Thioridazine requires calcium influx to induce *MLL-AF6* rearranged AML cell death

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SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

Patient selection and cell culture

All AML primary cultures (AIEOP AML 2002/01 - EUDRACT:2014-000976-25) were supplemented with recombinant human (rh) cytokines: interleukin-3 (rhIL-3; 20 ng/mL), interleukin-6 (rhIL-6; 20ng/mL), stem cell factor (rhSCF; 50 ng/mL), thrombopoietin (rhTPO; 50 ng/mL) and fms-like tyrosine kinase-3 ligand (rhFLT-3 ligand; 50 ng/mL, Milteny Biotec, B. Gladbach, Germany) for *in vitro* expansion.

Cell treatments.

Briefly, $3x10^4$ cells were seeded in 100 µl of medium, in a white flat corning opaque 96-well plate (Sigma-Aldrich–Merck), and after 24 hours cells were treated in triplicate using a 10 µM concentration of the different drugs tested [1]. Seventy-two hours after drug exposure, cell viability was measured. The ML-2-t(6;11) cell line underwent to treatment with the whole library (1280 compounds). Active compounds were further evaluated using a second t(6;11) rearranged cell line, namely SHI-1; at this point, we further refined selection by testing compounds in HL60, an AML cell line without t(6;11) translocation, and discarding those being active in this cell line. The remaining compounds were screened in two t(9;11)-*MLL* translocated cell lines, namely NOMO-1 and THP-1, using the same criteria and reducing the final number of candidates for being exclusively active in AML with t(6;11) aberrancy. Hit compounds such as TDZ, fluspirilene, and Ara-C, were sourced from Sigma-Aldrich–Merck for further experiments *in vitro* and *in vivo*.

For mitophagy investigation we used HL60 and SHI-1 cell lines and treated with 50 µM FCCP for 6 hours and then processed with "APC Annexin V Apoptosis Detection Kit with 7-AAD" (Biolegend, San Diego, CA, USA) to assess viable cells (annexinVnegative and 7-AADnegative) using BD FACS Celesta (Becton Dickinson, NJ, USA) and DIVA software.

In vitro treatment with TDZ and analogues was performed at 10 µM concentration.

Pipeline for target selection

All the 1280 compounds were used on the ML-2 with t(6,11) rearrangement cell line and identified 104 active molecules; all of them were then screened using the SHI-1 cell line, another cell line harboring the t(6;11) rearrangement, confirming the activity of 93 out of 104 compounds (Fig. 1A).

To reduce numbers, we tested these 93 compounds on the *non*-t(6;11) AML cell line HL60. This screening led to the exclusion of 73 out of 93 molecules resulted active in a cell line non *MLL-AF6* rearranged. Finally, the 20 remaining compounds were used to treat two t(9;11)*MLL-AF9*-rearranged cell lines, THP-1 and NOMO-1, identifying 10 compounds as toxic for both of them (Supplementary Table S123), which were finally selectively as active in t(6;11)-rearranged cells. Since two compounds, the fluspirilene (FLUS) and TDZ worked through the same mechanism of action, we selected them as primary candidates to be investigated for their repositioning from antipsychotics agents to anticancer drugs.

Cell viability assay

Cell viability was evaluated with CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI), following manufacturer's guidelines. For each treatment we performed 3 independent experiments, and cell viability was calculated as mean±SD. The compounds were classified as active when cell viability resulted ≤60% compared to DMSO control-treated cells.

Colonies forming assay

After 24 hour treatment, a total of 5×10^2 ML-2, SHI-1, HL60, SKNO-1 or NOMO-1 cells and 3×10^3 primary blasts from patients with t(6;11)-rearranged AML were seeded into 500 µl of MethoCult™ (H4230 and H4534 respectively, Stemcell Technologies, Meda MB, Italy), in 24-well plates and incubated at 37°C. Fourteen days after seeding, an adequate volume of a 1:6 solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich–Merck) in Hanks' was added to semisolid medium. Images were acquired by optical microscope with camera, and colonies were counted using ImageJ software.

Reactive oxygen species (ROS)

ROS production was monitored by flow-cytometry using CellROX Deep Red Reagent (C10422, Invitrogen, Thermo Fisher Scientific) following manufacturer's instructions, Cell ROX Deep Red Reagent was added at a final concentration of 5 µM to the cells and incubated for 30 minutes at 37°C. Cells were washed three times with PBS and analyzed by flow-cytometry using Cytoflex (Beckman Coulter).

Mitochondrial membrane potential

Mitochondrial membrane potential was measured by using TMRE Assay Kit (ab113852, Abcam), following manufacturer's instructions Briefly, TMRE 200 nM diluted in BSA 0.2%-PBS 1X was added for 20' at 37°C. Cells were analyzed by flow-cytometry using FC500.

Measurements of intracellular Ca^{2+}

Cells were washed in Hanks Balanced Salt Solutions, incubated with Fluo-4 AM 3 µM diluted in the working buffer (0.02% w/v Pluronic and 0.2 mM sulfinpyrazone, both provided by Sigma-Aldrich-

Merck, in 10 mM HEPES-buffered Hanks) for 30' at RT. After incubation, cells were washed and resuspended in working buffer with CaCl2 0.2 mg/l for 30', to allow the de-esterification of Fluo-4 AM.

Dopamine receptors measurement

Rabbit anti-dopamine receptors (DRs) D1, D2, D3, D4 and D5 antibodies were sourced from Millipore-Merck (Darmstadt, Germany). Anti-rabbit Alexa-Fluor-488 (Life Technologies – Thermo Fisher Scientific, Waltham, MA) was used as the secondary antibody. Briefly, $5x10^5$ cells incubated with anti-DR antibody diluted 1:100 in 80 µl of 1xPBS+0.1% Bovine Serum Albumin, (BSA, Sigma-Aldrich Merck) + 20 µl of FcR Blocking Reagent (Miltenyi), for 30' on ice. Then, cells were washed in 1xPBS and incubated with 488-conjugated secondary antibody diluted 1:2000 in 1xPBS for 15', and analyzed using Cytometer FC500.

Plasmids construction

pMIG plasmid was a gift from William Hahn (Addgene plasmid # 9044); pMIG-FLAG-MLL-AF9 was a gift from Daisuke Nakada (Addgene plasmid # 71443) [2].

To generate a chimeric construct by subcloning *MLL-AF6* into pMIG (Addgene plasmid # 9044), we amplified from SHI-1 cell line cDNA a fragment of 3074 bp starting from the beginning of *MLL* coding region and terminating at the end of the NCR domain of *AF6*, enough to confer leukemic features [3]. For the amplification reaction, we took advantage of Platinum™ SuperFi II DNA Polymerase (Invitrogen) and used a pair of primers including the restriction sites for HpaI and EcoRI (Forward primer-HpaI GTTAACGGGGCGAACATGGCGCACA, Reverse primer-EcoRI GAATTCCTACTGAAGGCGGTAAAGCTTTGGCTTATC). The PCR product was cloned into pCR XL-2-TOPO vector (Invitrogen) and transformed into OmniMAX 2 T1R chemically competent *E. coli* (Invitrogen) in order to amplify the region of interest. Then we excised *MLL-AF6* using HpaI and EcoRI restriction enzymes and cloned it into the HpaI and EcoRI sites of the pMIG vector, obtaining pMIG-MLL-AF6 plasmid. pMIG, pMIG-FLAG-MLL-AF9 and pMIG-MLL-AF6 were transiently transfected in AML cell lines for *in vitro* validations.

Cell transfection

Transient transfection was performed using the Nucleofector systems (Amaxa Biosystems, Lonza) according to manufacturer's instructions. Specific siRNAs (Invitrogen) were adopted to silence *S100A8, S100A9* and *ANXA6*, whereas silencing of *MLL-AF6* (sir MAF6) was obtained as previously reported [4]. We used the combination of the three siRNA for *S100A8, S100A9* and *ANXA6* (sirBOMB) to get a combinatorial gene silencing *in vitro*. Transfected cells were screened by RQ-PCR for *MLL-AF6*, *MLL-AF9*, *S100A8, S100A9 and ANXA6* expression, protein level was checked by flow cytometry, and cells were used for *in vitro* experiments.

Intracellular staining by flow cytometry

Cells were harvested, fixed in 1.5% paraformaldehyde and permeabilized with 100% Methanol. Samples were stored in 100% methanol at −20°C. After recovering, they were stained by anti-Annexin-6/ANXA6 (Abcam) primary antibody and then incubated with anti-mouse secondary antibody conjugated with Alexa Fluor 488 dye (1:500, Molecular Probes). Alexa Fluor 647 Mouse Anti-Human S100A8/A9 antibody (MRP-8/14, BD Biosciences) was also used. Samples were analyzed on Cytoflex flow cytometer (Beckman Coulter). Data are presented as fold change of Median Fluorescence Intensity (MFI) of positive-cells in the live subpopulation of silenced samples respect to sirneg. Overlay histograms were set up with Cytobank platform (www.cytobank.org).

Immunofluorescence

For mitophagy evaluation, cells were allowed to adhere to 40 µg/ml fibronectin (FN, Sigma Aldrich – Merck)-coated LabTek slides, and then treated. For cytoskeletal rearrangement evaluation, cells were seeded onto 40 µg/ml FN-coated chamber slides (Sacco, Como, Italy) 4 hours after treatment, and allowed to adhere for 1 hour or centrifugated by cytospinning at 350 g. Cells were fixed with 4% formaldehyde and then subjected to immunofluorescence using the following antibody: mouse anti-TOM20 (Santa Cruz, TX, USA), rabbit anti-PARKIN (Abcam), mouse anti-AF6 (BD Biosciences). All primary antibody incubations were followed by incubation with appropriated Alexa-Fluor-conjugated secondary antibodies (Life Technologies – Thermo Fisher Scientific); 1 hour at RT F-ACTIN staining was performed using fluorochrome-conjugated Phalloidin antibody (Sigma-Aldrich-Merck). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10000; Sigma-Aldrich-Merck) to label nuclei. For mitophagy evaluation, images were acquired using a Perkin Elmer Ultraview VoX upon 0.4 µm slices z-stack reconstructions: for PARKIN translocation evaluation, Manders' index of colocalization signal was calculated by Volocity software (n= 13-17 cells); mito-aggresomes formation was evaluated by counting cells with mito-aggresomes (n=27-94 cells).

Mitochondrial/cytosol fractionation; Immuofluorescence

Mitochondria isolation has been performed as previously described [5]. Briefly, cells were resuspended in Permeabilization Buffer (250mM sucrose, 10mM Hepes, 1mM EDTA, plus Protease Inhibitors) and membranes disrupted by mechanical dissociation using 100-hits glass homogenizer (Potter-Elvehjem). Cells were then centrifuged twice for 10 min at 600/1500g to remove cell nuclei and debris. Supernatant was centrifuged at 11000g for 15 min to separate the cytosol fraction (supernatant) and the mitochondria-containing pellet.

For mitophagy investigation we used HL60 and SHI-1 cell lines and treated with 50 µM FCCP for 6 hours and then processed with "APC Annexin V Apoptosis Detection Kit with 7-AAD" (Biolegend, San Diego, CA, USA) to assess viable cells (annexinVnegative and 7-AADnegative) using BD FACS

Celesta (Becton Dickinson, NJ, USA) and DIVA software. For mitophagy evaluation, cells were allowed to adhere to 40 µg/ml fibronectin (FN, Sigma Aldrich – Merck)-coated LabTek slides, and then treated. For cytoskeletal rearrangement evaluation, cells were seeded onto 40 µg/ml FN-coated chamber slides (Sacco, Como, Italy) 4 hours after treatment, and allowed to adhere for 1 hour or centrifugated by cytospinning at 350 g (see antibodies at supplementary methods). For mitophagy evaluation, images were acquired using a Perkin Elmer Ultraview VoX upon 0.4 µm slices z-stack reconstructions: for PARKIN translocation evaluation, Manders' index of colocalization signal was calculated by Volocity software (n= 13-17 cells); mito-aggresomes formation was evaluated by counting cells with mito-aggresomes (n=27-94 cells).

Western Blot

For Western Blot analysis, whole cells and cell fractions were lysed in RIPA buffer (Tris-HCl 50 mM pH8, NaCl 150 mM, Nonidet-P40 1%, Sodium deoxycolate 0.5%, SDS 0.1%) and processed for protein expression by Western Blot. The following primary antibodies were used: rabbit anti-PARKIN (Abcam, Cambridge, UK), rabbit anti-TOM20 (Santa Cruz), rabbit anti-ACTIN (Cell Signaling, Danvers, MA), mouse anti-TUBULIN (BD Pharmingen), rabbit anti-AF6 (BD), mouse anti-COXIV (Abcam), mouse anti-ATPb (Abcam), rabbit anti-MnSOD (Enzo Life Science, Farmingdale, NY, USA), ANXA6 (Abcam), S100A8/A9 (Novus Biologicals, Centennial, CO, USA), GAPDH (GeneTex, Irvine, CA, USA).

Flank injection xenograft experiments

Six NOD-SCID interleukin-2 receptor gamma null (NSG) mice between 6 and 8 weeks of age were injected subcutaneously with 2×106 SHI-1 cells per flank-injection resuspended in 200 µl of matrigel (Corning, Corning, NY, USA). Each mouse received two flank-injections. When tumors reached an area of 30 mm2, mice were randomized in 2 groups (control and TDZ-treated), and daily treated with intra-peritoneally (ip) administered TDZ 8 mg/kg, a lower dosage compared to the therapeutic window for schizophrenia [6]. For the combination treatment, twelve NSG mice were flank-injected as previously described, and randomized in 4 groups (control, TDZ, Ara-C and combination of TDZ with Ara-C) when tumors reached an area of 30 mm2, and daily treated with TDZ 8 mg/kg (ip), Ara-C 6.25 mg/kg (ip) or the combination of the two drugs. Experimental endpoint is reached when tumors reached an area of 250 mm2. The combination index (CI) was calculated using the Response Additivity approach [7]. For the twenty NSG mice tail vein-injected data analysis was performed using Living Image software, and the intensity of the signal was quantified in the Regions Of Interest (ROI), in triplicate and averaged. Radiance unit was expressed as number of photons (p) per second (sec) that leave a square centimeter of tissue (cm2) and radiate into a solid angle of one steradian (sr), p/sec/cm2/sr). When cell engraftment was verified, mice were randomly allocated to 4 groups

(control, TDZ, Ara-C and combination of TDZ with Ara-C), and treated daily with TDZ 8 mg/kg (ip), Ara-C 6.25 mg/kg (ip) or the combination of the two drugs. The combination index (CI) was calculated using the Response Additivity approach.

Xenograft experiments by bioluminescence imaging in NSG mice

Data analysis was performed using Living Image software, and the intensity of the signal was quantified in the Regions Of Interest (ROI), in triplicate and averaged. Radiance unit was expressed as number of photons (p) per second (sec) that leave a square centimeter of tissue (cm2) and radiate into a solid angle of one steradian (sr), p/sec/cm2/sr). When cell engraftment was verified, mice were randomly allocated to 4 groups (control, TDZ, Ara-C and combination of TDZ with Ara-C), and treated daily with TDZ 8 mg/kg (ip), Ara-C 6.25 mg/kg (ip) or the combination of the two drugs. The combination index (CI) was calculated using the Response Additivity approach. See Flank injection xenograft experiments at supplementary methods.

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) and affinity enrichment

SHI-1 cells were cultured in 2 conditions, namely light and heavy: for heavy condition, L-arginine-13C6 and L-lysine-13C615N2 were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). The cell culture media RPMI deficient in arginine and lysine was purchased from GIBCO-Thermo Fisher Scientific Dialyzed serum was obtained from Life Technologies – Thermo Fisher Scientific. After at least 6 passages in heavy medium, to perform affinity enrichment assay, proteins were extracted in two different in ice-chilled lysis buffer, the commercial FNN0011 cell extraction buffer (Invitrogen, Thermo Fisher Scientific) namely Buffer #1, and a second buffer containing 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA and PBS containing 1% β-OG, namely Buffer #2, described to extract preferentially membrane proteins [8], both of them supplemented with protease and phosphatase inhibitors (P2714, and P5726, respectively, Sigma-Aldrich–Merck). Solid phase-bound TDZ was used in both light and heavy pull-down, but 10-20 fold excess soluble TDZ3 was added only to the light set in order to competitively bind target proteins, and thus reducing the amount of target proteins on the bead surface. We finally identified and compared the relative abundance of proteins bound to solid phase-TDZ3 by mass spectroscopy (MS), and the differential SILAC ratio between the 2 conditions, heavy and light, to identify specific interactors to the soluble TDZ3. Briefly, to ensure efficient elution of bound proteins, a bead-equivalent volume of 1% SDS was added, the matrix boiled for 10 min and then 4x volume of dH2O added. The matrix was vortexed, the solution removed and then reduced to the original volume and SDS percentage (1%) using a SpeedVac. Proteins were reduced and alkylated, first by the addition of 10 mM DTT (boil for 10 min), and then by the addition of 50 mM iodoacetamide (incubate at room temperature in the dark for 30 min). A small aliquot of Laemmli sample buffer 4X was added and proteins were resolved on an 8-16%

gradient Mini-PROTEAN® TGX™ gel (Biorad, Hercules, CA, USA). Gel was Coomassie stained and de-stained overnight before excision of slices. Peptides resulting from in-gel digestion with trypsin (Promega) were extracted from the gel slices and analyzed by an ultra-high performance (UHPLC) system (Ultimate 3000, Thermo Fisher Scientific) coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) with an electrospray ion source (Thermo Fisher Scientific). Peptides were resolved at a flow rate of 0.2 mL/min on an AdvanceBio Peptide Map (2.7µ, 2.1 x 150 mm) column (Agilent Technologies, Santa Clara, CA, USA) with mobile phase A (water with 0.1% formic acid) and B (acetonitrile and 0.1% formic acid). An 85-min linear gradient from 3% of mobile phase B to 45% of B, followed by a linear increase to 90% B in 5 min and a washing step with 90% B for 10 min was used.

MS data were acquired using a data-dependent Top 5 method, dynamically choosing the most abundant precursor ions from the survey scan (300-1500 m/z) using HCD fragmentation. Full MS resolutions were set to 70 000 and full MS AGC target was 3E6 with an injection time of 100 ms. AGC target value for fragment spectra was set at 1E5, and intensity threshold was kept at 2E5. The isolation window was set to 2.0 m/z. Normalized collision energy was set at 27 and isotope exclusion was on. Data were acquired using Xcalibur software.

Spectral raw files were processed with the SequestHT search engine on Proteome Discoverer™ 2.3. Data were searched against the Homo sapiens database (Uniprot TaxID = 9606), at 1% spectrum level FDR criteria using Percolator (University of Washington). Precursor mass tolerance was constrained to 10 ppm and fragment mass tolerance was set to 0.02 Da. Trypsin was set as the digestion enzyme with a maximum of two allowed missed cleavages. Cysteine carbamidomethylation was set as fixed modification. Variable modifications used were oxidation (M), deamidation (NQ), phosphorylation (STY), arginine-13C6, and lysine-13C615N2. Peptide spectral matches (PSM) were validated using the Percolator algorithm, based on q-values of 1% FDR. Precursor ion quantitation was conducted using Feature Mapper and Precursor Ions Quantifier nodes in the Consensus Step and Minora Feature Detector node in the Processing Step.

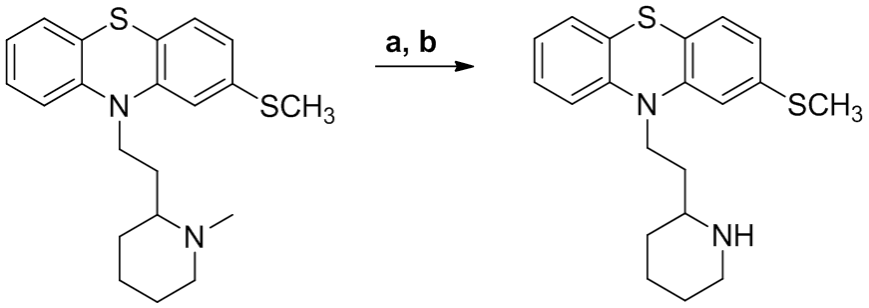
SILAC validation by pull down-immunoblotting

Total protein was extracted from SHI-1 cells using lysis buffer and quantified with the BCA assay. Total protein extracts (1 mg) were mixed with solid phase bound to TDZ3 (SP+TDZ3) for the pull down experiment; briefly, to ensure efficient bound of target proteins to solid phase, we incubated the solid phase with the protein lysate overnight (16 h) with gentle rotation at 4°C; to recover the target proteins the solid phase was washed, boiled for 10 min and then 4x volume of dH2O was added. In order to confirm the specificity of TDZ3 we also performed a pull down experiment with a solid phase empty (SP). Finally, eluate from solid phase was added to small aliquot of Laemmli sample

buffer 2X and resolved on an gradient gel (See "Western Blot" section). SHI-1 total lysate and GAPDH antibody were used as loading control and solid phase specificity control, respectively. Anti-ANXA6 (Abcam) and anti-S100A8/A9 (Novus Biologicals) primary antibodies were used for target detection (see "Western Blot" section).

TDZ analogues synthesis

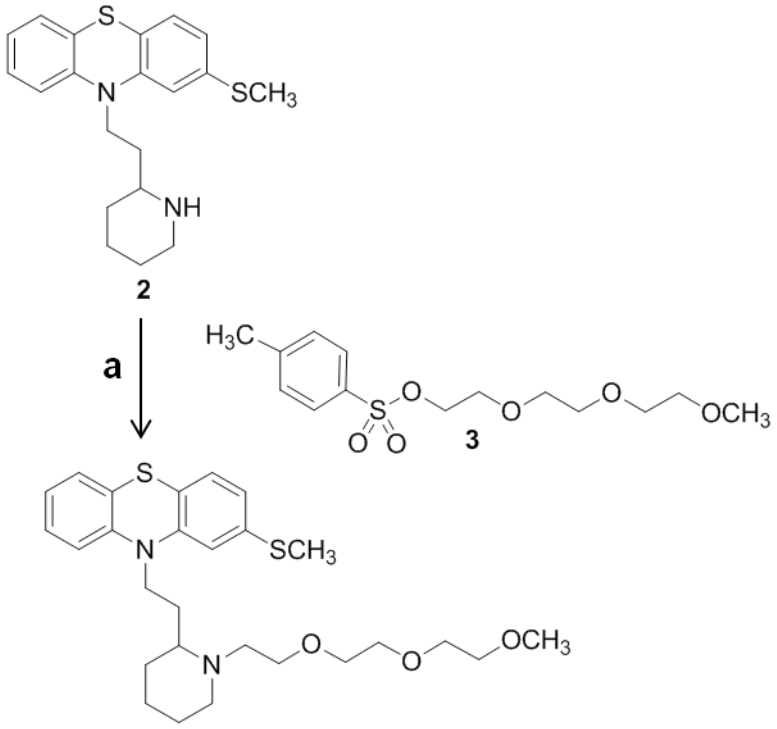
- *Procedure:* Following a previously reported synthesis [9], commercially available racemic thioridazine was *N*-demethylated by treatment with 1-chloroethyl-chloroformate in refluxing DCE followed by hydrolysis with MeOH under reflux, leading to derivative **2**.



Thioridazine (**1**) **2**

Reagents: a) 1-chloro-ethylchloroformate, 1,2-dichloroethane, reflux, 12 h; b). MeOH, reflux, 12 h.

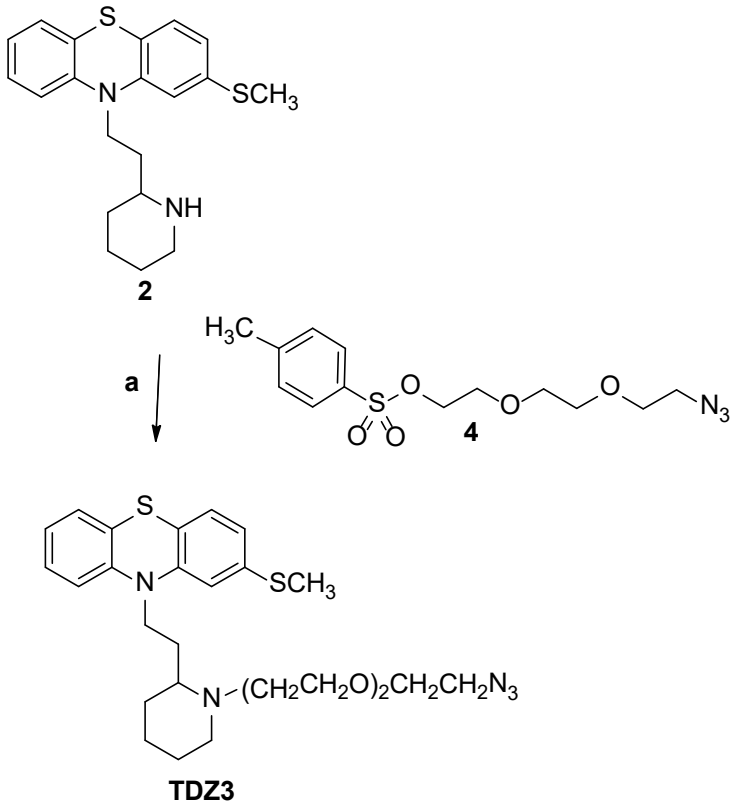
TDZ2: Preparation of compound TDZ2: The reaction of compound **2** with tosyl triethylene glycol methyl ether derivative **3** in refluxing acetonitrile, in presence of potassium carbonate as base, furnished product TDZ2. The bifunctional polyethylene glycol (PEG) linker **3** was prepared as previously described [10].



TDZ2

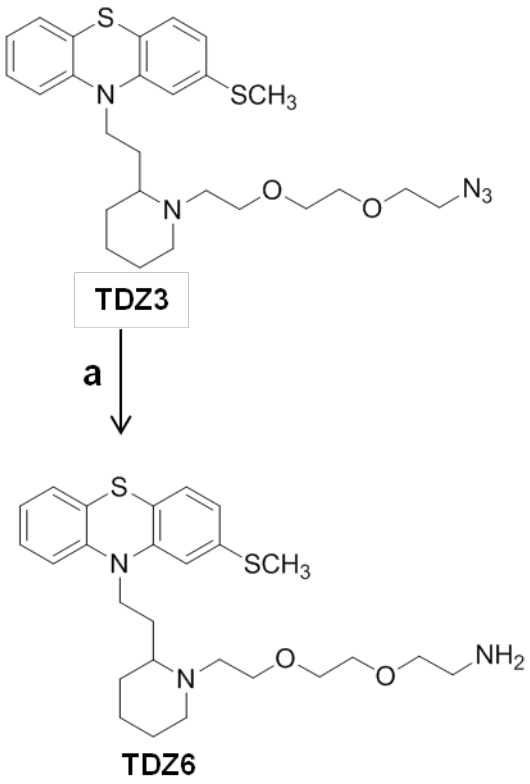
Reagents: a) tosyl triethylene glycol methyl ether (**3**), K_2CO_3, CH_3CN, reflux, 48 h.

TDZ3: Preparation of TDZ3: The condensation of des-methylthioridazine **2** with azido tosylate derivative **4** in refluxing acetonitrile, in presence of potassium carbonate as base, yielded the N-alkylated piperidine derivative TDZ3. The bifunctional polyethylene glycol (PEG) linker **4** was prepared as previously described [11].



Reagents: a) 2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**3**), K_2CO_3, CH_3CN, reflux, 48 h.

TDZ6: Synthesis of TDZ6: The efficient Staudinger reduction at room temperature with triphenylphospine allowed the preparation of amine TDZ6 in excellent yield starting from the previously described azide derivative TDZ3[11].



Reagents: a) Ph_3P, water, room temperature, 24h

- *Chemistry:* A Varian VXR 200 spectrometer was used to obtain ^{1}H NMR; a Varian Mercury Plus 400 spectrometer was used to obtain ^{13}C NMR data. Peak positions are provided in ppm (□) downfield, and *J* values in hertz. Mass spectra were obtained on a Waters ZQ 2000 ESI single quadrupole mass spectrometer, with values given as $[M+1]^{+}$. Melting points (mp) (uncorrected) were obtained on a Buchi-Tottoli instrument. Purity (≥ 95%) was verified by combustion elemental analyses performed at the Microanalytical Laboratory of the Department of Chemistry and Pharmaceutical Sciences of the University of Ferrara using a Yanagimoto MT-5 CHN recorder elemental analyzer. Thin layer chromatography (TLC) was performed on glass plates from Merck coated with silica gel 60 F_{254}, with compounds visualized by UV detection or with aqueous $KMnO_4$. Flash column chromatography was performed with 230-400 mesh silica gel and solvents as indicated. Organic solutions were dried over anhydrous Na_2SO_4. Commercial solvents and reagents were from Sigma-Aldrich–Merck or Scharlab (Barcelona, Spain) and were used as supplied.

TDZ2: *10-(2-(1-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)piperidin-2-yl)ethyl)-2-(methylthio)-10H-phenothiazine.* 2-(Methylthio)-10-(2-(piperidin-2-yl)ethyl)-10H-phenothiazine **2** (71 mg, 0.2 mmol) and K_2CO_3 (55 mg, 0.4 mmol, 2.0 equiv.) were mixed in dry MeCN (6 mL) under argon. After tosylate **3** (182 mg, 0.4 mmol, 2.0 equiv.) dissolved in CH_3CN (2 mL) was added, the reaction mixture was heated to reflux for 24 h. After this time, an additional amount of tosylate **3** (182 mg, 0.4 mmol, 2.0 equiv.) dissolved in CH_3CN (2 mL) was added and the reaction mixture heated for additional 24 h at reflux. The mixture was cooled to room temperature, filtered on Celite and the filter cake rinsed with dicloromethane. The collected organic solution was concentrated under reduced pressure and the residue dissolved in dichloromethane (15 mL). The organic phase was washed with water (5 mL), brine (5 mL) and dried over Na_2SO_4. The volatiles were removed under reduced pressure and the crude mixture purified by flash column chromatography on silica gel using EtOAc/MeOH/Et_3N (9.5:5:0.1) as eluent, to afford the product (65 mg, 65%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.33 (m, 1H), 1.65 (m, 3H), 1.76 (m, 3H), 2.01 (m, 1H), 2.46 (s, 3H), 2.74 (m, 1H), 2.96 (m, 1H), 3.38 (m, 5H), 3.62 (m, 8H), 4.02 (m, 4H), 6.78 (m, 2H), 6.92 (t, J=7.2 Hz, 2H), 7.02 (d, J=7.0 Hz, 2H), 7.22 (d, J=7.0 Hz, 2H). MS (ESI): $[M+1]^{+}$=503.63.

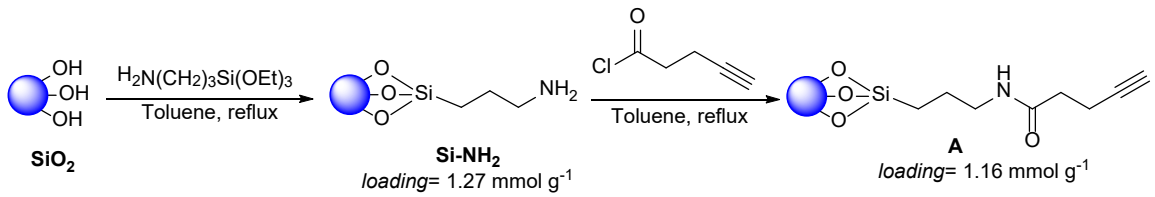
TDZ3: *10-(2-(1-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)piperidin-2-yl)ethyl)-2-(methylthio)-10H-phenothiazine*. 2-(Methylthio)-10-(2-(piperidin-2-yl)ethyl)-10H-phenothiazine **2** (107 mg, 0.3 mmol) and K_2CO_3 (123 mg, 0.9 mmol, 3.0 equiv.) were mixed in dry MeCN (6 mL) under N2. After tosylate **3** (197 mg, 0.6 mmol, 2.0 equiv.) dissolved in CH_3CN (2 mL) was added, the reaction mixture was heated to reflux for 24 h. After this time, an additional amount of tosylate **3** (197 mg, 0.6 mmol, 2.0 equiv.) dissolved in CH_3CN (2 mL) was added and the reaction mixture heated 24 h at reflux. The

mixture was cooled to room temperature, filtered on Celite and the filter cake rinsed with dicloromethane. The collected organic solution was concentrated under reduced pressure and the residue dissolved in dichloromethane (15 mL). The organic phase was washed with water (5 mL), brine (5 mL) and dried over Na_2SO_4. The volatiles were removed under reduced pressure and the crude mixture purified by flash column chromatography on silica gel using DCM/MeOH/Et_3N (92.5:7.5:0.1) as eluent, to afford the product (80 mg, 52%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.32 (m, 1H), 1.63 (m, 3H), 1.76 (m, 3H), 2.02 (m, 1H), 2.46 (s, 3H), 2.72 (m, 1H), 2.96 (m, 1H), 3.36 (t, J = 5.0 Hz, 2H), 3.59 (m, 8H), 4.03 (m, 4H), 6.81 (m, 2H), 6.92 (t, J=7.2 Hz, 2H), 7.03 (d, J=6.9 Hz, 2H), 7.22 (d, J=6.9 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 16.39, 22.95, 24.16, 28.25, 29.69, 44.19, 50.65, 50.74, 52.43, 58.21, 69.33, 69.97, 70.06, 70.37, 70.47, 114.56, 114.66, 120.79, 122.64, 125.92, 127.44, 127.54, 127.63, 138.12, 144.41, 145.77. MS (ESI): $[M]^+$=514.65.

TDZ6: To a solution of **TDZ3** (102 mg, 0.2 mmol) in THF (4 mL) was added triphenylphospine (105 mg, 0.4 mmol, 2 equiv.) at 0 °C. The mixture was stirred at room temperature for 2h and then water (43.2 mg, 0.043 mL, 2.4 mmol, 12 equiv) was added at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 22 h. The reaction mixture was quenched by adding 2M aqueous HCl (5 mL) e diluted with EtOAc. The aqueous layer was washed with EtOAc (3x5 mL), and then basified with 2M aqueous NaOH to pH 12. The aqueous layer was extracted with dichloromethane (3x5 mL). The organic phase was washed with brine (5 mL) and dried over Na_2SO_4. The volatiles were removed under reduced pressure and the crude mixture purified by flash column chromatography on silica gel using EtOAc/MeOH/Et_3N (8.0:2.0:0.1) as eluent, to afford the product **TDZ6** (65 mg, 67%) as a brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.34 (m, 1H), 1.66 (m, 3H), 1.72 (m, 3H), 2.02 (m, 1H), 2.39 (s, 3H), 2.70 (m, 1H), 2.88 (m, 1H), 3.38 (t, J = 5.2 Hz, 2H), 3.59 (m, 8H), 4.02 (m, 4H), 5.11 (bs, 2H), 6.76 (m, 2H), 6.88 (t, J=7.2 Hz, 2H), 7.06 (d, J=7.0 Hz, 2H), 7.24 (d, J=7.0 Hz, 2H). MS (ESI): $[M]^+$=488.6.

Solid Phase Immobilization for SILAC.

The procedure for the preparation of alkyne-functionalized silica followed several steps. The alkyne-functionalized silica **A** was prepared according to [12].

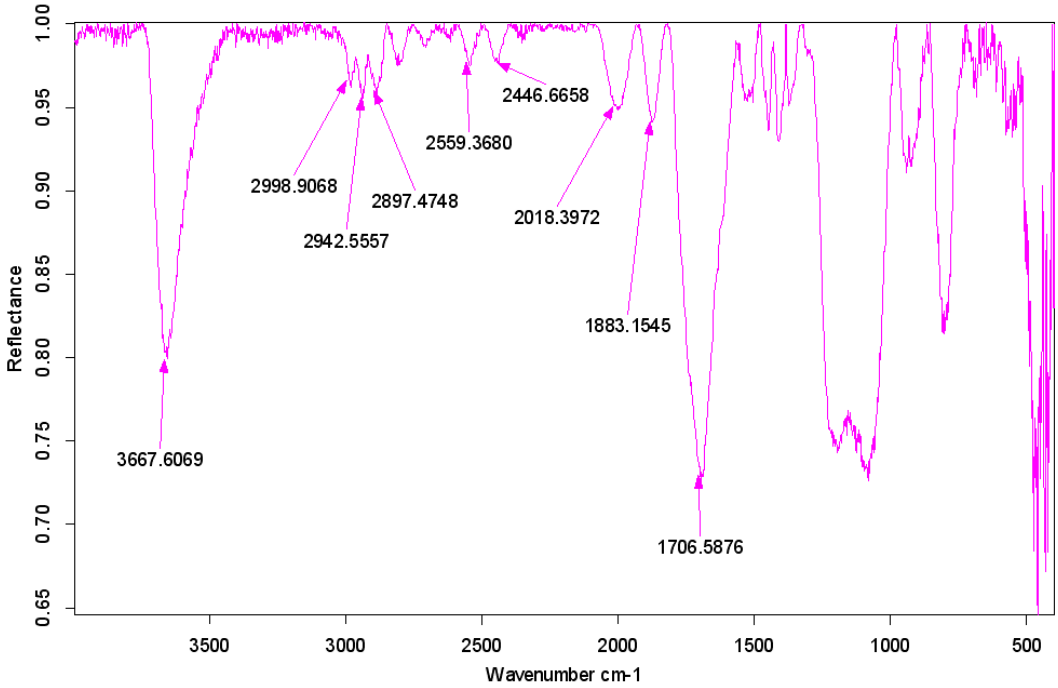


A mixture of SiO_2 (1.0 g) and 3-aminopropyltriethoxysilane (1.0 mL, 4.3 mmol) were loaded into a 20 mL vial and suspended in anhydrous toluene (10.0 mL). The stirred solution was degassed under vacuum and saturated with argon (by an Ar-filled balloon) three times and heated at reflux for 24 h. After cooling, the resulting white solid mixture was centrifuged with portions of cyclohexane (2 × 10.0 mL) and methanol (2x 10.0 mL), and dried under vacuum to collect the amino-functionalized silica (**Si-NH_2**). Lately, 4-pentynoyl chloride (0.11 g, 0.94 mmol) and Si-NH_2 (0.44 g, 0.56 mmol of -NH_2) were suspended in 20 mL of dry toluene under an argon atmosphere. After 24 h of refluxing, the product was centrifuged with portions of cyclohexane (2 × 10.0 mL) and methanol (2 × 10.0 mL), and dried at reduced pressure (0.1 mbar, 40°C, 12 h) to afford silica **A**.

The loading value is evaluated by elemental analysis and it is expressed as mmol of ligand per gram of functionalized support using nitrogen as the reference atom [loading= N(%) * 10/(14)].

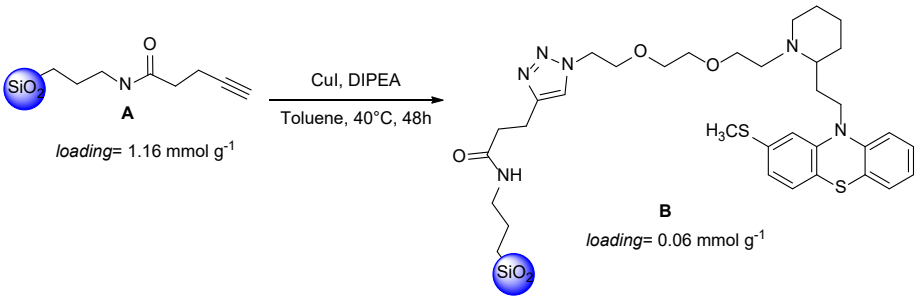
Elemental analysis (%) found: N 1.63 (loading = 1.16 $mmolg^{-1}$).

FT-IR (KBr): ν 3667, 2998, 1706 cm^{-1}.



FT-IT spectrum of alkyne-functionalized silica **A**

The procedure for the preparation of silica-supported TDZ B via Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC):



TDZ3 (41 mg; 0.08 mmol) and alkyne-functionalized silica **A** (35 mg, 0.04 mmol; loading= 1.16 mmol g^{-1}), were loaded into a 5 mL vial and suspended in anhydrous toluene (1.0 mL). The stirred solution was degassed under vacuum and saturated with argon (by an Ar-filled balloon) three times. CuI (8 mg, 0.04 mmol) and *N*,*N*-Diisopropylethylamine (17 µL, 0.1 mmol) were next added, followed by heating at 40 °C for 48 h. After cooling to room temperature, the mixture was centrifuged with portions of THF (3 × 5.0 mL), 0.05M aqueous EDTA disodium salt solution (4 × 5.0mL), H_2O (2 × 5.0 mL), and acetone (2 × 5.0 mL). The resulting silica-supported TDZ **B** was finally dried at reduced pressure (0.1 mbar, 40°C, 12 h).

For the silica-supported TDZ **B** the loading value is evaluated by elemental analysis and it is expressed as mmol of ligand per gram of functionalized support using sulfur as the reference atom [loading= S(%) * 10/(32*n)] where n is the number of the sulfur atom in the molecule (n = 2).

Elemental analysis (%) found: S 0.39 (loading = 0.06 $mmolg^{-1}$).

FT-IR (KBr): ν 3664, 2943, 2108, 1998, 1878, 1221 cm^{-1}.

FT-IR spectrum of silica-supported TDZ **B**

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SUPPLEMENTAL TABLES

Compound	Description
GSK1210151A	inhibitor of the BET (bromodomain and extra terminal domain protein) family proteins
Sunitinib malate	tyrosine kinase inhibitor. Targets: VEGF-R1, VEGF-R2, VEGF-R3, PDGF-Ra, PDGF-Rß, KIT, FLT3, CSF-1R, and RET
Quinacrine dihydrochloride	Monoamine oxidase (MAO) inhibitor; antimalarial
PD 0325901	MKK1 (MEK1) and MKK2 (MEK2) inhibitor
5-(N,N-hexamethylene) amiloride	Na+/ H+ antiport inhibitor
GBR-12909 dihydrochloride	Selective dopamine reuptake inhibitor
L-703,606 oxalate salt hydrate	non-peptide NK-1 tachykinin receptor antagonist
Pentamidine isethionate	NMDA glutamate receptor antagonist
Pyridostatin trifluoroacetate salt	stabilizes G-quadruplexes, targeting the proto-oncogene SRC and telomeric G-quadruplexes
Tyrphostin AG 879	Tyrosine kinase nerve growth factor receptor (TrkA) inhibitor

SUPPLEMENTAL TABLE 1. Compounds resulted active in t(6;11) and t(9;11) rearranged AML cell lines.

Compound	Description
Arvanil	activator of cannabinoid and vanilloid receptors
CP-100356 monohydrochloride	specific inhibitor of MDR1 (P-Gp)
Fluspirilene	Dopamine receptor antagonist; antipsychotic
CID2858522	specifically inhibits NF-kB activation downstream of protein kinsase C (PKC)
Eupatorin	antiproliferative in cells expressing the CYP1A- family. It induces G2/M block follow by apoptosis
ANA-12	TrkB ligand that prevents activation of the receptor by BDNF
BAY 61-3606 hydrochloride hydrate	Spleen tyrosine kinase (Syk) inhibitor; anti-inflammatory
Ara-G hydrate	inhibitor of DNA synthesis; antineoplastic; and antimetabolite; inducer of apoptosis
Tyrphostin 47	EGFR tyrosine kinase inhibitor
Thioridazine hydrochloride	Dopamine receptor antagonist; Ca2+ channel antagonist; antipsychotic

SUPPLEMENTAL TABLE 2. Compounds resulted selectively active in t(6;11) rearranged AML cell lines.

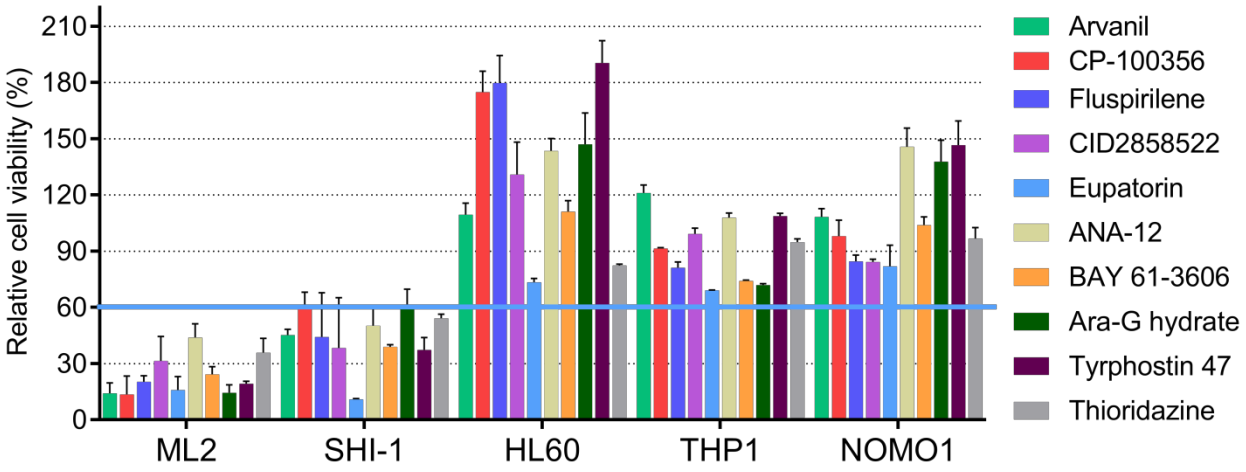
Compound	GI50 on t(6;11) AML cell lines (μM)
Arvanil	1 – 10
CP-100356	3 – 4
Fluspirilene	5 – 10
CID2858522	5 – 7
Eupatorin	3 – 4
ANA-12	5 – 10
BAY 61-3606	1 – 6
Ara-G hydrate	5 – 10
Tyrphostin 47	5 – 10
Thioridazine	6 – 10

SUPPLEMENTAL TABLE 3. Growth inhibition 50 (GI_{50}) measurement of the 10 drugs resulted active and selective in t(6;11)-AML cells.

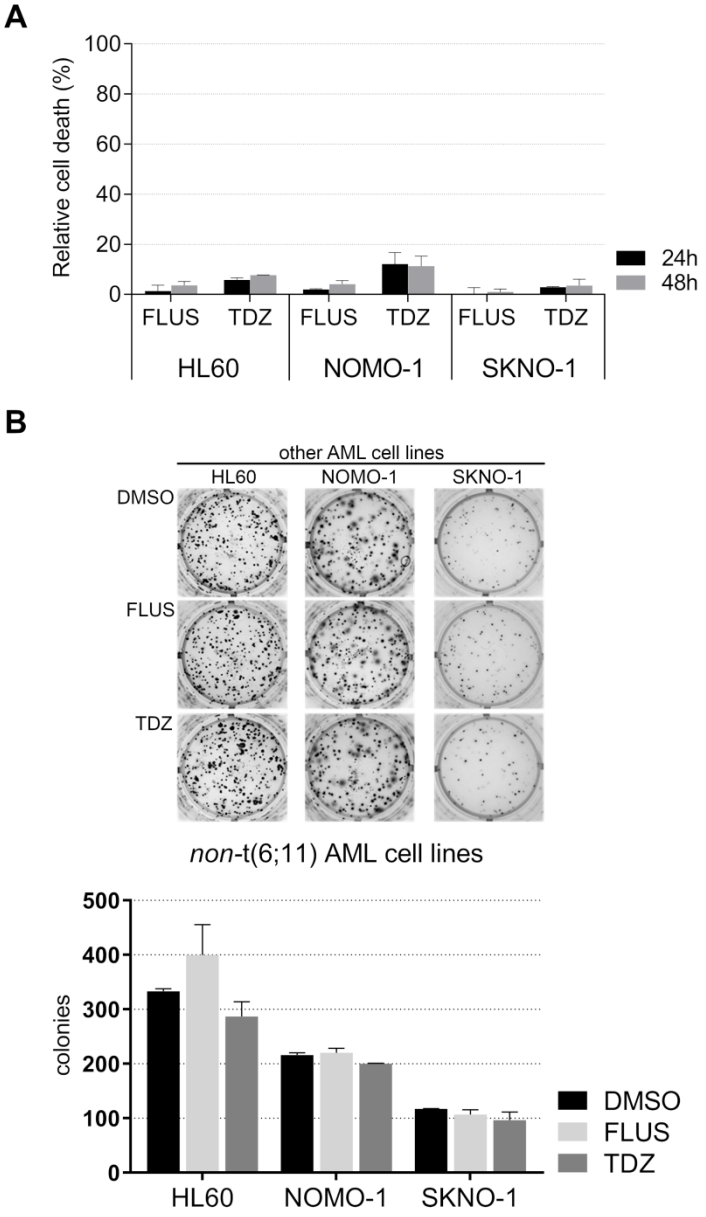
GI_{50} (µM)	t(6;11) **SHI-1**	t(6;11) **ML-2**	**HL60**	**K562**	t(4;11) **MV4;11**	t(9;11)**MO LM-13**	t(9;11) **NOMO-1**	t(9;11) **THP-1**	t(10;11) **U937**	t(8;21) **SKNO-1**
Mean	**7.31**	**8.72**	**12.96**	**11.76**	**11.25**	**13.39**	**11.93**	**16.36**	**12.98**	**15.22**
SEM	0.45	0.27	0.65	0.63	0.80	0.03	0.38	1.69	0.02	0.41
adjusted p-value		**n.s.** 0.3889	0.0002	0.0009	0.0033	<0.0001	0.0009	<0.0001	0.0002	<0.0001

SUPPLEMENTAL TABLE 4. TDZ GI_{50} in AML cell lines. GI_{50} values calculated in n=10 AML cell lines with different genetic markers after 72h of treatment with TDZ, measured by ATP assay. Data are indicated as mean ± SEM. Adjusted p-value for SHI-1 compared with the other cell lines is reported (one-way ANOVA, Dunnett's multiple comparison test).

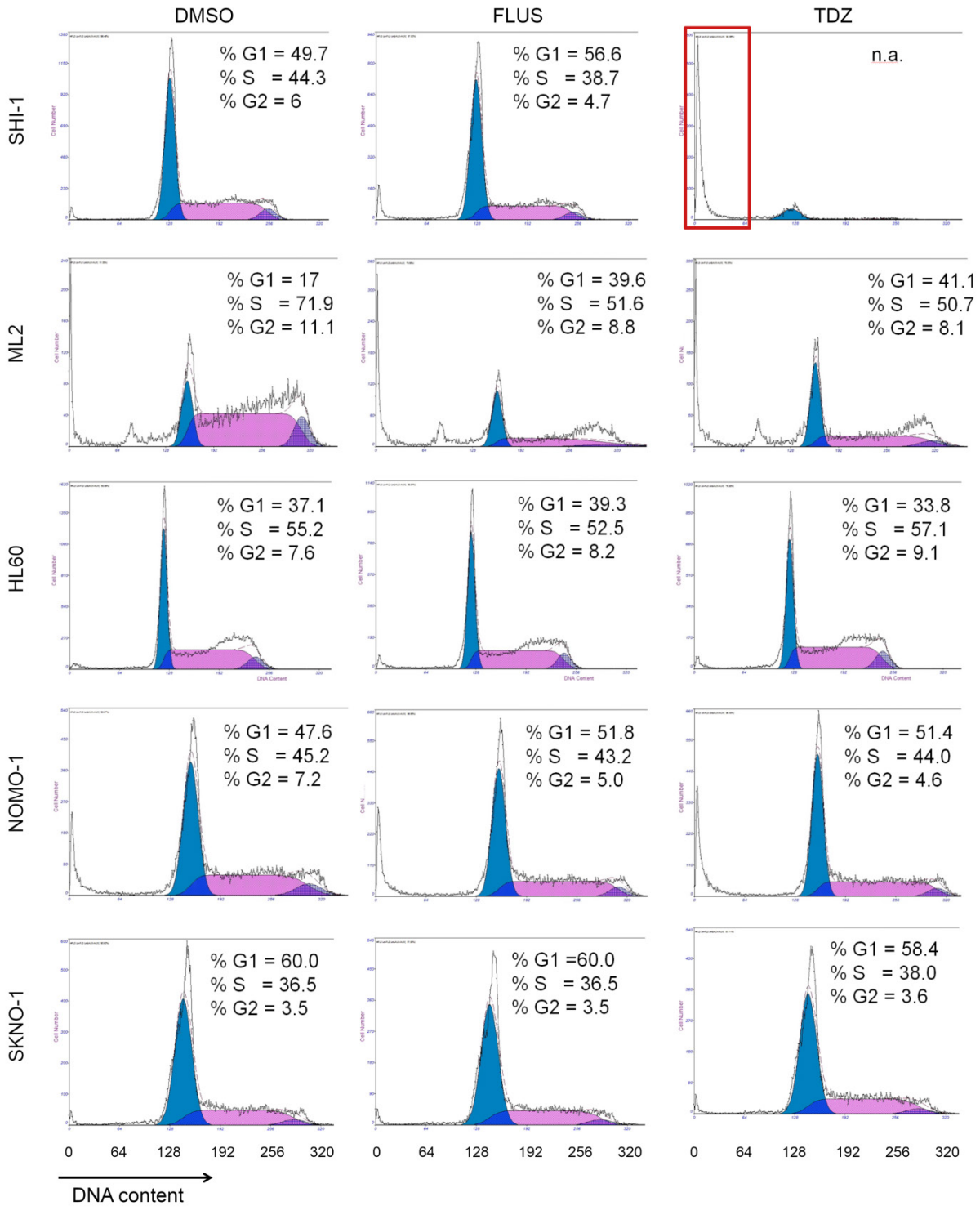
SUPPLEMENTAL FIGURES:



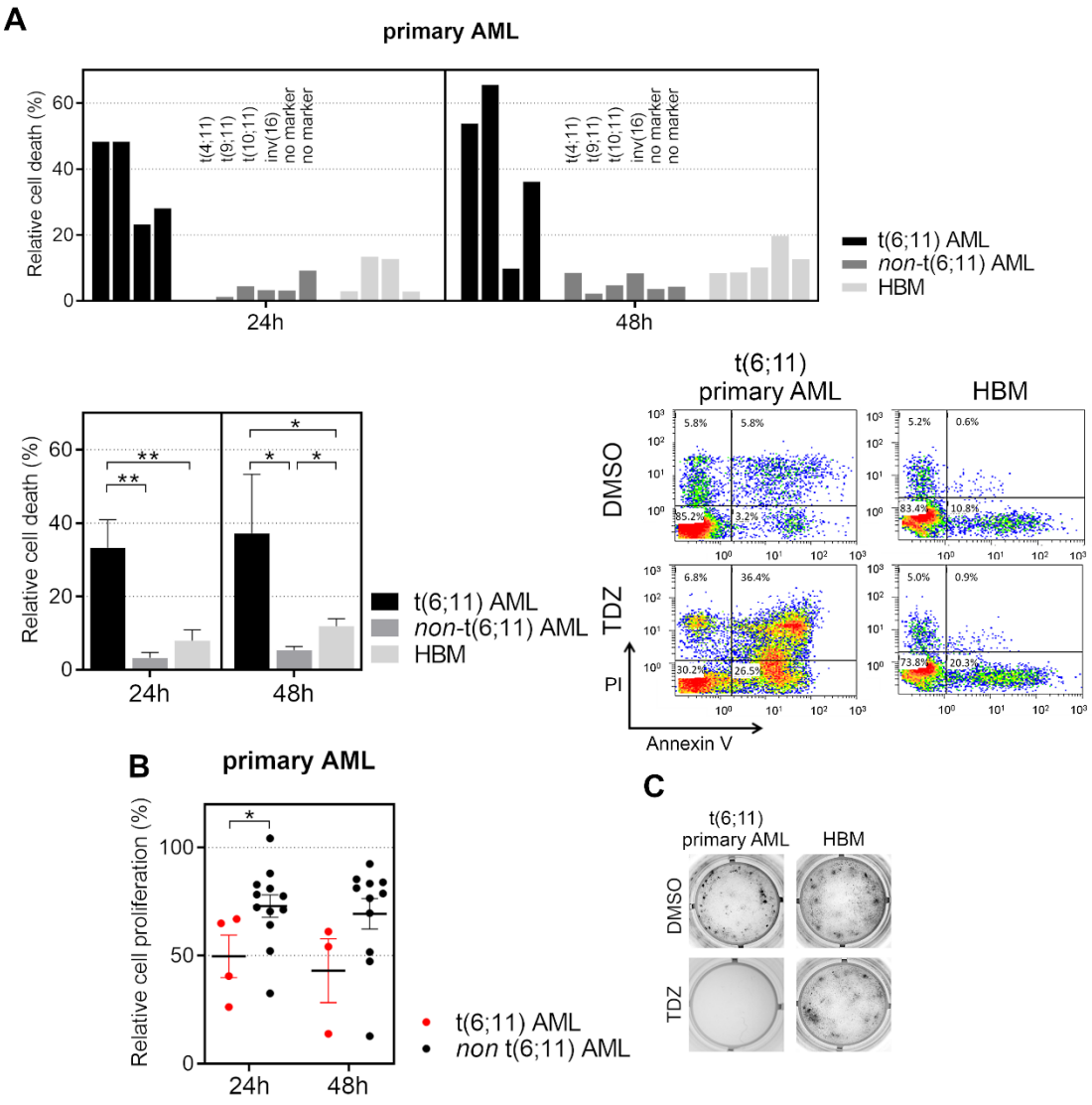
SUPPLEMENTAL FIGURE 1. **IC50 of the 10 candidate compounds.** Cell viability of t(6;11) (ML-2 and SHI-1) and *non*-t(6;11) (HL60, THP-1 and NOMO-1) cells, measured by ATP assay, 72h after treatment with the selected 10 compounds, relative to DMSO value. Blue bar represents the arbitrary threshold of 60% cell viability. Data are presented as mean ± SEM.



SUPPLEMENTAL FIGURE 2. TDZ induced apoptosis and clonogenicity in *non*-t(6;11) rearranged AML cell lines. To confirm selectivity, we treated different AML cell lines, namely HL60, NOMO-1, SKNO-1. (**A**) Cell death (Annexin V+, PI+ and Annexin V+/PI+) induced by FLUS and TDZ in HL60, NOMO-1 and THP-1 cell lines 24 and 48h after treatment (n=3), relative to DMSO value. (**B**) Colony-forming assay performed on viable HL60, NOMO-1 and THP-1 cells seeded 24h after FLUS or TDZ treatment (n=3). Data are presented as mean ± SEM.



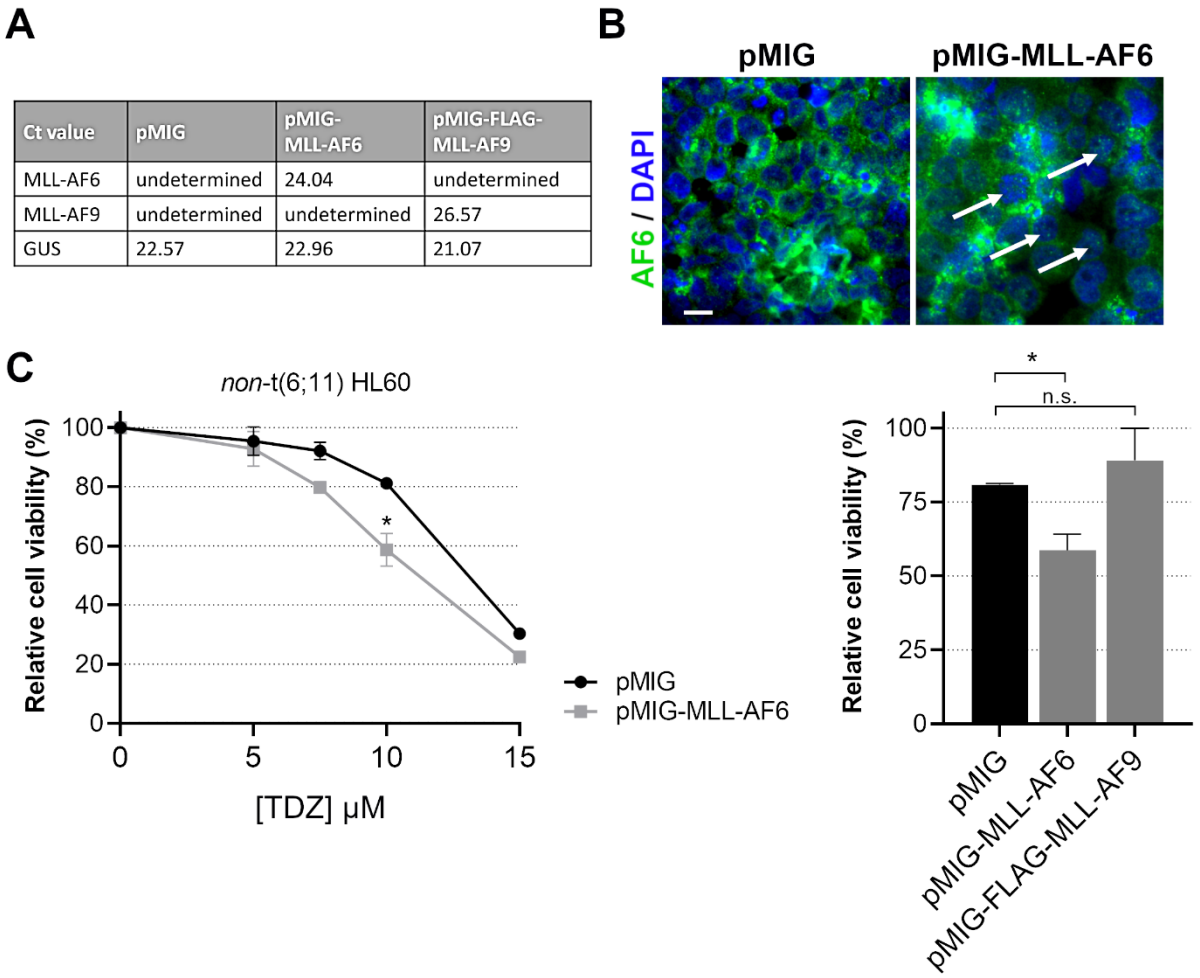
SUPPLEMENTAL FIGURE 3. Cell cycle analysis after 24 h TDZ treatment. t(6;11) ML-2 and SHI-1 cell lines and *non*-t(6;11) cell lines HL60, NOMO-1 and SKNO-1 were analyzed for cell cycle after 24h of drug treatment. Red box shows sub-G0 cells.



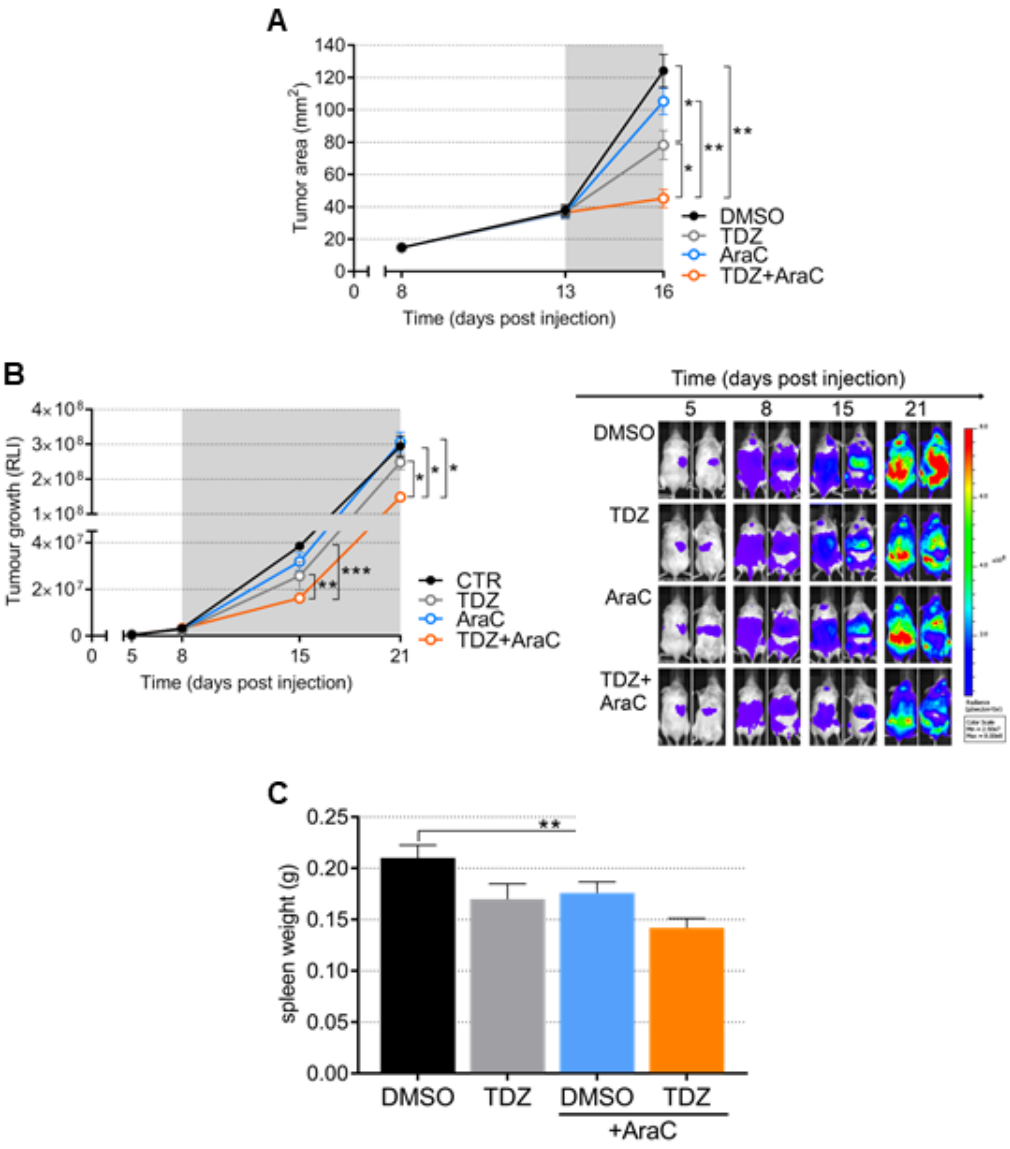
SUPPLEMENTAL FIGURE 4. TDZ treatment over AML-primary cells. (**A**) Histograms showing cell death (Annexin V+, PI+ and Annexin V+/PI+) of t(6;11), *non*-t(6;11) primary AML samples (n=4 and 6, respectively), and HBM (n=5), 24 and 48h after TDZ treatment, relative to DMSO value. Single values (upper panel) and mean ± SEM (lower left panel) are reported. In the lower right panel, representative AnnexinV/PI plot are shown. (**B**) Cell proliferation of t(6;11) (n=4) and *non*-t(6;11) (n=12) patients, measured by ATP assay and relative to DMSO, 24h and 48h after TDZ treatment (10 µM). (**C**) Colony-forming assay performed on viable t(6;11) primary AML samples and HBM (n=2). Data are presented as mean ± SEM. *p < 0.05; **p < 0.01.

A

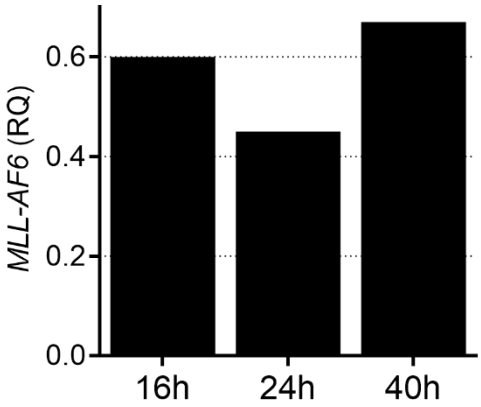
Ct value	pMIG	pMIG-MLL-AF6	pMIG-FLAG-MLL-AF9
MLL-AF6	undetermined	24.04	undetermined
MLL-AF9	undetermined	undetermined	26.57
GUS	22.57	22.96	21.07



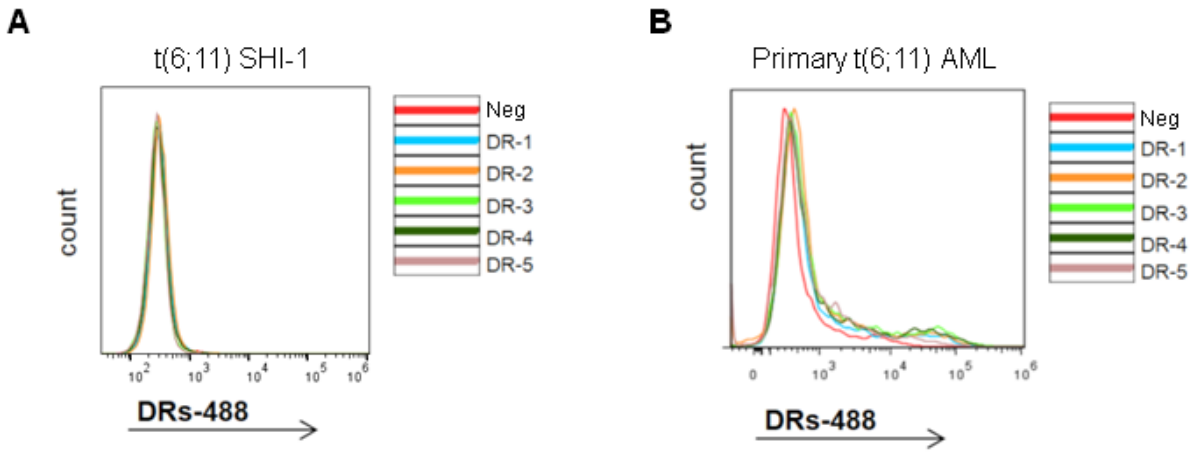
SUPPLEMENTAL FIGURE 5. MLL-AF6 expression in *non*-t(6;11) cell line. (**A**) Chimera expression detected by RQ-PCR, reported as Ct value measured after the HL60 cell line transfection with empty pMIG, pMIG-MLL-AF6 and pMIG-FLAG-MLL-AF9. (**B**) Immunofluorescence analysis showing AF6 (green) localization after MLL-AF6 expression. Arrows indicate AF6 nuclear foci with respect to cytoplasmic expression of the pMIG. DAPI (blue) was used for staining cell nuclei. Original magnification 40x. Scale bar 10µm. (**C**) Dose-response curve of pMIG/pMIG-MLL-AF6 transfected cells after TDZ treatment, with respect to DMSO (left panel). Cell viability of pMIG/pMIG-MLL-AF6 and pMIG-FLAG-MLL-AF9 transfected cells after TDZ treatment at 10µM, with respect to DMSO (right panel). Cells were treated with TDZ after 24h from transfection and cell viability was measured by ATP assay after additional 72h. Data are presented as mean ± SEM. *p < 0.05.



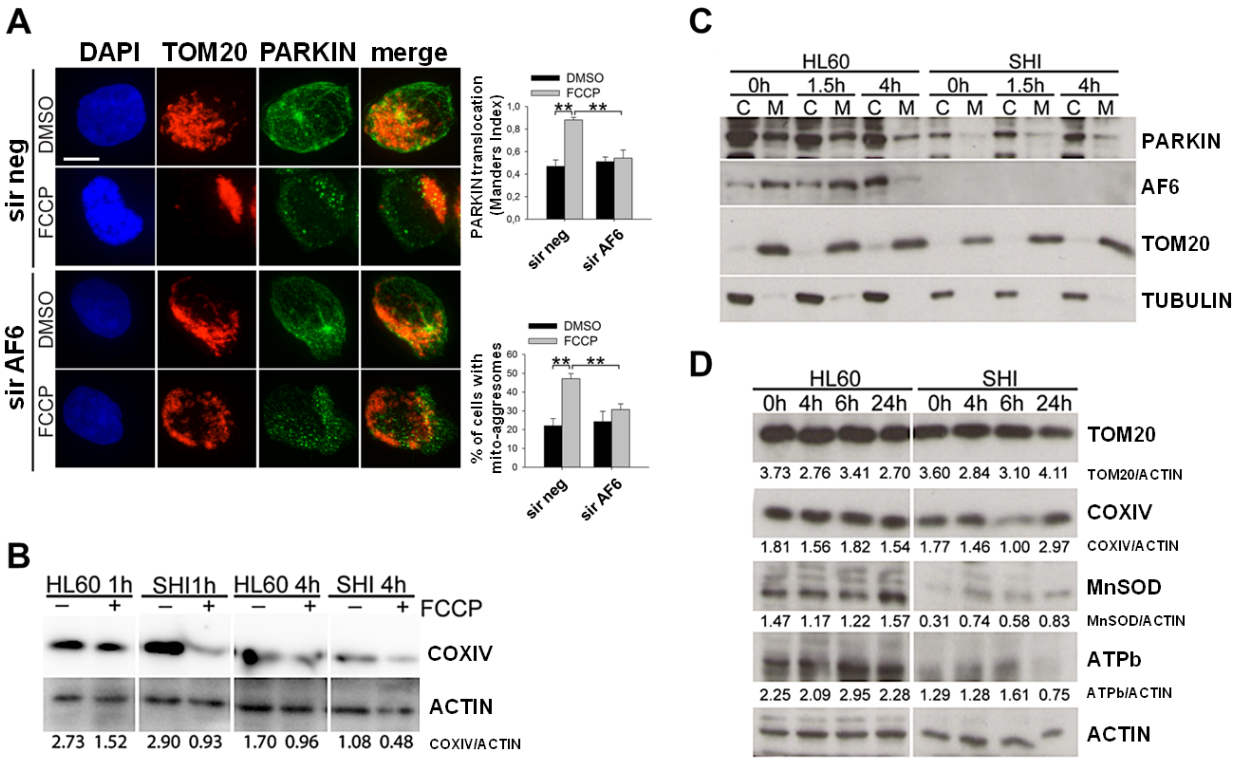
SUPPLEMENTAL FIGURE 6. Tumor growth in mice treated with TDZ. (**A**) Tumor growth in mice flank-injected with SHI-1 and daily treated with TDZ at 8 mg/kg, AraC 6.25 mg/kg or the combination of the two drugs compared with the control group treated with DMSO (n=4). Grey area indicates treatment interval. (**B**) Tumor growth (measured by relative light intensity, RLI) in mice engrafted with intravenous injection of LUC-transduced SHI-1 cells, and daily treated with TDZ at 8 mg/kg, AraC 6.25 mg/kg or the combination of the two drugs compared with the control group treated with DMSO (n=4). Grey area indicates treatment interval. In the right panel, representative images of luciferase activity in mice measured 5, 8, 15 and 21 days after treatment are reported. (**C**) Mice were treated with 8 mg/kg, AraC 6.25 mg/kg or the combination of the two drugs and after being sacrificed (n=4) spleens were extracted and weighed at the experimental endpoint. Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.



SUPPLEMENTAL FIGURE 7. *MLL-AF6* silencing. RQ-PCR of *MLL-AF6* fusion gene, measured at 16, 24 and 40h after *MLL-AF6* silencing, relative to sir neg (RQ=1). Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$.



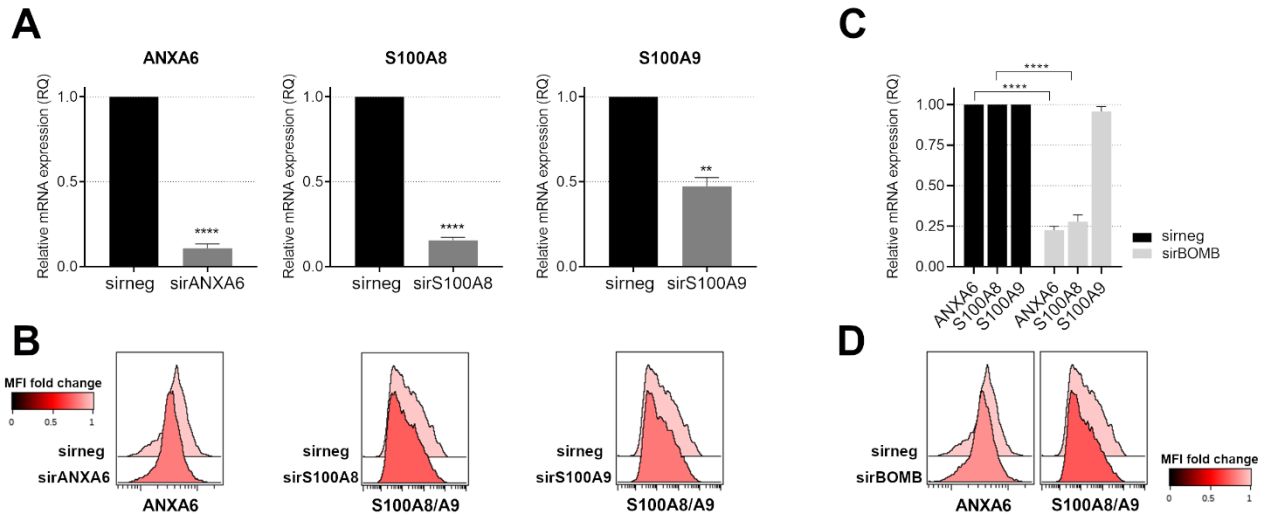
SUPPLEMENTAL FIGURE 8. DR receptors expression. DR receptors expression was monitored in (**A**) SHI-1 AML cell line and (**B**) primary AML patient with t(6;11) rearrangement by flow cytometry; a clear positivity after isotype control was not revealed for all the five DRs.



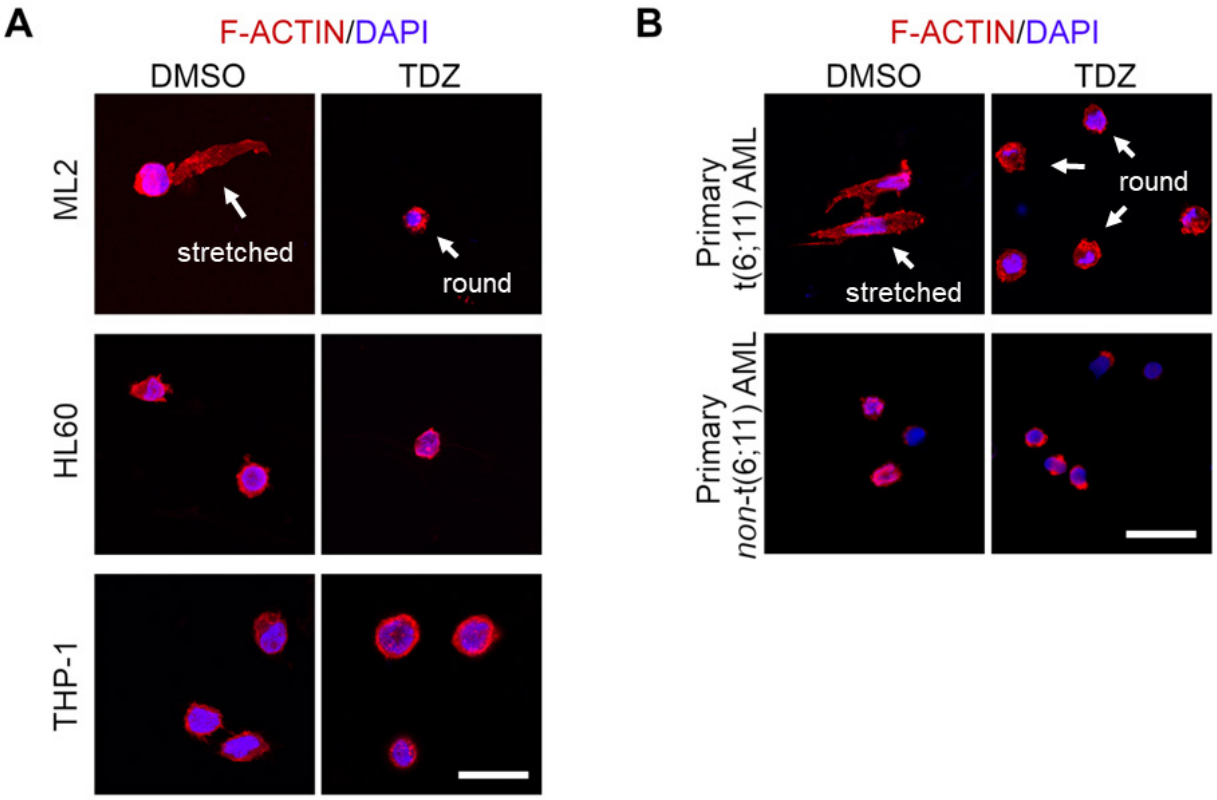
SUPPLEMENTAL FIGURE 9. Mitophagy evaluation after TDZ treatment. (**A**) We tested PARKIN recruitment to mitochondria after treatment with a mitophagy inductor, namely Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP): HL60-AF6-expressing cells underwent to mitophagy as expected, but this phenomenon was reduced by AF6 silencing due to impaired PARKIN translocation which resulted in less mito-aggresome formation. Representative z-stack reconstruction of confocal immunofluorescence images of HL60 cells transfected with either sirNeg or sirAF6 and then treated with FCCP or DMSO for 1h. Cells are stained with TOM20 (red) and PARKIN (green) antibodies, and DAPI (blue) as nuclear counterstain to evaluate PARKIN translocation to mitochondria (by Manders' colocalization index, upper histogram, n=13 to 17 cells), and mito-aggresomes quantification (lower histogram, n=27 to 94 cells). Data are presented as mean ± SEM. ** $p < 0.01$. Scale bar 6µm. (**B**) We asked whether the absence of functional AF6 in SHI-1 cells may promote cell death upon TDZ treatment through an impairment of PARKIN-dependent mitophagy. Figure showes that SHI-1 cells underwent mitochondrial clearance (COXIV reduction) upon FCCP treatment, indicating mitophagy occurrence by other PARKIN-independent mechanisms: COXIV and ACTIN expression by western blot in HL60 and SHI-1 cells treated with FCCP for the indicated time. The relative COXIV/ACTIN expression ratio is reported below each line. (**C**) Expression of PARKIN, AF6, TOM20 and TUBULIN in mitochondrial (M)/cytosolic (C) fractions of HL60 and SHI-1 cells treated with TDZ for the indicated time. (**D**) Expression of TOM20, COXIV, MnSOD,

ATPb and ACTIN in HL60 and SHI-1 cells treated with TDZ for the indicated time. The relative marker/ACTIN expression ratios are reported below each protein.

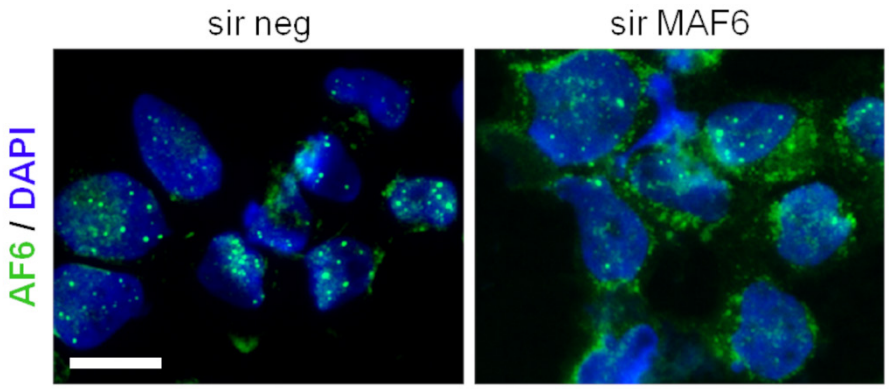
SUPPLEMENTAL FIGURE 10. TDZ3 analogue formula for SILAC experiment. (**A**) Chemical structure of TDZ, and of its derivative TDZ3; TDZ3 was then used to be covalently linked to the SiO_2 solid phase (lower structure) for SILAC experiment to determine TDZ target protein(s). (**B**) Cell death (Annexin V+, PI+ and Annexin V+/PI+) evaluated 24h after TDZ3 analogue treatment, compared with DMSO value (n=3). Data are presented as mean ± SEM. **p < 0.01.



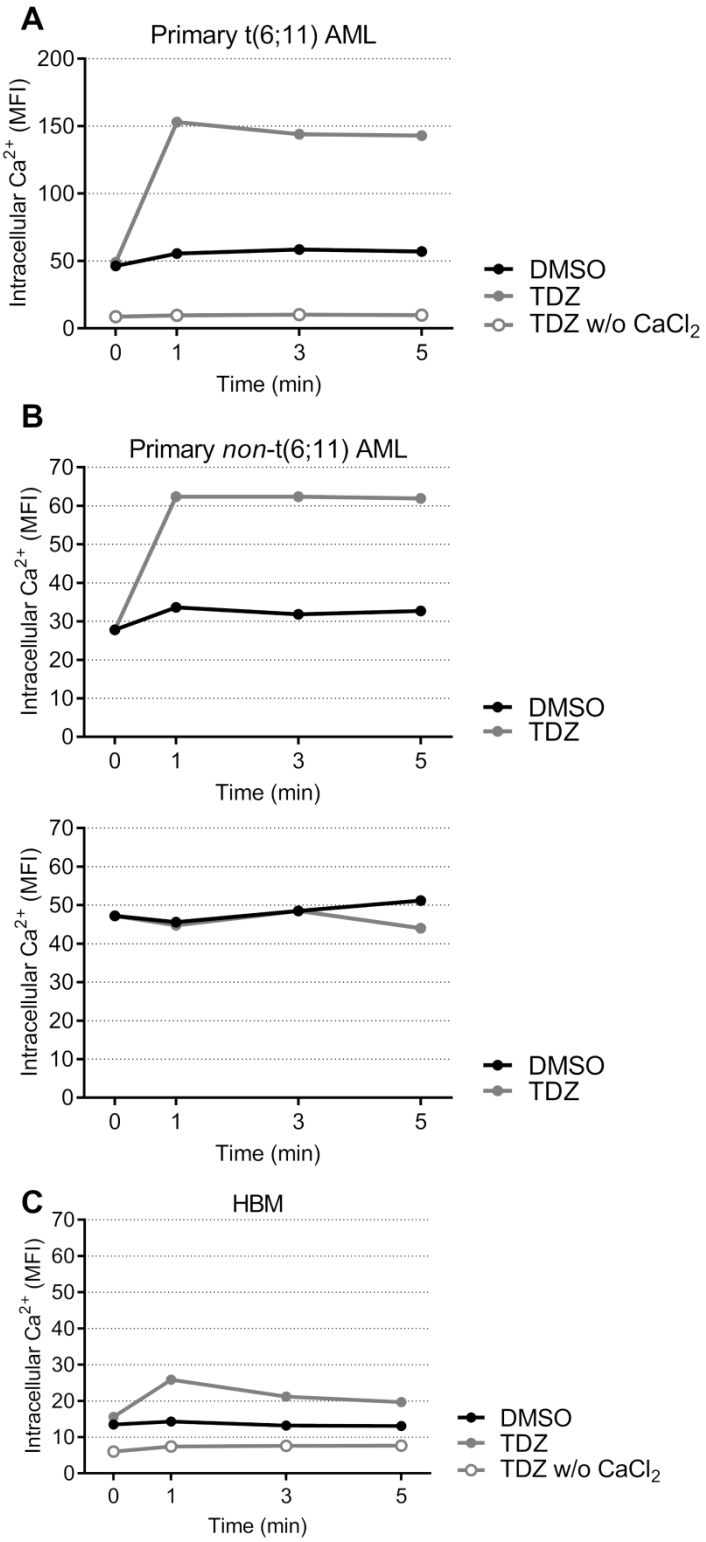
SUPPLEMENTAL FIGURE 11. Silencing of TDZ targets. **S**ingle gene (**AB**) or all together BOMB **(CD**) gene silencing of ANXA6, S100A8, S100A9 were evaluated by mRNA expression at 24h post-transfection by RQ-PCR and by protein level detection in flow cytometry expressed as MFI fold change (protein levels were reduced from 14 to 29%; data are presented as mean ± SEM. **p < 0.01, ****p < 0.0001).



SUPPLEMENTAL FIGURE 12. F-ACTIN on a series of different AML cell lines and primary AML cultures. The non-t(6;11)-rearranged AML exhibited a small and round cytoskeleton when compared to the stretched t(6;11)-rearranged cells before and after treatment independently from their genetic asset. (**A-B**) Immunofluorescence of t(6;11) ML-2 and non-t(6;11) HL60 and THP-1 cell lines (**A**) and primary t(6;11) and *non*-t(6;11) AML (**B**) seeded onto FN-coated slides 4h after TDZ treatment, stained with F-ACTIN antibody (red), and with DAPI (blue) as nuclear counterstain. Original magnification 63x. Scale bar 10µm.

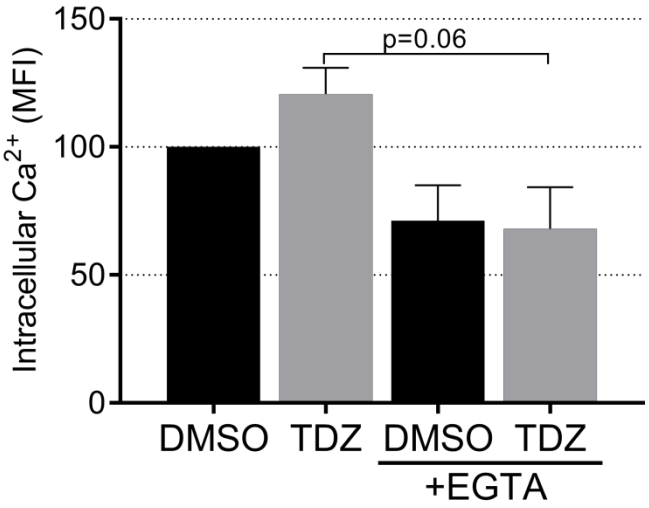


SUPPLEMENTAL FIGURE 13. Immunofluorescence of centrifuged t(6;11) SHI-1 cells 24h after *MLL-AF6* silencing. Cells were stained with AF6 antibody (green), and with DAPI (blue). Original magnification 40x. Scale bar 10µm.

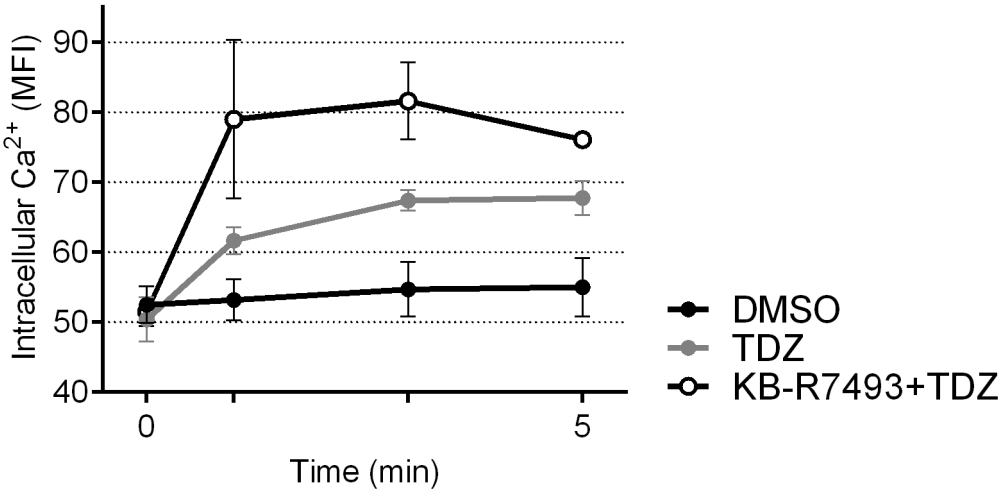


SUPPLEMENTAL FIGURE 14. Live intracellular Ca^{2+} measurement in primary t(6;11)AML. (**A**, n=3) primary *non*-t(6;11) AML (**B**, n=3) or HBM (**C**, n=2). Cells were loaded with Fluo-4 AM Ca^{2+} indicator and measured by flow cytometry in Ca^{2+}-containing, or in Ca^{2+}-free buffer (**A** and **C**),

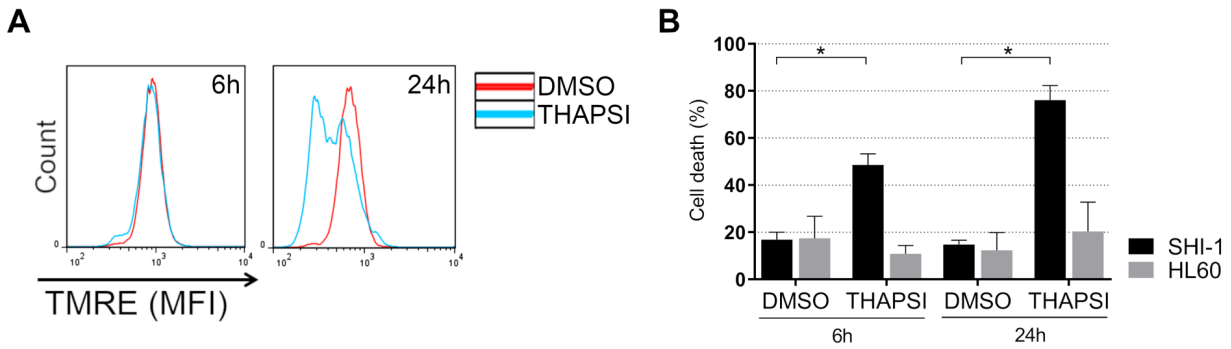
where TDZ (or DMSO as control) was added after the basal measurement at t=0, and the fluorescence was detected 1', 3' and 5' after treatment. MFI: mean fluorescence intensity.



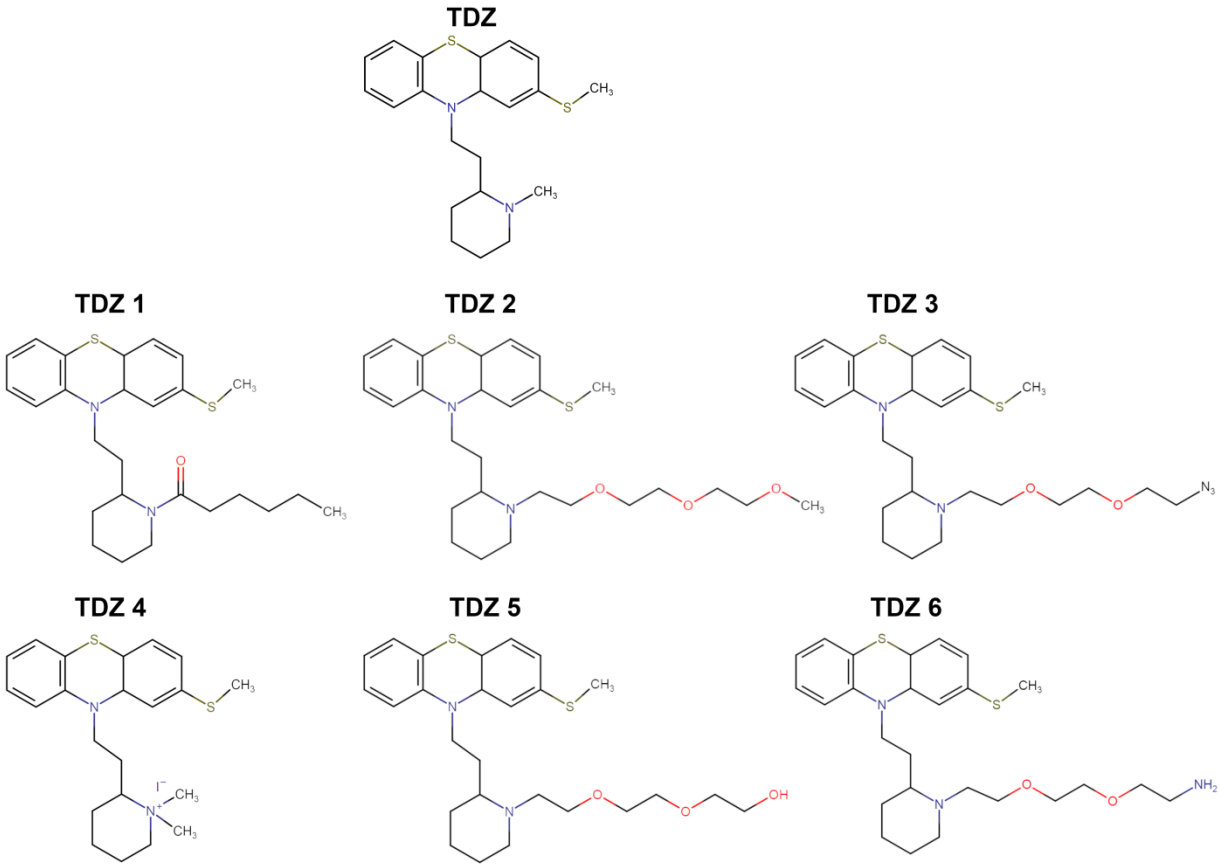
SUPPLEMENTAL FIGURE 15. Intracellular Ca^{2+} measurement in SHI-1 cells. Cells were loaded with Fluo-4 AM Ca^{2+} indicator, 1.5h after TDZ, EGTA or EGTA+TDZ treatment. Data are presented as mean ± SEM. MFI: mean fluorescence intensity.



SUPPLEMENTAL FIGURE 16. Live intracellular Ca^{2+} measurement in t(6;11)-SHI-1. We exacerbated Ca^{2+} cytoplasmic levels by co-treating cells with TDZ and KB-R7943 (10 μM), a drug that inhibits both the mitochondrial Calcium Uniporter (mCU) and plasma membrane Na^{+}-Ca^{2+} exchanger. Cells were loaded with Fluo-4 AM Ca^{2+} indicator and Ca^{2+} was measured by flow cytometry where drugs (or DMSO as control) were added after the basal measurement at t=0, and the fluorescence was detected 1', 3' and 5' after treatment. Data are presented as mean ± SEM. MFI: mean fluorescence intensity.



SUPPLEMENTAL FIGURE 17. AML cells treatment with Thapsigargin. To definitively establish that Ca^{2+} homeostasis was pivotal for t(6;11) blast survival, we treated AML cells with thapsigargin (THAPSI) (2 µM), a specific inhibitor of endoplasmic reticulum (ER) ATPase, which depletes Ca^{2+} ER storage, pouring its cargo into cytosol. (**A**) Representative histograms overlay showing mitochondrial depolarization evaluated through TMRE measurement, 6 and 24h after THAPSI treatment (light blue) compared with DMSO (red), in SHI-1 cells. (**B**) Histogram showing cell death (Annexin V+, PI+ and Annexin V+/PI+) of SHI-1 and HL60 cells, 6 and 24h after treatment, compared with DMSO value. Data are presented as mean ± SEM. *$p < 0.05$. MFI: mean fluorescence intensity.



SUPPLEMENTAL FIGURE 18. TDZ analogues formulae. Analogues were obtained by different substitutions at the heterocyclic N-piperidine.

SUPPLEMENTAL VIDEOS:

Supplemental Video 1.

Cytosolic Ca^{2+} levels upon TDZ treatment, measured by 2-photon microscope, in SHI-1 cell line.

Supplemental Video 2.

Intracellular Ca^{2+} upon TDZ treatment in primary t(6;11) cells, measured by 2-photon microscope.