Supplemental Methods

Cell line and compounds. HT is a human DLBCL cell line obtained from the DMSZ Cell Line Bank. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% glutamine, 100 units/ml penicillin/streptomycin (Life Technologies_Bethesda Research Laboratories) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. HT-SC cells or bioluminescent HT-Luc-SC cells were obtained from HT or HT-Luc subcutaneous tumors, respectively (see “Animal experiments”).

Chemical structure of the compound E7123 is 4-(5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-1-yl) benzenesulfonamide. E7123 was synthesized by the HSCSP Pharmacy Department and Laboratories Esteve S.A. The stock solution of E7123 was reconstituted in PEG-400:FBS 3:1 before its oral administration in mice.

Lentiviral infection of HT cells. A plasmid containing Luciferase gene (Luc) was constructed by subcloning and replacing the GFP1 gene of the plasmid pSIN-DUAL-GFP1-GFP2, kindly provided by Mary Collins (College Medical School, Cleveland, OH). Luciferase cDNA was obtained from the plasmid pPK-CMV-F3 (C-Luc) (Promokine, Heidelberg, Germany). Lentiviral particles containing the pSIN-DUAL-Luciferase-GFP2 vector, were used to infect HT cells as described previously by our group. To obtain a homogenous population expressing the vector, GFP positive cells were sorted out using a BD FACSria cell sorter (BD Pharmingen, San Jose, CA, USA). Sorted cells, termed HT-Luc, were used for in vivo studies in order to non-invasively monitor the dissemination of the lymphoma in mice and to evaluate the antitumor effect of E7123.

Animal experiments. Female NOD/SCID mice (4 week-old) were obtained from Charles River Laboratories. They were housed in micro isolator units and had access to sterile food and water ad libitum throughout the studies. All procedures involving mice were approved by the Hospital Sant Pau Animal Ethics Committee according to established guidelines. Ten NOD/SCID mice were subcutaneously injected with 10x10⁶ HT cells per flank. When tumors achieved a 600-900 mm³ volume, mice were euthanized by cervical dislocation. Tumor pieces were excised, placed in a sterile Petri dish with RPMI 1640 culture medium, and manually disaggregated with forceps.
until a single-cell suspension was obtained. Cells were filtered through a cell strainer and cultured for 15 hours. Cells obtained after the subcutaneous passage were denominated HT-SC cells. On continuation, two groups of 17 and 10 NOD/SCID mice were injected intravenously with 2 x 10^6 HT-SC or HT cells, respectively. Mice injected with HT-SC cells were randomly divided in two groups and received vehicle or 75 mg/kg of E7123. They were treated every day since day 10 post-injection until they were sacrificed for weight loss or signs of sickness (Supplementary Figure S1). Following the same procedure, luminescent HT-Luc cells were also injected in mice flanks. Grown tumors were extracted and disaggregated until obtaining a single-cell suspension (HT-Luc-SC). 20 x 10^6 HT-Luc-SC cells were intravenously injected in 20 mice, which were randomly divided in two groups receiving vehicle PEG:FBS (n=10) or 75 mg/kg of E7123 (n=10) as described for the first experiment. Bioluminescence images were acquired as described below (Supplementary Figure S2).

**Animal monitoring and necropsy.** Mice injected subcutaneously with human HT or HT-Luc cells were monitored every other day for tumor growth. Tumors were measured using a caliper and the formula width^2 x length x 0.5 was used to calculate tumor volume. Palpable tumors resulted after 24 days and reached 600-900 mm^3 at 46 days, time at which mice were sacrificed by cervical dislocation. Mice injected intravenously with HT, HT-SC or HT-Luc-SC cells were monitored every other day for changes in weight or signs of sickness. A loss of 15% in body weight or the appearance of motor problems was considered as the endpoint. Tumor involvement was documented by color photography of gross pathologic lesions. Brain, lymph nodes, spleen, liver, kidneys, and bone marrow (from both femurs) were collected, fixed in buffered formaldehyde and paraffin embedded for histopathologic or immunohistochemical studies. Tissue sections were stained with Hematoxylin and Eosin (H&E) staining. In vivo BLI imaging was performed as previously described\(^{27}\). Briefly, mice were anesthetized with ketamine-xylazine and intraperitoneally injected with D-luciferin (Xenogen) at 2.25 mg/mouse. After 10 minutes, bioluminescence was captured. Images were taken once a week from day 7 after injection of cells. For dorsal image acquisition, animals were placed at 140 mm from the camera objective in the detection chamber of a high-resolution ORCA-II Deep Cooling BTW Imaging System (Hamamatsu Photonics, Hamamatsu City, Japan). Animals were exposed for 2
minutes in order to acquire the BLI images. To locate the luminescence signal, a second image of each animal was obtained using white light. Quantification and analysis of photons recorded for each image was performed using the Wasabi 1.5 image analysis software (Hamamatsu Photonics, Hamamatsu City, Japan). Quantification and analysis of BLI recorded in images was performed using Living Image, v4.2, Caliper Life Science software (Hopkinton, MA, USA). The number of recorded photons was expressed as Photon Counts (PHCs). For image quantification, the area of interest was selected and total number of PHCs was obtained. The net number of PHC was calculated using the formula: [Total number of PHC in the area of interest - (Number of pixels in the area of interest x Average background PHC per pixel)].

**Paraffin-embedded HT cells.** HT and HT-SC cells were paraffin-embedded to perform immunohistochemical analyses. Thus, 20 x 10^6 cells were centrifuged for 10 minutes at 1200 rpm. The pellet was suspended adding equal portions of human plasma and thrombin (from human plasma, SIGMA-ALDRICH, St.Louis, USA) and gently mixed until obtaining a cell lump. The lump was fixed in buffered formaldehyde, paraffined and routinely processed in the pathology laboratory.

**Immunohistochemistry.** Paraffin-embedded tissue sections of HT-SC or HT infiltrated brains were used to detect CD20, CD10, Ki67 (DAKO, Carpinteria, CA, USA), p130Cas (Neomarkers, Fremont, CA, USA) and β1-integrin (Upstate, Billerica, MA, USA) proteins. 4µM thick sections were incubated at 58°C for 1 h and then dewaxed in xylene, rehydrated through a graded ethanol series, and washed with phosphate-buffered saline. Heat-induced epitope retrieval was done by immersing the sections in sodium citrate buffer (pH 6.0) and incubating them at 97°C for 20 minutes using a DAKO PTLink. The immunohistochemical reactions were visualized following the biotin-free EndVision system (DAKO, Carpinteria, CA, USA) using diaminobenzidine. The reactions were performed in a DAKO Autostainer Link48. The percentage of cells stained with Ki67 was determined in ten high-power fields by two different observers. All slides were viewed with an Olympus BX51 microscope. Images were acquired using an Olympus DP72 digital camera and processed with the Olympus Cell^D^ Imaging 3.3 software (Olympus Corporation, Tokyo, Japan).
**Western Blot Analysis.** HT-SC cell lysates were performed on the day of disaggregation of the subcutaneous tumors and repeated one week later. HT and HT-SC protein extracts were prepared and Western Blots were performed as previously described\(^2\). The following primary anti-human antibodies diluted in TBS-T, containing 0.1% BSA, were used: mouse anti-p130Cas, anti-AKT, anti-FAK, anti-FAK Tyr397, anti-Pyk2, anti-Lyn, anti-Mcl1, anti-integrinβ1 (BD Pharmingen, San Jose, CA, USA), rabbit anti-AKT Thr308 and anti-Src (Cell Signalling, Danvers, MA, USA) and goat anti-β-actin (Santa Cruz Biothecnology, CA, USA). The secondary antibodies used were anti-mouse-IgG and anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA).

**Statistical analysis.** Differences in survival between control HT or HT-SC groups and between HT-SC or HT-Luc-SC mice receiving vehicle or drug were calculated using Kaplan-Meier curves and Log-rank test. Fisher exact test was used to calculate differences between categorical variables (percentage of mice with CNS, lymph node or bone marrow infiltration). \(p\) values lower than 0.05 were considered statistically significant.
Supplemental Figure 1. Diagram of the experimental design

**OBJECTIVE 1:**
Confirm whether sc passage of HT cell line increase cell aggressivity

**OBJECTIVE 2:**
Evaluate the in vivo antitumour effect of E7123 in a disseminate xenograft model of DLBCL generated with HT cell line.

*PEG:FBS-treated group served as experimental group to achieve objective 1 and as control group to achieve objective 2.*
Supplemental Figure 2. Diagram of the validation experiment

OBJECTIVE 3:
Validation of E7123 antitumour effect in a bioluminescent xenograft model of DLBCL with CNS infiltration

HT CELLS

Lentiviral infection with pSIN-DUAL-GFP-Luciferase vector

HT-Luc cells

SC INJECTION (HT-Luc)

SC tumour growth

HT-Luc-SC cells

HT-SC IV INJECTION

(n= 20)

PEG:FBS (n=10)

E7123 (75 mg/kg) (n=10)

VEHICLE

DRUG