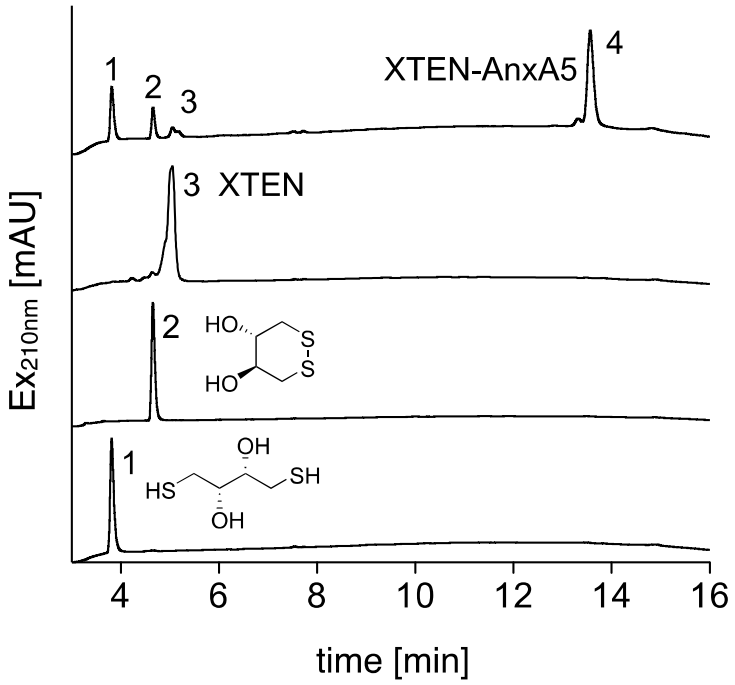
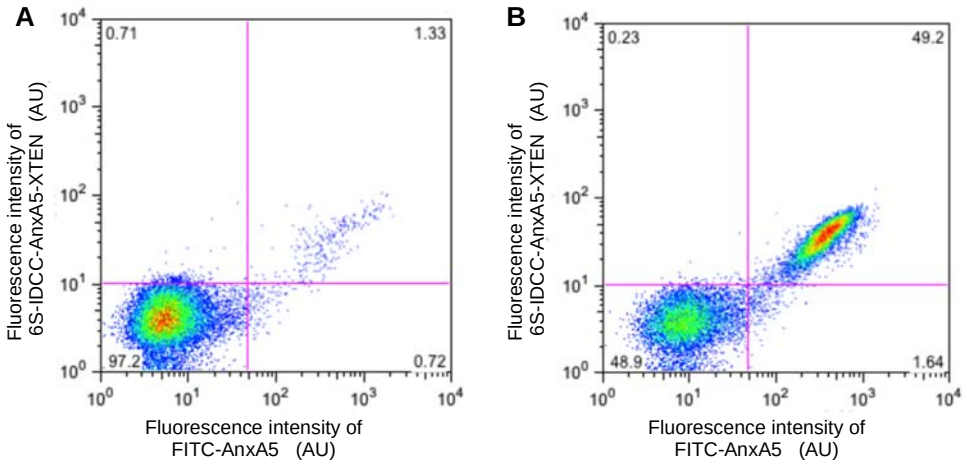


Supplemental Figure 1: RP-HPLC of DTT, XTEN and XTEN-AnxA5. Upper graph: XTEN-AnxA5 (4) samples were treated with DTT (1, 2) before purification by a hydrophobic interaction column to reduce dimerized XTEN-like XTEN-AnxA5 fragments (3, t_{Ret} = 5.1 min). Lower graphs: (1) freshly dissolved DTT, t_{Ret} = 3.8 min; (2) oxidized DTT, t_{Ret} = 4.7 min; (3) XTEN fragment, t_{Ret} = 5.1 min; (4) XTEN-AnxA5, t_{Ret} = 13.6 min.



Supplemental Figure 2: Flow cytometry of double-stained Jurkat cells. Flow cytometry demonstrates similar binding of fluorescein-labeled wild-type AnxA5 and 6S-IDCC-labeled XTEN-AnxA5 to apoptotic cells in untreated Jurkat T-cells with low apoptosis rate (A) in comparison to camptothecin-treated cells (B).

MATERIALS AND METHODS

Chemicals

Kanamycin was purchased from Carl Roth (Karlsruhe, Germany). LB-Agar, LB-Medium, MagicMedia™ e.coli expression medium, Novex® 4-12% Bis-Tris gradient gels, Coomassie SimplyBlue™ SafeStain, WesternBreeze® chromogenic western-blot immunodetection kit, fetal bovine serum, penicillin and streptomycin were obtained from Life Technologies (Darmstadt, Germany). BugBuster® protein extraction reagent and Benzonase® endonuclease were purchased from Merck Millipore (Darmstadt, Germany), and Proteinase Halt™ protease inhibitor cocktail as well as bicinchoninic acid (BCA) protein assay were obtained from Thermo Fisher Scientific (Schwerte, Germany). UnosphereQ and BioGel P6 were from Bio-Rad Laboratories GmbH (Munich, Germany). Diethylaminoethyl (DEAE) cellulose, Octyl-Sepharose® 4 Fast Flow and camptothecin were bought from Sigma-Aldrich (Steinheim, Germany). Maleimide-DTPA was obtained from CheMatech (Dijon, France). Maleimide-6S-IDCC was purchased from Mivenion GmbH (Berlin, Germany). $^{111}InCl_3$ was obtained from Covidien Deutschland GmbH (Neustadt, Germany). Cys-Annexin A5 was purchased from TAU Technologies (Kattendijke, The Netherlands). Fluorescein-5-Maleimide was purchased from Thermo Scientific™.

Europium Standard for ASS, dithiothreitol (DTT), 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES), ethylenediamine tetraacetic acid (EDTA), trifluoroacetic acid (TFA) and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

Expression and purification of XTEN-AnxA5

To design the fusion protein, an XTEN sequence of 288 amino acids (XTEN288) (*1*) was joined on to cDNA of the human AnxA5 gene (NP 1145.1). On the N-terminus, one cysteine was added at the first position to allow subsequent labeling with thiol reactive fluorophores or metal chelators for radioactive tracers. The endogenous cysteine of AnxA5 at position 316 was mutated to serine to avoid unspecific labeling (*2*). Two stop codons were added to eliminate C-terminal tag expression of the plasmid. Gene synthesis of XTEN alone and XTEN-AnxA5 fusion as well as subcloning of them into pET 30a(+) vector at NdeI and XhoI sites was performed by Genscript USA Inc. (NJ, USA).

Heat shock-competent *E. coli* BL21(DE3) Gold cells (Agilent Technologies, Boeblingen, Germany) were transformed with the pET30(+) plasmid carrying sequence of the XTEN or fusion protein and spread to grow on agar plates containing 100 µg/ml kanamycin. A single colony was picked to produce 10 ml overnight culture in LB medium with the same concentration of antibiotic growing at 30 ºC. Further, the overnight culture was diluted 1:40 to inoculate 0.2 l of MagicMedia

E.coli expression medium as a main culture. This culture was let grow at 30 ºC, 300 rpm for 7 h and was further cultivated at 23 ºC for 24 h by 300 rpm shaking intensity.

Bacterial cells were then collected by centrifugation, and 8 g wet cell pellet was lysed in 40 ml BugBuster protein extraction reagent containing Benzonase endonuclease at the recommended concentration and Proteinase Halt protease inhibitor cocktail. Bacterial cell lysate was loaded onto a 50 ml weak anion exchange column with DEAE cellulose, equilibrated with starting buffer (20 mM Tris, 50 mM NaCl, pH 6.8). The protein of interest was eluted using a gradient to end buffer (20 mM Tris, 0.5 M NaCl, pH 6.8) with a flow rate of 1 ml/min using a BioLogic LP system (BioRad, Munich, Germany). The fractions containing the fusion protein were determined by SDS-PAGE (Novex 4-12% Bis-Tris gradient gel) with subsequent Coomassie Simply Blue SafeStain, then pooled, and the elution buffer was exchanged to the equilibration buffer (20 mM Tris, 50 mM NaCl, pH 6.8). The solution was loaded onto a 50 ml strong anion exchange column with UnosphereQ, (equilibrated with 20 mM Tris, 50 mM NaCl, pH 6.8) and the protein of interest was again eluted using a gradient to end buffer (20 mM Tris, 1 M NaCl, pH 6.8) with a flow rate of 1 ml/min. XTEN-AnxA5 fractions were selected as described above, then pooled, and the concentration of NaCl in the buffer was increased to 3M. This solution was added to 30 ml Octyl−Sepharose 4 Fast Flow hydrophobic interaction column, which was equilibrated with high salt buffer (20 mM Tris, 3 M NaCl, pH = 7.5), and the desired protein was eluted using decreasing gradient to end buffer (20 mM Tris, 135 mM NaCl, pH = 7.5). XTEN-AnxA5 fractions were selected again as described above, then pooled and desalted against storing buffer (10 mM HEPES, 135 mM NaCl, pH 7.5). XTEN288 was expressed, purified and analyzed as described by V. Schellenberger et al. (*1*) (sequence in supplement). The protein solution was passed through 0.22 µm filter devices, and concentrations of XTEN and XTEN-AnxA5 were measured using BCA protein assay.

Since XTEN lacks the amino acids tryptophan and tyrosine, which are mainly responsible for the colorimetric reaction of the BCA assay, a correction factor for XTEN and XTEN-AnxA5 protein concentration measurements in comparison to the albumin standard was calculated. The determination of the factor of the XTEN polypeptide concentration, measured using BCA assay, in comparison with the weight of lyophilized XTEN polypeptide revealed a correction factor of 4.0 for XTEN288 and 1.4 for XTEN-AnxA5.

Western-blot analysis

Proteins were loaded onto 4-12% Bis-Tris Novex Gel, and SDS-PAGE was performed under recommended conditions. After electrophoresis, the proteins were transferred onto nitrocellulose membrane using iBlot gel transfer system (Life Technologies, Darmstadt, Germany). The mem-

brane was stained with rabbit annexin V antibody (Abcam, Cambridge, UK) and developed using WesternBreeze chromogenic western-blot immunodetection kit.

HPLC

The resulting samples were analyzed by reversed phase high-performance liquid chromatography (RP-HPLC). Two solvents were used for elution: Phase A (0.05% TFA in water) and Phase B (0.05% trifluoroacetic acid in acetonitrile). The following gradient was applied, 0-100% B in 20 min. The flow rate was 1.0 ml/min on a Dionex-Ultimate 3000 system using a PDA-100 Photodiode Array Detector (Dionex GmbH, Idstein, Germany) with an Acclaim 300 RP-C18 column (4.6 x 150 mm, 3 µm, 300 Å). Samples were maintained at 25°C and observed at 210 nm.

MALDI-TOF/MS

For mass spectrometry analysis, XTEN-AnxA5 samples were desalted against 0.1% TFA using 10 kDa Amicon Ultra 0.5 Centrifugal Filters (Millipore, Billerica, MA, USA) and diluted in 10 g/l sinapinic acid in 60% acetonitrile / water with 0,2% TFA. Samples were analyzed using an AutoFlex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen) in positive high mass linear mode. The measurements were performed by the Core Facility Proteomics of the Charité.

Coupling of XTEN-AnxA5 with DTPA and 6S-IDCC

Prior to labeling, 1100 µl XTEN-AnxA5 with a concentration of 1.8 mg/ml was treated with DTT (10 mM final concentration) to reduce potential cysteine dimers at the N-terminus. XTEN-AnxA5 was therefore incubated with DTT for 90 min at 37 °C. To remove DTT, the solution of reduced XTEN-AnxA5 was dialyzed for 24 h at 4 °C in buffer containing 10 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4. Maleimide-DTPA or the near-infrared fluorescence (NIRF) dye maleimide-6S-IDCC (Em_{Max}=695nm, Ex_{Max}=675 nm) was dissolved in water at a concentration of 10 g/l and then added to the protein solution in a 20-fold molar excess. After 24 h reaction time at 4 °C, unbound maleimide-DTPA or maleimide-6S-IDCC was removed by two rounds of gel filtration using centrifugal spin columns with BioGel P6. The labeling of AnxA5 was done accordingly (6S-IDCC-AnxA5). 10 ml BioGel P6 was equilibrated with 10 mM HEPES, 140 mM NaCl, pH 7.4; centrifugation steps included 3 min (1st round) and 5 min (2nd round) at 1500 x g. The final protein concentration was determined by BCA protein assay. The labeling ratio of protein with maleimide-6S-IDCC was calculated after measuring the dye absorption at 675 nm (ε= 240 000 $M^{-1}cm^{-1}$). The labeling ratio of the maleimide-DTPA was determined employing complexation of Eu^{3+}. Unbound Eu^{3+} was removed using centrifugal spin columns and the concentration of remaining Eu^{3+} was calculated after measuring the fluorescence of the europium chelate formed using an enhancer solution (*3*).

Labeling of XTEN-Anx-DTPA with $^{111}In^{3+}$

121.2 µg of XTEN-AnxA5-DTPA (52 µl) was mixed with 463 µl buffer (270 mM NaOAc/ HOAc, 79 mM Gentisin, pH 5.0). The resulting solution was added to 500 µl $^{111}InCl_3$ solution (0.02 M HCl, 291 MBq, 157.3 ng indium), thoroughly mixed and incubated for 70 min at room temperature. Thereafter, the labeled protein was purified by three rounds of ultrafiltration with 10 kDa Amicon Ultra 0.5 Centrifugal Filters (Millipore, Billerica, MA, USA) by adding 1 ml buffer (10 mM HEPES, 140 mM NaCl, pH 7.4) each time. The radioactivity of both column and filtrate was measured after each step. Procedure yielded 206 MBq of XTEN-AnxA5-^{111}In in 160 µl buffer solution (71% yield related to the used $^{111}In^{3+}$).

Flow cytometry and fluorescence microscopy imaging

Human T-cell leukemia (Jurkat) cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures and grown in RPMI 1640 medium containing GlutaMAX, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% CO_2.

To induce apoptosis, the cells were treated with camptothecin at final concentration of 9 mM for 5 h at 37 °C with 5% CO_2, then harvested, centrifuged at 194 x g for 5 min and washed in cold phosphate-buffered saline (PBS, pH 7.4) with 1% fetal bovine serum. After a repeated centrifugation step, the cells were resuspended in cold binding buffer (1.8 mM $CaCl_2$, 10 mM HEPES, 150 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$, pH 7.4) and aliquoted at a density of 5 x 10^5 / 500 µl. Further, the cells were incubated with 0.2 µg XTEN-AnxA5-6S-IDCC or wild-type AnxA5-FITC (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, Heidelberg, Germany) for 30 min in the dark on ice. Subsequently cells were centrifuged again, supernatant was removed, and cells were fixed for 30 min with 1% paraformaldehyde at room temperature. Afterwards, paraformaldehyde was exchanged against binding buffer. The measurements were performed using FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) at excitation length of λ = 488 nm (FITC) and λ = 633 nm (NIFR dye). The examination of results was done with FlowJo 8.7 software (Tree Star, Inc., USA).

For the microscopy imaging, a small amount of sample was taken before FACS analysis. A drop of cell suspension was applied onto a glass slide and examined using a Observer.Z1 fluorescence microscope (Carl Zeiss AG, Jena, Germany).

Calcium Titration Curves of Binding to Aged Red Blood Cells (RBC)

Cys-Anx-117 mutant or XTEN-AnxA5 were coupled with Fluorescein-5-Maleimide at their singular cysteins (Thermo Scientific™) similar to the 6S-IDCC (see above). The RBC assay was performed as described by Tait et al. (2004) (*4*). Briefly, aged erythrocytes from human RBC concen-

trate (Charité blood bank, about $5x10^7$ cells in 1 ml assay buffer) were incubated with 1 nM Fluorescein-labeled AnxA5 (cys-Anx-117 mutant, NeXins Research / TAU Technologies) or XTEN-AnxA5 in 50 mM HEPES, 100 mM NaCl, 1 mg/ml BSA, pH 7.4 and varying concentrations of $CaCl_2$ (0-7 mM) for 8 min at room temperature. After incubation the cells were centrifuged for 3 min at 7800 x g and the supernatant was removed. The cells were washed in 1 ml assay buffer with the same concentration of calcium used during the incubation step. After a repeated centrifugation step the cells were resuspended in 0.65 ml assay buffer supplemented with 5 mM EDTA to release the bound annexins. The fluorescence of the supernatant, obtained after removing of RBCs by centrifugation, was analyzed using a VICTOR™ X3 Multilabel Plate Reader (Perkin Elmer). The analysis (One site binding with Hill slope) was done with Prism 5 software (GraphPad Software, Inc.).

SPECT/CT

All animal experiments were approved by the institutional animal care and use committee in accordance with laboratory animal care guidelines (protocol number G0246/08). Four- to five-week-old Balb/c female mice were obtained from Charles River Laboratories (Sulzfeld, Germany).

The anesthesia was induced and maintained with 1-1.5% isoflurane during the entire experimental procedure. The mice were injected with approximately 100 MBq of ^{111}In-XTEN-AnxA5 (80 µl) via the tail vein. The body temperature was maintained throughout the studies with a warming bed. Measurements were acquired with a NanoSPECT/CT small animal imager (Bioscan Europe, Paris, France) at four time points. The signal intensities for blood half-life determination were measured using a monitor-defined, circular region of interest (ROI) of the left ventricle of the heart and other organs. The size and location of the ROI were kept constant for subsequent measurements and corrected by the ^{111}In decay. The blood half-lives were calculated by averaging the half-life of 5 mice using Prism 5 software (GraphPad Software, Inc., one phase exponential decay).

For the *ex vivo* biodistribution the animals (n=3) were sacrificed under anesthesia after the 24 h-SPECT/CT-measurements. The retrieved organs were weighted and measured in an activimeter ISOMED2010 (Nucmed, Dresden, Germany).

NIRF Imaging

EL4-cells (mouse lymphoma cell line) were subcutaneously inoculated at $1x10^6$ cells in 100 µl DEMEM into both flanks of female nude mice (Charles River Laboratories, Sulzfeld, Germany). About one week after transplantation when the tumors reached a size between 5 and 10 mm, the mice were treated intravenously with 19 mg/kg etoposide and 25 m/kg cyclophosphamide (5). One day later, the NIRF imaging was performed 3 h after intravenous application of 6S-IDCC-XTEN-AnxA5 or 6S-IDCC-AnxA5 as control probe. The concentration of the probes was 50 nmol dye /

kg. The *ex vivo* optical imaging of the explanted organs was performed in a custom made NIRF-imager (excitation with 670 nm laser, emission filter 710 nm high pass) with an exposure time of 1 sec. The images were quantified using ImageJ software (National Institutes of Health USA). The ROIs included either whole organs or selected hotspot regions of the tumors. The statistical comparison was done with Prism 5 (GraphPad Software) employing a two-tailed Wilcoxon-Mann-Whitney-Test.

After *ex vivo* NIRF imaging, the tissue probes were snap-frozen and stored at -80 °C.

Immunohistology

The *ex vivo* microscopy of 6S-IDCC-XTEN-AnxA5 and 6S-IDCC-AnxA5 in tumors were performed in unfixed 10 µm slices counterstained with Hoechst33258.

For apoptosis detection, sections (10 µm) were fixed with 4% paraformaldehyde at room temperature, then blocked with 10% goat serum and incubated in primary antibody (anti-active caspase 3, Abcam, Cambridge, UK) at 4 °C overnight. After washing with PBS, sections were incubated with a secondary antibody (goat polyclonal to rabbit IgG coupled with Cy5®, Abcam, Cambridge, UK) for 1 h. Then, sections were counterstained with Hoechst33258. All images were acquired with a fluorescence microscope (Zeiss Observer.Z1, Carl Zeiss AG, Jena, Germany) under the same condition and displayed at the same magnification (200x).

Molecular Modelling

The molecular model of XTEN-AnxA5 was done using Pymol (*6*) software and Marvin (ChemAxon Software, Budapest, Hungary) based on the Sequence of the unstructured XTEN288 (*1*) and the crystal structure of AnxA5 (7).

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Protein Sequence of XTEN288-AnxA5:

CGTSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSGSETPGTSESATPESGPGTST
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Protein Sequence of XTEN288:

GTSESATPESGPGSEPATSGSETPGTSESATPESGPGSEPATSGSETPGTSESATPESGPGTSTE
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