAFF alone was expressed using pQE-80L expression vector in *E. coli* DH5α as described previously.[17] All the proteins were His-tagged, and detected with a mouse anti-His monoclonal antibody. The proteins were then purified from the supernatants of bacterial lysates by Ni^{2+}-NTA affinity chromatography under native conditions, and subsequently isolated by Sepharcryl S200 gel filtration chromatography.

Binding and internalization of the fusion proteins

Raji, Namalwa, Daudi, JeKo-1, THP-1 and Jurkat cells were treated with 250 nM fusion proteins for 1 h. Thereafter, the cells were briefly washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min at room temperature. After rinsing with PBS, the fixed cells were permeabilized for 5 min in PBS containing 0.2% Triton X-100, washed three times with PBS and blocked with PBS containing 3% bovine serum albumin for 1 h at room temperature. Following a brief wash with PBS, the cells were incubated with anti-His monoclonal antibody diluted 1:500 in PBS containing 0.1% Tween 20 (PBST) and 0.2% bovine serum albumin for 1 h at room temperature. The cells were washed three times in PBST for 15 min and incubated with a 1:100 dilution of FITC-conjugated goat antimouse IgG. After three washes with PBST, the cells were washed once in PBS for 10 min. Thereafter, the cells (50 000) were adhered onto slides using cytospin, mounted in media containing DAPI (Sigma, St Louis, MO, USA) and analyzed with laser scanning confocal microscopy.

Cell proliferation assay and competitive inhibition assay

To determine the growth inhibition effects of the fusion proteins on Raji, Namalwa, Daudi, JeKo-1, THP-1 and Jurkat cells, proliferation assays measuring [^{3}H]-thymidine incorporation were performed. Briefly, cells (1×10^4 per well) were seeded into a 96-well flat-bottom plate, and the fusion proteins at

various concentrations were added to the wells in triplicate. Thereafter, the cells were incubated at 37 °C for 5 days, and [^{3}H]-thymidine (GE Healthcare, Chalfont St Giles, UK) was added to the wells (1 μCi/well) during the last 16 h of incubation. Subsequently, the cells were collected on glass fiber filters, washed, dried and counted using standard scintillation methods. Each assay was performed in triplicate on at least three independent occasions.

Competitive inhibition assays were conducted to determine the specificity of the fusion proteins. Briefly, Raji cells were pretreated with 10 μg/ml mBAFF for 1 h, followed by treatment with 250-nM fusion proteins in quadruplicate wells. The cells were then incubated for 5 days, and proliferation was measured by [^{3}H]-thymidine uptake as described above.

Analysis of apoptosis using annexin-V

Raji and Jurkat cells (1×10^4 per well) were seeded into a 96-well flat-bottom plate, and the fusion proteins at 250-nM were added to the wells in triplicate. After incubation at 37 °C for 5 days, apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed with PBS and resuspended in 200 μl of 1 × annexin-binding buffer at a density of 1×10^6 cells/ml. Thereafter, 5 μl of FITC Annexin V and 5 μl of Propidium Iodide (PI) were added and the cells were incubated in the dark for 15 min at room temperature. The labeled cells were analyzed immediately using a FACSCalibur flow cytometer.

Telomerase activity and telomere restriction fragment (TRF) length assays

To examine the effects of the fusion proteins on telomerase activity *in vitro*, Raji cells were lysed in lysis buffer, and the telomerase-containing fraction was prepared and then the telomerase activity was assayed with the TeloTAGGG Telomerase Detection kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. Briefly, fusion proteins were incubated with telomerase-containing fraction for 10 min at 4 °C and then subjected to telomerase extension. Telomerase products were separated on 10% polyacrylamide gels and then the gels were stained with chemiluminescence reagent. Telomerase activity was semiquantified by normalizing the band intensities of the characteristic 6-bps telomerase-specific ladder to that of the 216-bps internal standard using NIH image software (http://rsb.info.nih.gov/nih-image/about.html).[13]

TRF length was measured as described.[13] Briefly, Raji cells (2×10^5/well) were seeded into a 24-well plate and the fusion proteins (100-nM) were added to the wells in triplicate. Subsequently, the cells were continuously maintained in culture by splitting the cells and seeding them at 2×10^5 per well in the presence of 100-nM fusion proteins for 30 population doublings. Thereafter, genomic DNAs were prepared and telomere lengths were assayed using the TeloTAGGG Telomere Length Assay kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. Briefly, genomic DNA was digested with *Hin*fI and *Rsa*I and then separated on 0.7% agarose gels (2 μg per lane). The gels were dried and hybridized with a digosin-labeled telomeric DNA probe, and hybridization signals were detected by horseradish peroxidase-conjugated antidigosin antibody and developed with an enhanced chemiluminescent reagent. To obtain reliable results, the signal strength must be within the linear range of the X-ray film. The loss of telomeric sequence was evident from the shortening of TRF length (based on the reduction in the TTAGGG hybridization signal).

In vivo *efficacy studies*

Female SCID/Beige mice were obtained from Beijing Vital River Experimental Animals Co. Ltd (China), housed and maintained in accordance with institutional guidelines. At 4–6 weeks of age, the mice were injected intravenously (i.v.) with 5×10^6 Raji cells, following the model described by Ghetie *et al.*,[18] in which they used Daudi cells (both Daudi and Raji cells grow similarly[19]). The mice were monitored daily for hindleg paralysis, which was observed in 12–19 days in control mice without treatment with the fusion proteins. For therapy, the mice were treated with multiple injections of 50 μg of PinX1C–G_4S–9R–G_4S–mBAFF i.v. on days 1, 4, 7, 10 and 13. Because this Raji substrain always metastasizes to the central nervous system resulting in hindlimb paralysis, the mice were killed when paralysis was observed. Animals were judged terminally if they died or if hindlimb paralysis occurred.

Statistical analysis

Statistical analysis was performed with the SPSS13.0 software package for Windows. Survival rates were analyzed by the Kaplan–Meier method with comparisons between treatment groups made by the log-rank test. Two-way ANOVA and multiple comparisons of mean values were conducted. A *P*-value of <0.05 was considered as statistically significant.

Results

Fusion proteins are successfully prepared

In this study, 13 recombinant proteins were generated, and their schematics are shown in Figure 1. Because the *C*-terminal of BAFF is important for preserving the function of the protein, we designed the fusion proteins with mBAFF at *C*-terminal. It is suspected that the G_4S linker may be important to avoid steric hindrance and enable simultaneous binding of fusion proteins; therefore, we prepared two kinds of fusion proteins including PinX1/C/N–G_4S–9R–G_4S–mBAFF and PinX1/C/N–9R–mBAFF. The proteins PinX1/C/N–mBAFF, PinX1/C/N and mBAFF alone were used as controls in this study. Western blot analysis with anti-His antibody indicated the presence of the fusion proteins (Supplementary Figure 1A), and the purified fusion proteins migrated on sodium dodecyl sulfate (SDS)-PAGE gels as monomers at the expected molecular weight under reducing conditions (Supplementary Figure 1B).

mBAFF-containing fusion proteins specifically bind and internalize into BAFF receptor-expressing cells

We used reverse transcriptase (RT)-PCR to examine the mRNA expression profile of the three BAFF receptors including BAFF-R, TACI and BCMA. As shown in Supplementary Figure 2, Raji, Namalwa and JeKo-1 cells expressed all three BAFF receptor mRNAs. Daudi cells expressed only BAFF-R and BCMA mRNA (no detectable TACI mRNA). THP-1 cells showed relatively low levels of BAFF-R mRNA (no detectable TACI and BCMA mRNA). Jurkat cells showed no detectable mRNA of any BAFF receptor. As all lymphoma cells express BAFF receptors, we next evaluated the binding and internalization of fusion proteins into cancer cells using the immunofluorescence method under a confocal microscope after treatment with 250-nM recombinant proteins for 1 h. As illustrated in Figure 2 and Supplementary Figure 3, all the

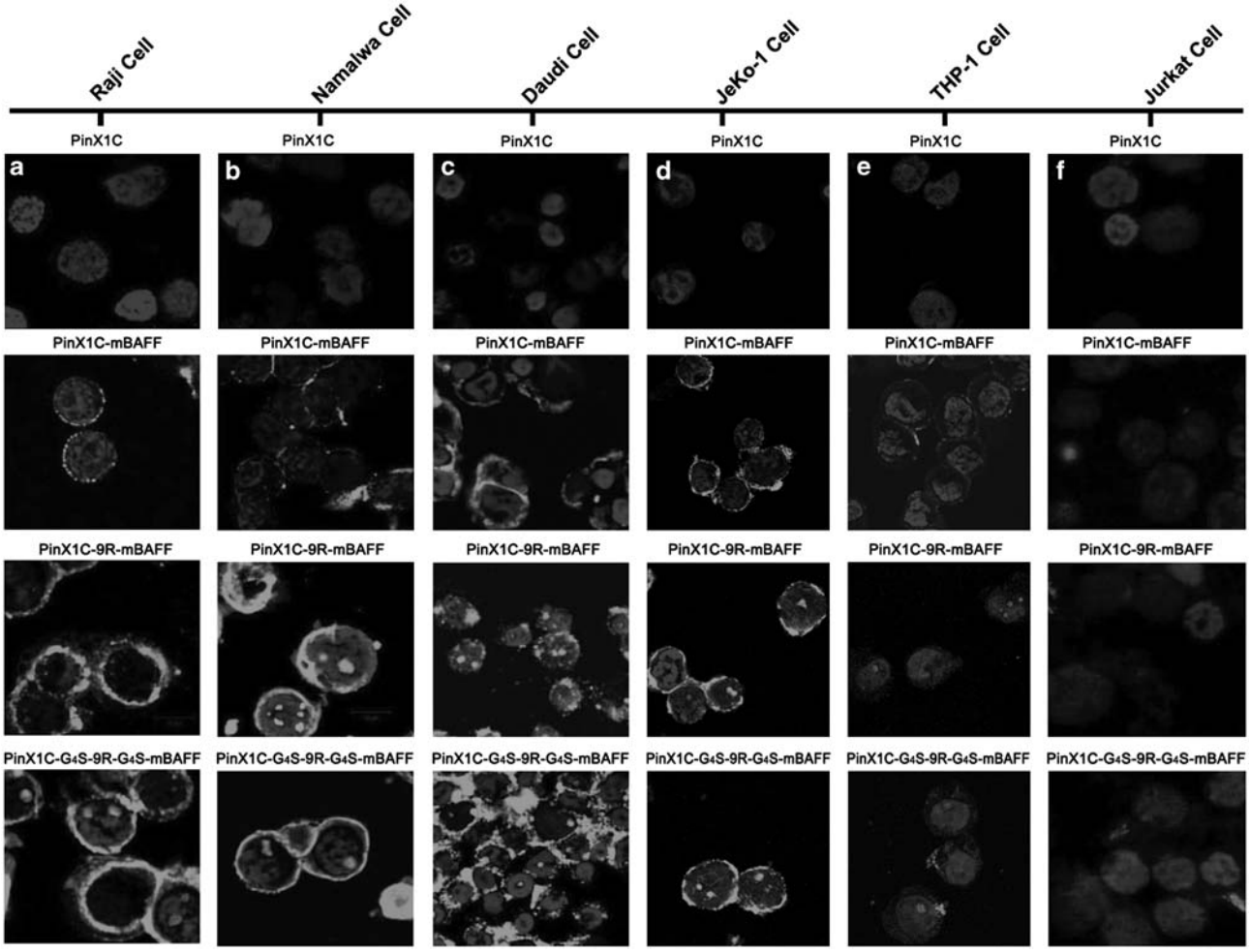

Figure 2 mBAFF mediates the specific binding and internalization of PinX1C-containing fusion proteins into BAFF receptor-positive cells. The BAFF receptor-positive cells including Raji (**a**), Namalwa (**b**), Daudi (**c**), JeKo-1 (**d**) and THP-1 (**e**), and BAFF receptor-negative Jurkat cells (**f**) were treated with 250-nM fusion proteins for 1 h. The cells were then adhered onto a microscope slide and fixed in 4% paraformaldehyde, followed by a brief rinse with PBS. Subsequently, the cells were incubated with an anti-His monoclonal antibody. After a brief wash with PBS, cells were incubated with FITC-conjugated goat antimouse IgG. After washing with PBS, slides were mounted in mounting medium and then analyzed by laser scanning confocal microscopy.

mBAFF-containing proteins (but not PinX1/C/N alone) could bind and internalize into BAFF receptor-positive cells (Raji, Namalwa, Daudi, JeKo-1 and THP-1 cells), but not BAFF receptor-negative cells (Jurkat cells), indicating that the specific binding is mediated by mBAFF and the internalization is a BAFF receptor-mediated endocytosis. After internalization, PinX1/C/N–9R–mBAFF and PinX1/C/N–G_4S–9R–G_4S–mBAFF were primarily localized in the nucleus (especially in nucleoli) in BAFF receptor-expressing cells. Because the nucleus makes up the majority of the lymphoma cell volume, whereas the cytoplasm is minimal and on the periphery, PinX1/C/N–mBAFF–FITC appeared as a peripheral stain in BAFF receptor-expressing cells, suggesting that the furin site(s) existing in 9R is/are necessary for PinX1/C/N delivery to the cytosol and even to the nucleolar region.

The fusion proteins PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF specifically induce growth inhibition and apoptosis of BAFF receptor-expressing cells in vitro

To determine the ability of PinX1/C/N–G_4S–9R–G_4S–mBAFF and PinX1/C/N–9R–mBAFF to induce the growth inhibition of BAFF receptor-expressing cells, the fusion proteins were tested in Raji, Namalwa, Daudi, JeKo-1 and THP-1 cells using proliferation assays measuring [^{3}H]-thymidine incorporation. As shown in Figure 3A and Supplementary Figure 4A, PinX1C–G_4S–9R–G_4S–mBAFF could more efficiently inhibit the proliferation of BAFF receptor-expressing cells as compared with PinX1C–9R–mBAFF, which suggests that the 5-aa G_4S linker is important for the fusion proteins to function. Similar results were obtained with PinX1–G_4S–9R–G_4S–mBAFF and PinX1–9R–mBAFF, although PinX1-mediated growth inhibition was not as potent as that by PinX1C. Conversely, PinX1N–G_4S–9R–G_4S–mBAFF and PinX1N–9R–mBAFF were far less effective than PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF, suggesting that the *C*-terminal of PinX1 is crucial for the fusion proteins to inhibit cell growth. Compared with PinX1/C–9R–mBAFF, PinX1/C–mBAFF showed a very low inhibition effect, indicating that the 9R tract is necessary for the proteins to function efficiently. Furthermore, PinX1/C alone showed minimal activity against cancer cells. Therefore, linking PinX1/C to 9R and mBAFF augmented its toxicity toward B-lymphoma cells, and simultaneously conferred a high degree of selectivity. Of all the BAFF receptor-expressing cells tested, THP-1 cells

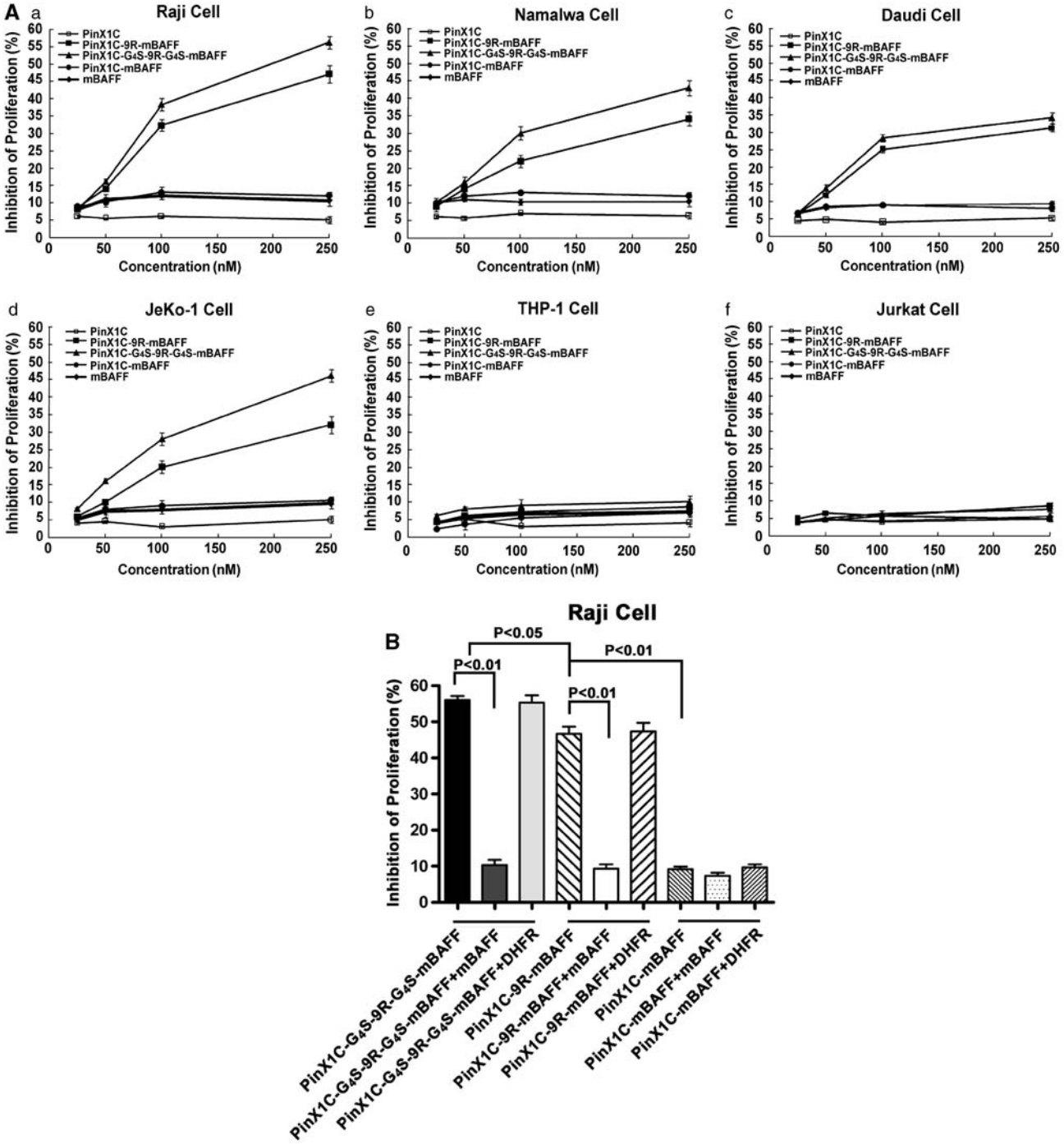

Figure 3 The fusion proteins PinX1C–G_4S–9R–G_4S–mBAFF and PinX1C–9R–mBAFF selectively induce growth inhibition of BAFF receptor-expressing cells. (**A**) Cell proliferation assay was performed using the [^{3}H]-thymidine incorporation method. BAFF receptor-expressing cells including Raji (**a**), Namalwa (**b**), Daudi (**c**), JeKo-1 (**d**) and THP-1 (**e**), and BAFF receptor-negative Jurkat cells (**f**) were seeded into a 96-well flat-bottom plate (1×10^4 cells per well) and treated in triplicate with the fusion proteins at various concentrations. Thereafter, the cells were incubated for 5 days and [^{3}H]-thymidine was added to the wells (1 μCi/well) during the last 16 h of incubation. Subsequently, the cells were collected on glass fiber filters, washed, dried and counted using standard scintillation methods, and percentages of the inhibition of proliferation were calculated. (**B**) Blocking assay was conducted to confirm the importance of the fused mBAFF for the activity of the fusion proteins. Raji cells were pretreated with 10 μg/ml of mBAFF for 1 h, followed by treatment with 250-nM of the fusion proteins in quadruplicate wells. The cells were then incubated for 5 days, and proliferation assays measuring [^{3}H]-thymidine incorporation were conducted. DHFR, which does not bind BAFF receptors, serves as a negative control. All data in **A** and **B** are expressed as mean ± s.e.

were found to be less sensitive to the fusion proteins than the other cell lines, which may be because of the low level of BAFF receptor or a different intracellular fate for internalized fusion proteins.[1] To confirm the specificity of PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF, we tested the effects of fusion proteins on the proliferation of BAFF receptor-negative

Jurkat cells. As shown in Figure 3Af and Supplementary Figure 4B, Jurkat cells were completely resistant to all these reagents.

The biological effects of BAFF are mediated by three cell surface receptors including BAFF-R, TACI and BCMA. Therefore, pretreatment of cells with mBAFF may block the binding of PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF to these BAFF receptors. To test this hypothesis, 10 μg per ml of mBAFF was added to Raji cells before treatment with fusion proteins. Dihydrofolate reductase (DHFR), which does not bind BAFF receptors, serves as a negative control. Indeed, pretreatment with mBAFF blocked 90–95% of the growth inhibition effects in PinX1/C–G_4S–9R–G_4S–mBAFF-treated and PinX1/C–9R–mBAFF-treated lymphoma cells (Figure 3B and Supplementary Figure 4C), indicating that the growth inhibition effects of the fusion proteins are mediated by their direct binding to BAFF receptors.

To ensure that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF were in fact killing the BAFF receptor-expressing cells and not merely inhibiting proliferation, we measured the apoptosis of the target cells. We used Raji cells for these experiments because they exhibited a significant growth inhibition response to treatment with the fusion proteins. The ability of fusion proteins to induce apoptosis was tested by monitoring the binding of Annexin V to Raji cells treated with 250-nM fusion proteins. As shown in Figure 4 and Supplementary Figure 5, PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF efficiently induced apoptosis in Raji cells (but not in Jurkat cells). Consistent with the above results in the cell proliferation assay, PinX1C–G_4S–9R–G_4S–mBAFF resulted in the most obvious apoptosis. These data showed that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF are capable of recognizing and destroying BAFF receptor-positive cells but not BAFF receptor-negative cells.

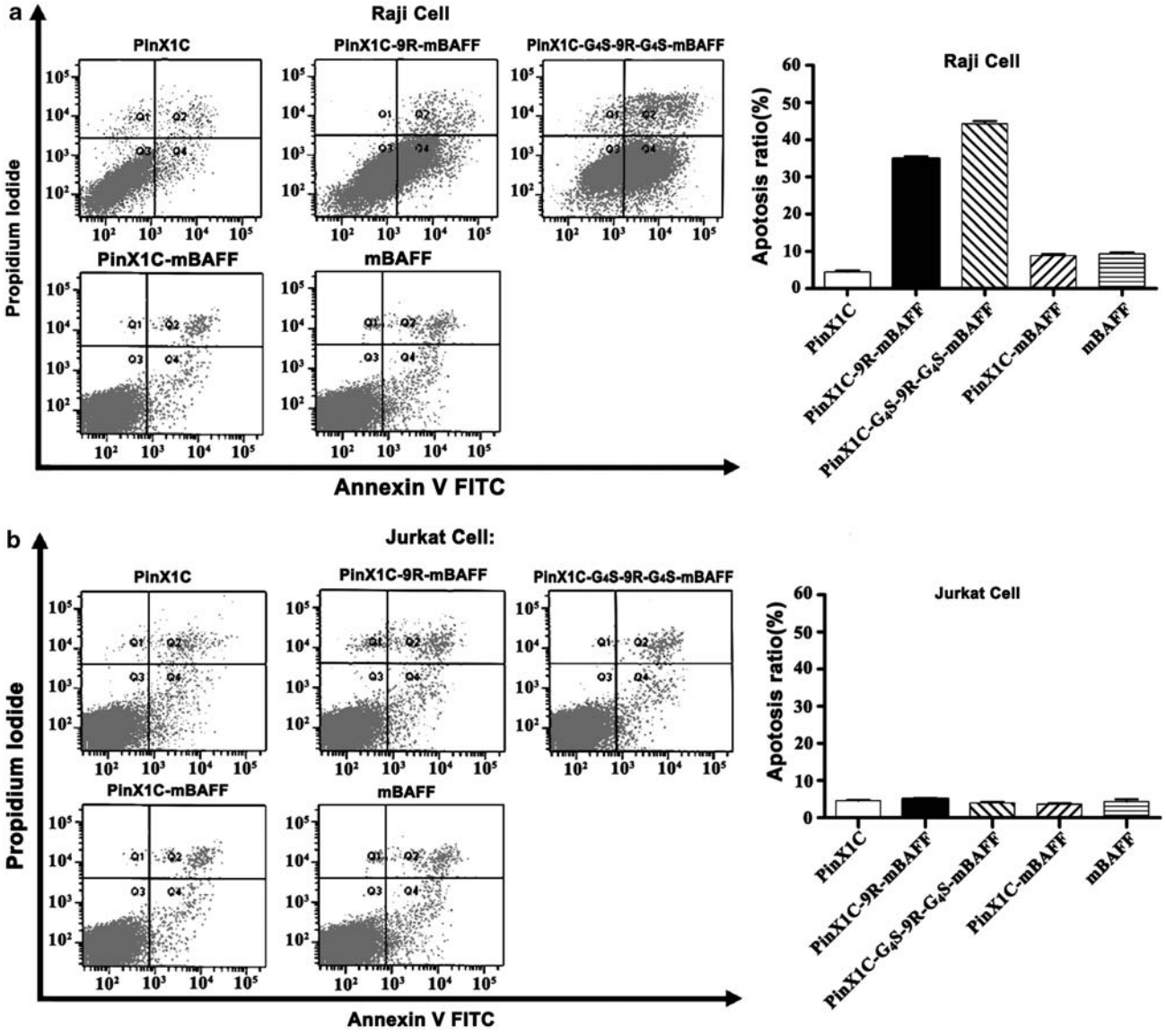

Figure 4 Fusion proteins PinX1C–G_4S–9R–G_4S–mBAFF and PinX1C-9R-mBAFF selectively induce apoptosis of BAFF receptor-expressing cells. Raji (**a**) and Jurkat (**b**) cells were treated in triplicate with 250-nM fusion proteins for 5 days, and apoptosis was then measured using flow cytometry after staining cells with annexin V-PI. Specific apoptosis in comparison with control cell death was calculated and presented as a percentage of control. The results are expressed as mean ± s.e.

PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF inhibit telomerase activity and shorten telomeres of Raji cells in vitro

The above studies have demonstrated that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF specifically killed BAFF receptor-expressing cells. To investigate the mechanism of action, we performed *in vitro* telomerase activity assays to determine the correlation between the fusion proteins and telomerase. As shown in Figure 5a, telomerase activity was readily detected in Raji cell extracts, but not in the extracts that were pretreated with RNase. PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF potently inhibited telomerase activity in a concentration-dependent manner, whereas PinX1/C, PinX1/C–mBAFF, PinX1N-containing fusion proteins and mBAFF alone had no significant effect. Most strikingly, telomerase activity was almost undetectable in cells treated with PinX1C–G_4S–9R–G_4S–mBAFF. Together with the results in Figures 3 and 4, the difference in cellular telomerase activity inhibition correlates with the ability of these proteins to kill cells.

To confirm that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF regulate cellular telomere length, we measured TRF length in Raji cells. As shown in Figure 5b, the untreated Raji cells maintained relatively long telomeres, with an average TRF length of 10.4 kb. The similar TRF length was also detected in cells treated with free PinX1/C, PinX1/C–mBAFF, PinX1N-containing fusion proteins and mBAFF alone. However, the TRF length was shortened in cells treated with PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF. Most importantly, in cells incubated with PinX1C–G_4S–9R–G_4S–mBAFF, the telomeres were shortened, with TRF length reaching the minimal length (8.1 kb). These results indicate that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF shorten telomeres and that PinX1C–G_4S–9R–G_4S–mBAFF produces a much more potent effect.

PinX1C–G_4S–9R–G_4S–mBAFF effectively prolongs survival in SCID mice with systemic cancer

Because PinX1C–G_4S–9R–G_4S–mBAFF has much more potent antitumor activity *in vitro*, we chose it to analyze the therapeutic effect *in vivo* in a model of non-Hodgkin's lymphoma by administering 5×10^6 Raji cells i.v. into SCID mice. One advantage of this model is that i.v. injection of tumor cells into SCID mice leads to the formation of systemic tumors that infiltrate all major organs and is more reminiscent of human leukemia. As shown in Figure 6, mice treated five times (on days 1, 4, 7, 10 and 13, 50 μg per time) with PinX1C–G_4S–9R–G_4S–mBAFF lived significantly longer than control mice that were treated with PinX1C–mBAFF, PinX1C or mBAFF alone using the same regimen ($P<0.01$). In addition, the untreated control mice had a similar survival rate as PinX1C-treated mice ($P>0.05$). Furthermore, there were no statistically significant differences between PinX1C–mBAFF- and mBAFF-treated groups with respect to survival times ($P>0.05$), but mice in both the groups had a significantly longer life span ($P<0.05$) compared with the untreated control groups. These data indicate that PinX1C–G_4S–9R–G_4S–mBAFF can effectively target and suppress Raji cells in a highly aggressive SCID model.

Discussion

In this study, we have described the generation and functional characterization of novel fusion proteins containing PinX1/C/N linked to mBAFF and a 9R tract. The fusion proteins PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF specifically killed BAFF receptor-positive lymphoma cells but not BAFF receptor-negative cells *in vitro*. Furthermore, PinX1C–G_4S–9R–G_4S–mBAFF also proved to be a functional and specific therapeutic agent for disseminated B-cell lymphoma in a mouse xenograft model. These cooperative functions highlight the importance of targeting tumors as an efficacious therapeutic strategy. Moreover, we demonstrated that the fusion proteins specifically kill BAFF receptor-expressing BL cells through a mechanism that involves the inhibition of telomerase activity and the consequent shortening of telomeres.

Successful development of tumor-targeted therapeutic agents is dependent, in part, on the site-specific delivery of therapeutic agents and also on the biological activity of the delivered agent.[1,2,20–23] BAFF is crucial for B-cell survival, and the biological effects of BAFF are mediated by three receptors including BAFF-R, TACI and BCMA. These receptors are desirable therapeutic targets because of their increased expression in numerous B-cell malignancies.[1,2] Kern *et al*.[24] reported that BAFF expression mediates the apoptotic resistance of B-chronic lymphocytic leukemia cells through an autocrine pathway. Therefore, therapeutic targeting of BAFF and its receptors may disrupt the important autocrine growth processes that are at work in B-cell malignancies. In this study, we used mBAFF (a BAFF mutant) as a targeting molecule, which can efficiently bind to the BAFF receptors but fail to stimulate B-lymphocyte proliferation.[12] The *in vitro* and *in vivo* studies showed that mBAFF can target the fusion proteins to kill BAFF receptor-positive cancer cells. Besides serving as a targeting ligand, mBAFF itself may have therapeutic potential as it can compete with wild-type BAFF for the same receptors. We found that mBAFF and PinX1/C/N–mBAFF were far less effective than PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF, but still toxic to lymphoma cells (the efficiency of mBAFF alone and PinX1/C/N–mBAFF is similar), indicating that mBAFF alone or the fused mBAFF may function as an inhibitor of wild-type BAFF and block the BAFF autocrine pathway to some extent. To compare the antitumor effects of the *C*- and *N*-termini of PinX1, we expressed different PinX1 fragments as mBAFF fusion proteins. The *in vitro* and *in vivo* studies showed that PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF (but not PinX1N–G_4S–9R–G_4S–mBAFF and PinX1N–9R–mBAFF) caused selective cell growth inhibition and apoptosis, and PinX1C–G_4S–9R–G_4S–mBAFF produced the most obvious effect, suggesting that the *C*-terminal of PinX1 is crucial for the fusion proteins to kill cells. In brief, the cytotoxic effects of PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF were dependent on the presence of PinX1/C and mBAFF molecules. Linking PinX1/C to the 9R tract and mBAFF augmented its toxicity toward B-lymphoma cells, and simultaneously conferred it with a high degree of selectivity.

The short sequence 9R, which contains potential furin-sensitive sites, exhibited relatively high cleavage efficiency.[3] Because arginine-rich sequences have been shown to function as protein transduction domains,[25] we suspected that, following furin cleavage, cleaved 9R may help in translocating the delivered agents from the endosomes to the cytosol and facilitate the localization of the delivered agents to the nucleolar regions and subsequent biological activity. As anticipated, PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF are able to selectively kill B-lymphoma cells *in vitro*, and the former is more effective. The apparent difference between these fusion proteins is not due to their purity, but may be the result of their refolded conformation, which can influence how they are processed intracellularly. Indeed, a previous report has

shown that the particular intracellular processing of the endocytosed fusion proteins may be critical in determining potency.[26] Presumably, translocation of the fusion proteins into the cytoplasm is a time-dependent process, and if they are rapidly degraded within lysosomes, then the probability of translocation of a biologically active protein is reduced. In addition, we also noticed that our 9R conjugates did not show nonspecific cellular uptake as protein transduction domain

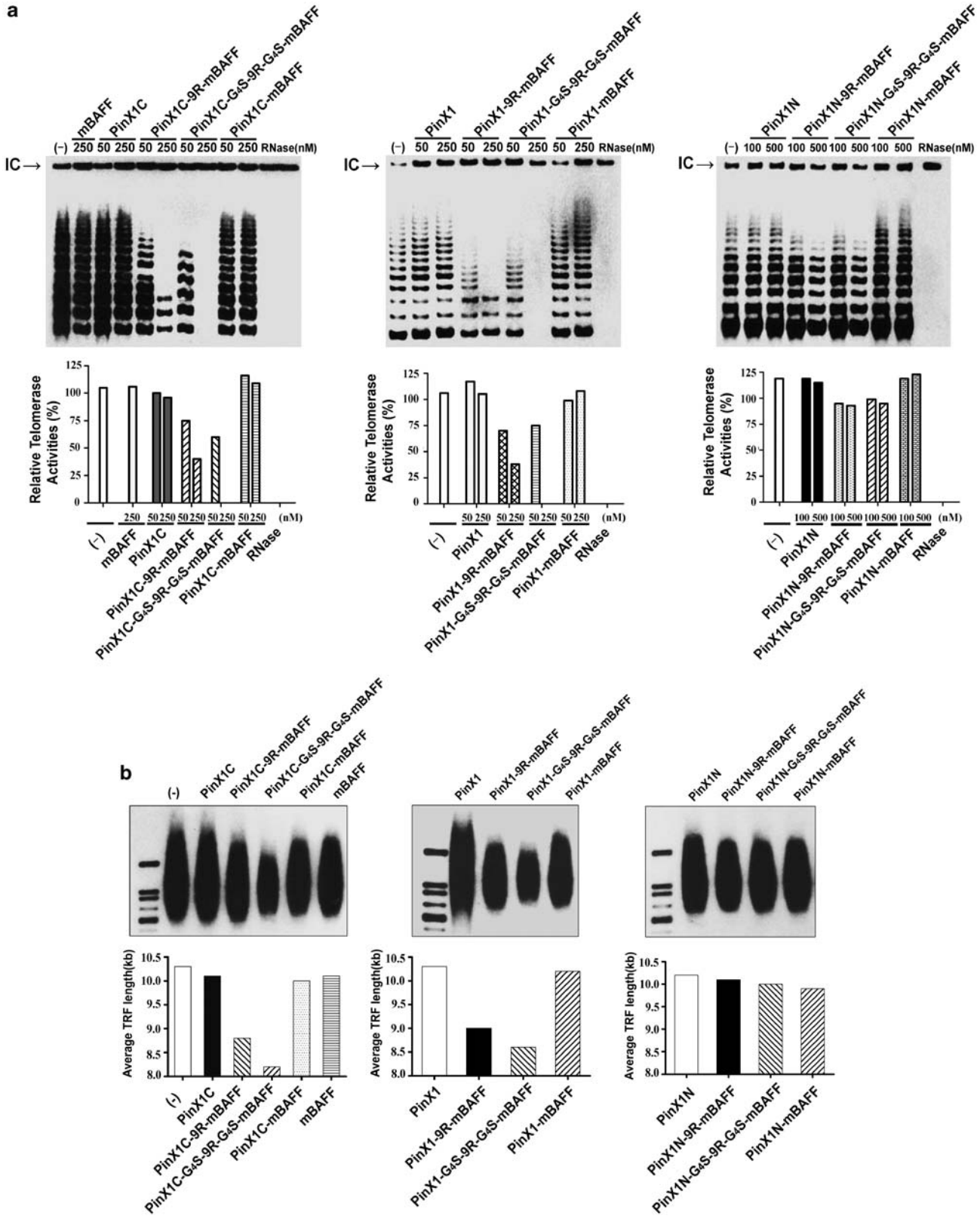

Figure 5 The fusion proteins inhibit telomerase activity and shorten telomeres of Raji cells. (**a**) PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF inhibit telomerase activity. Telomerase-containing extracts from Raji cells were incubated with various concentrations of fusion proteins for 10 min at 4 °C and then subjected to telomerase extension. Telomerase products were separated on 10% polyacrylamide gels, after which the gels were stained with chemiluminescence reagent. Telomerase activity was semiquantified by normalizing the band intensities of the characteristic 6-bp telomerase-specific ladder to that of the 216-bp internal control (IC) using NIH image software. Arrows indicate 216-bp IC. −, telomerase-containing extracts without treatment with any protein; Rnase, telomerase-containing extracts pretreated with RNase. (**b**) PinX1/C–G_4S–9R–G_4S–mBAFF and PinX1/C–9R–mBAFF shorten telomere length. Raji cells were continuously maintained in culture by splitting the cells and seeding them at 2×10^5 per well in the presence of 100-nM fusion proteins for 30 population doublings. Genomic DNA was isolated and digested with *Hin*fI and *Rsa*I, and Southern blot analysis was performed using a TTAGGG repeat as a probe. The average TRF length of Raji cells was quantified using ImageQuant. −, Raji cell without treatment with protein.

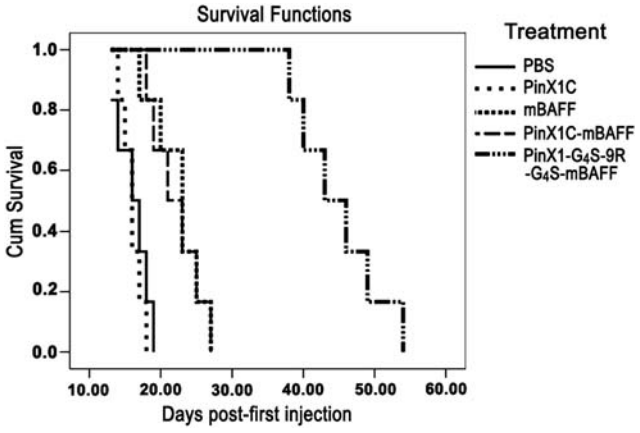

Figure 6 Antitumor effects of PinX1C–G_4S–9R–G_4S–mBAFF on disseminated Raji xenografts in SCID mice. A model of disseminated Raji xenografts was established by injecting 5×10^6 Raji cells i.v. into SCID mice ($n = 6$ per group). The mice were then given 50 μg of PinX1C–G_4S–9R–G_4S–mBAFF five times (on day 1, 4, 7, 10 and 13). The proportions of surviving mice are graphed over time. Animals were judged terminal if they died or if hindlimb paralysis occurred. Data were graphed as proportion surviving versus time. Statistical analysis was performed using the log-rank test, and the PinX1C–G_4S–9R–G_4S–mBAFF-treated group was compared with the PinX1C–mBAFF-, PinX1C- and mBAFF-alone-treated groups.

usually does when fused to either the *N*- or *C*-terminal of a protein.[3] This may be due to the inaccessibility of the 9R tract to the surface of the cell membrane as a result of steric hindrance by the mBAFF and PinX1 moieties.

We finally analyzed the effects of PinX1C–G_4S–9R–G_4S–mBAFF on systemic cancer in SCID mice treated i.v. with high doses of Raji cells, which causes hindlimb paralysis, indicating the malignant nature of the cancer.[19] Our results showed that PinX1C–G_4S–9R–G_4S–mBAFF exhibits significant antitumor effect *in vivo*. Although none of the animals were completely cured in our model, the antitumor effects of the fusion protein may be improved by altering the regimen and dose schedule or by reducing the dose of Raji cells administered to the mice,[19,27] which need to be further studied.

This study has demonstrated that the fusion protein PinX1C–G_4S–9R–G_4S–mBAFF has impressive and specific cytotoxic effects on BL cells. Moreover, the expression profile of BAFF receptors (including BAFF-R, TACI and BCMA) in BL cell lines in this study is consistent with that in some clinical specimens of BL,[28–31] suggesting the potential of PinX1/C–G_4S–9R–G_4S–mBAFF in targeted therapy of BL. In addition, it has come to our attention that BAFF receptors exist on normal B cells,[32] which means that the fusion protein can enter normal B cells and induce apoptosis. However, a previous study has shown that resting B lymphocytes generally possess little or no telomerase activity.[33] Therefore, if the PinX1C–G_4S–9R–G_4S–mBAFF fusion protein is brought into clinical trials, resting B cells would generally not be depleted. Furthermore, BAFF receptors are present on mature B cells, but not on either pre- or pro-B cells,[32] and telomerase activity has been found in some somatic lymphoid tissues and activated B cells.[34] Therefore, if the fusion protein is applied in clinical trials, the somatic lymphoid tissues and activated B cells (including the cancerous ones), but not pre- and pro-B cells, would be depleted, allowing a new population of healthy B cells to be developed from pre- or pro-B cells.[35] Therefore, this novel fusion protein did not completely cure cancer in the animal model used in this study, but its superior activity makes it a good candidate for the treatment of BL or other B-cell malignancies with high levels of BAFF receptor(s) and high telomerase activity.[28,36] To determine its therapeutic potential, further studies on the toxic side effects and detailed functional mechanism(s) of this novel fusion protein are warranted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)