Inhibitors of ADAM10 reduce Hodgkin lymphoma cell growth in 3D microenvironments and enhance brentuximab-vedotin effect

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SUPPLEMENTARY MATERIAL

Inhibitors of A DisintegrinAnd Metalloproteinases-10 reduce Hodgkin lymphoma cell growth in 3D microenvironments and enhance brentuximab-vedotin effect.

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SUPPLEMENTARY METHODS

Cell cultures. The HL cell line L428, from pleural effusion, and L540, from bone marrow of classic Hodgkin lymphoma (HL) patients (DSMZ GmbH, Braunschweig, Germany) and the RS773 cell line obtained from a HL lymph node (LN) were maintained in RPMI 1640 complete medium supplemented with 10% FCS and 2mM glutamine (GIBCO, Thermo Scientific).[14] The LN-MSC773, LN-MSC16412 and LN-MSC23724 were obtained from HL patients lymph nodes (LN) and cultured in MEM-αcontaining desossiribonucleotidessupplemented with 2mM glutamine, 1% penicillin/streptomycin (GIBCO, Thermo Scientific) and 1% Chang medium (Irvine). Stabilization of primary HL and MSC cultures was performed as described.[14] Conventional co-cultures were performed between HL cell lines (10^4 cells/well) and LN-MSC (10^3 cells/well) at the ratio of 10:1 in flat bottom multiwell plates, with six replicates for each experimental condition.

*ADAM10 inhibitors.*LT4 and MN8, showing a IC_{50} selective for ADAM10, FC130, FC143, FC410with a IC_{50} selective for ADAM17, or MN8 conjugated with a linker followed by cyanine 5.5 (Cy5.5, CAM36) were synthesized and characterized as published.[16,17,46,47] As reported in our previous publications, LT4 and MN8 arylsulfonamido-based hydroxamates that inhibit ADAM10 by interacting with its catalytic domain and chelating the zinc ion, critical for both substrate binding and cleavage.[17,30] In vitro enzymatic activity of these compounds (Suppl. Table 1) shows that LT4 is a nanomolar inhibitor of ADAM10 (IC_{50}=40 nM) with a 37-fold selectivity over ADAM17 (IC_{50}=1500 nM), inactive on MMPs (MMP-1, -9 and -14) at 10μM. MN8 is more active than LT4 on ADAM10 (IC_{50}=9.2 nM) but less selective over ADAM17, still retaining selectivity over MMPs.Therefore LT4 can be considered selective for ADAM10 vs ADAM17 in low concentrations, up to 2μM, but it can be considered selective for ADAMs over MMPs also at 10 μM.

Compound ST178, a Cy5.5-*N*-isopropyl amide unable to bind ADAM10, was used as a negative control for the fluorescent probes (not shown).[17,30] The ADAM10 inhibitors used on the different HL cell lines, or on isolated LN-MSC, at 10μM concentration for 24h (and the solvent DMSO at the same dilutions and time points) did not exert direct toxic effect, assessed evaluating the mitochondrial potential upon staining with the dual emission fluorescent probe JC-1 (Molecular Probes, Life Technologies Italia, Monza),[30] even in 3D cultures. The reference commercial compound GI254023X (from now on GIX, Sigma-Aldrich) was used for comparison.All inhibitors were used in functional assays at the concentrations of 10μM or at serial dilutions from 10 to 1μM, as indicated in the Results section. See also Suppl. Table 1 for further characterization of each compound.

In preliminary experiments, performed under conventional culture conditions, the HL cells RS773 or L428 or L540 ($4x10^5$) were cultured for 96h (time point chosen on the basis of pilot experiments at 48, 72, 96h, not shown) with the control vehicle DMSO, LT4, MN8 or the commercial inhibitor GIX (10 to 2.5μM) and ATP content was measured as described in the corresponding paragraph. In

addition, 200µl cell suspension were collected from each culture well and the number of cells/µl counted with the MACS Quant Analyzer 10 (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).In some experiments on repopulated scaffolds (see the specific section) 10µM LT4 or MN8 were used in combination with the anti CD30 brentuximab-vedotin (BtxVed, 20 to 2µg/ml, left-over of the infused drug, obtained from the Pharmacy of the Policlinico San Martino, Genoa, Italy).

*Spheroids generation and culture.*Mixed spheroids of LN-MSC16412 and HL cells were prepared according to the method described with some modifications.[31] Briefly, 2x10^4 LN-MSC16412 were seeded in flat-bottom 96-well plates (Ultra-Low attachment plates, Corning®Costar®, NY, USA) with the appropriate culture medium as above. On day 4, when all LN-MSC16412 were alive, on the basis of ATP content measured on parallel samples, and the diameter of spheroids was of about 200µm, 2x10^5 HL cells were added to the cultures, and the medium replaced with fresh RPMI 1640 complete medium, to favor HL cell growth over LN-MSC. After 2 days, 10µM LT4 or MN8were added and the cultures were kept at 37°C, 5% CO_2 humidified atmosphere for further 4 days. Generation of spheroids was monitored along time, and proliferation and dimension (perimeter, area and volume) were analyzed in each culture well 48h, 72h, or 96h after addition of the inhibitors. At least triplicates were analyzed for each culture condition and the number of spheroids measured is indicated in each figure (a minimum of 50 single spheroidsfor each of at least three independent experiment).[31]

Images and measurement of spheroid size. Cell cultures were analyzed with Olympus IX70 bright field inverted microscope equipped with a CCD camera (ORCA-ER, C4742-80-12AG, Hamamatsu, Japan), associated to the CellSens software (version 1.12, Olympus, Tokyo, Japan). Images were taken with 10x objective NA 0.30 or 20x objective NA 0.40 (100x or 200x magnification). After calibration of the plate, a grid corresponding to each well was created and analyzed; at least five focal points were taken for each well of interest to obtain optimal images to be measured. Images of the entire well were then takenthrough an automated x-y axis motorized table SCAN IM 120x100-

2mm (Marzhauser WetzlarGmBH, 35579 Wetzlar, Germany). Spheroids weremeasured by applying the "Count and Measure" tool of the CellSens software, after creating circular regions of interest (ROI) that represent the spheroid perimeter. Data were then transferred to GraphPad Prism computer software (version 5.03) for subsequent statistical analysis and generation of graphs, as described.[30] To avoid counting and measuring very small spheroids or single cells or small cell/debris aggregates, only spheroids with a diameter greater than 50μm were selected.

*LN-matrix scaffold preparation.*LN from classic HL patients wereobtained from the Unit of Pathology, IRCCS Policlinico San Martino, Genoa, under conventional diagnostic procedures, provided informed consent and approval by the institutional ethical committee (IRB approval 0026910/07, renewal 03/2009, and 14/09/15). Extracellular matrix (ECM) from the LN was prepared according to a decellularization protocol previously described.[31] Some experiments were performed using, as scaffolds, the Avitene™ microfibrillar collagen sponges (3mm thick, Davol Inc., Warwick, RI, USA) washed twice in PBS and soaked o.n. in complete culture medium before use.

3D cultures of repopulated scaffolds. 3D cultures were performed by suspending $2x10^5$LN-MSC16412 in 50μl of culture medium and seeding onto a fragment of ECM or onto Avitene™ sponge, cut with sterile biopsy punches (Kai Medical, Japan) into 5mm (3mm thick) fragments, dipped in 1ml MSC culture medium 1h before cell seeding. Samples (triplicates) were placed in a 24w plate and kept 2h at 37° in a 5% CO_2 humidified incubator, then 1ml of complete MSC medium was added and samples cultured for 48h, with medium replacement after 24h. On day 3, $4x10^5$HL cells (either L428 or L540) were added to each ECM/LN-MSC16412 or Avitene™/LN-MSC16412 scaffold (some experiments were performed with Avitene™ scaffolds repopulated with LN-MSC23274) and cultured for further 2 days before addition of ADAM10 inhibitors. At this time, 10μM LT4 or MN8 were added to the culture for additional 72h or 96h. Culture supernatants (SN) were recovered after 48h, 72h or 96h to determine either TNFα or soluble CD30 content.In other experiments, to enhance scaffold repopulation and favour lymphoma cell colonization of the

3D structures, a mixture of $2x10^5$LN-MSC16412 and $4x10^5$HL cells were seeded onto Avitene^{TM}scaffolds, kept 2h at 37° in a 5% CO_2 humidified incubator, and cultured in 50% MSC medium and 50% RPMI 1640 for 3 days. Then, 20 to 2µg/ml BtxVed was added, either alone or in combination with LT4 or MN8 and samples were cultured for further 96h or 120h. At the end of the indicated time periods, a selection of 3D cultures was embedded in paraffine for immunohistochemistry, immunofluorescence or scanning electron microscopy.

Confocal microscopy and Scanning Electron Microscopy (SEM). Samples of mixed spheroids were incubated with 10µM CAM36 for 1h at 37°C, followed by1µM Syto16 (ThermoFisher Scientific, Monza, Italy), seeded onto glass slides and analyzed by FV500 (FluoView Confocal Laser Scanning Microscope System, Olympus Europe GMBH, Hamburg, Germany) equipped with an Argon laser to excite carboxyfluoscein and a He-Neon red laser at 633nm to excite cyanine 5 dye associated to a IX81 motorized microscope (Olympus).Compound ST178 was used as negative control in parallel samples (not shown). Samples were observed with PlanApo 40x NA1.00 oil objectives and data analyzed with FluoView4.3b computer software (Olympus). Each image has been taken in sequence mode to avoid cross-contribution of each fluorochrome. Results are shownas bright field or pseudocolor images.Z-stack sections were taken every 2µm.

For SEM, matrices or Avitene^{TM} empty or repopulated scaffolds were fixed with 4% paraformaldehyde in phosphate buffer (PB) for 5h, postfixed in 1% osmium tetroxide in PB for 1h, dehydrated in ascending degree of ethanol (70-80-95-100%), dried for 30min in xylene and embedded with paraffin after evaporation of xylene. Serial 10µm thick sections (Leica microtome) were immersed in xylene for 2h and then hydrated in descending degree of ethanol (100-95-80-70%) and H_2O. Ultrathin sections were collected on glass cover slips, mounted on aluminium stubs and sputter-coated 3min with gold.Morphology and ultrastructure were investigated by SEM on a Hitachi TM3000 Benchtop SEM instrument operating at 15kV acceleration voltage.

Immunohistochemistry (IHC) and immunofluorescence (IF) .3D cultures were fixed in HistoChoice (Amresco, Solon, OH) o.n. at 4°C. After dehydratation in ethanol, samples were clarified in xylene

and paraffin embedded. For each scaffold 5µm serial sections were cut up to a depth of about 50µm, dried o.n. at 37° C and analyzed by IHC or IF. IHC was performed on repopulated ECM after treatment with Peroxo-Blok (Novex, Life Technologies) to quench endogenous peroxidase, followed by Ultra Blok reagent (Ultravision Detection System, BioOptica, Milan Italy). The following antibodies were added: polyclonal rabbit anti-Ki67 antiserum (1:100, Ventana Hoffman-La Roche Ltd.), anti-CD30mAb (2µg/mL, Ventana), polyclonal rabbit anti-TGII antiserum (1:100, Thermo Scientific), the rabbit monoclonal antibody (mAb) anti-caspase-3 (1:1000, Cell Signaling) and an isotypic unrelated antibody as negative control (DakoCytomation). Biotinylated goat anti-mouse (Biot-GAM) or anti-rabbit antiserum (Biot-GAR, BioOptica) was then added, followed by HRP-conjugated avidin (HRP-Av, Thermo Scientific) and the reaction developed using 3,3′-diaminobenzidine (DAB, Sigma) as chromogen. Slides were counterstained with hematoxylin, cover-slipped with Eukitt (Bio Optica), and analyzed under a Leica DM-MB2 microscope equipped with a charged coupled device (CCD) camera (Olympus DP70 with a 40x objective). To standardize cell staining and analyses, the sections from repopulated Avitene™ sponges were processed with the above-mentioned antibodies using the BOND RXm (Leica) autostainer.

IF was performed with the anti-CD30 Ber-H2 mAb and the anti-Ki67 antiserum, using the BOND RXm (Leica), according to the manufacturer instructions (Leica Biosystems) as follows: (1) deparaffinization with Bond Dewax Solution (Leica Biosystems) at 72°C for 30min; (2)incubation with a cocktail of mouse anti-human CD30 mAb (1:2 dilution, Ventana) and rabbit anti-human Ki67 (1:5, Ventana) for 45min at 37°C; (3) washing with Bond Wash for 4min; (4) incubation with a cocktail of anti-mouse Alexa Fluor 594 (1:1000, ThermoFisher) and anti-rabbit-Alexa Fluor 488 (1:1000, ThermoFisher) for 30min at 37°C; (5) washing with Bond Wash and distilled water for 4min. Then, slides were counterstained with DAPI at 5µg/ml in PBS for 30sec and mounted withFluoromount ™ (Diagnostic BioSystems).

Scanning and computerized imaging. Slides obtained as described above, were analyzed with the Aperio VERSA (IF) or the Aperio AT2 (IHC) Digital Pathology Scanner (Leica Biosystems). IF

images were captured at 20x enlargement in three channels: DAPI for cell nuclei, CD30-Alexa Fluor 594 for membrane stain, and Ki67 Alexa Fluor 488 to identify cycling cells. At least 3 sections/scaffold, cut at 15µm distance, were acquired. Score classification criteria were defined according to the manufacturer instruction. Image data were then analyzed with the Aperio Cellular IF Algorithm (Leica Biosystems) and the number of $CD30^{+}/Ki67^{+}$cells was calculated accordingly.[33] The method of recognition and count of single and double positive cells is described in Suppl.Fig.S1. IHC images (3 section/scaffold cut at 15µm distance) were analyzed with the Genie recognition pattern (Leica Biosystems) and the nuclear count V9 macro of Image-Scope software (Leica Biosystems) trained and combined to obtain the count of caspase-3^{+} cells, excluding the biomaterial (scaffold); HL cells were recognized by morphology in HE staining.[33]

ATP, LDH, soluble CD30, TNFα and glucose measurement. ATP content was determined using the CellTiter-Glo® Luminescent Cell Viability Kit (Promega Italia Srl, Milan, Italy) following manufacturer instruction using the luciferase reaction consisting in mono-oxygenation of luciferin catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen.Luminescence was detected with the VICTORX5 multilabel plate reader (Perkin Elmer, Milan, Italy) expressedas luminescence arbitrary units (a.u)[33]LDH was measured after lysing cells with 10% NP40 in PBS, using the CytoTox96 Kit (Promega) according to the manufacturer instructions; reaction was developed for 30min at RT in flat-bottom 96w plates, read at 490nm and results expressed as $O.D._{490}$. In some experiments, cells were harvested and counted at the MACS Quant Analyzer 10 (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).Conditioned culture media were collected as supernatants (SN) from cell cultures (either LN-MSC or HL cell lines, spheroids or 3D cultures), untreated or after 48h, 72h or 96h exposure to the ADAM10 inhibitors (LT4, CAM29, 10µM). Soluble CD30 was measured by thespecific ELISA detection kit (Human CD30 Picokine ELISA kit) from Boster Bio (TebuBio, Milan, Italy). Plates were developed with 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, Milan, Italy) and read at a OD_{450}nm with the VICTORX5 reader. Results are expressed as pg/ml and referred to a standard curve obtained with the standard

CD30 contained in the specific kit. TNFαwas measured after treatment for 1h of SN with 1N HCl followed by 1N NaOH with a TNFα specific kit (PeproTech, London, UK). Results normalized to a standard curve are expressed as pg/ml.[34]Glucose was measured in the SN recovered from repopulated scaffolds every 24h up to 120h, untreated or treated with BtxVed and/or LT4 or MN8 as above, with the D-glucose Assay Kit (Megazyme Int. Ltd., Wicklow Ireland) and referred to a standard curve. Glucose was also measured in the SN recovered at 48h or 95h from 2D cultures of either L428 or L540 cells in culture medium containing serial dilutions of DMSO, 1:1000 (10^3) dilution (used as vehicle for ADAM10 inhibitors) showing no effects (Suppl.Fig. S5F and G).

*Statistical analysis.*Data are presented as mean±SEM or ±SD. Statistical analysis was performed using two-tailed unpaired Student's *t* test, with Welch correction, using the GraphPad Prism software 5.0. The cut-off value of significance is indicated in each figure legend.

LEGEND TO SUPPLEMENTARY FIGURES

Suppl. Figure S1. Effects of ADAM10 inhibitors on ATP contentand proliferation of HL cells.

Panel A: RS773 (panel Aa) or L428 (panels Ab) or L540 (panels Ac) cells ($4x10^5$) were cultured for 96h in medium containing DMSO solvent at the same dilution of the highest inhibitor concentration (1:1000) or with LT4, or MN8 or GIX (serial dilutions from 10µM to 2.5µM). Then, 100µl of cell suspension were collectedfrom each culture well and ATP was measured with the CellTiterGlo kit. Results are expressed as luminescence arbitrary units (a.u./10^3cells) and are the mean±SD of triplicates from three independent experiments.*$p<0.01$ and **$p<0.001$ vs DMSO.Panel B: RS773(panel Ba) or L428 (panel Bb) or L540 (panel Bc) cells (10^5)were cultured for 96h in the absence (DMSO 1:1000 or medium in the graphs) or presence of LT4 (1), or MN8 (2) or GIX (3), all at 5µM. Then 200µl cell suspension were collected from each culture well and counted with a counted at the MACS Quant Analyzer 10 (Miltenyi Biotech GmbH). Results are the mean±SD of triplicates from three independent experiments.*$p<0.01$ vs medium or vs DMSO.

Suppl. Figure S2. Reactivity and localization of the ADAM10 inhibitor CAM36 (Cy5.5-MN8) in mixed spheroid. Mixed spheroids of LN-MSC16412 ($2x10^5$) and L540 cells ($4x10^5$)were prepared as described.[31] Panel A: confocal microscopy of a mixed spheroid incubated with 1µMSyto16 (blue) to stain nuclei and 10µM CAM36 (red): single pseudocolor or merged images and bright field as indicated. Slides were analyzed under a FV500 confocal Laser Scanning Microscope System Olympus Europe), with PlanApo 40x NA1.00 oil objectives and data analyzed with FluoView4.3b software (Olympus). Imageshave been taken in sequence mode to avoid cross-contribution of each fluorochrome and shown in pseudocolor or bright field.Bar: 100µm.Panel B: Images of four z-stack sections taken in sequence mode every 2µm and shown in pseudocolors.

Suppl. Figure S3. ADAM10 inhibitor effects on LDH release, ATP contentand size of LN-MSC spheroids. Spheroids of LN-MSC16412 ($2x10^5$) were prepared as described[31]and cultured for 48h, 72h, 96h. Panels A-C: at the indicated time points, 100µl SN (for LDH) or cell suspension (for ATP content) were collected from each culture well and LDH (panel A) or ATP (panel B) measured by specific assays. Results are expressed as $O.D._{490}$ (a.u/10^4cells, panel A) or luminescence arbitrary units (a.u./10^4cells, panel B) and are the mean±SD of quadruplicates from three independent experiments. Panel C: spheroid images weretaken from each culture well and dimension (area: $µm^2$) evaluated as described in Materials and Methods. At least triplicates (mean±SD) were analyzed for each culture condition and a minimum of 150 single spheroids for each independent experiment.Panel D-E: LN-MSC16412 spheroids were cultured in the absence (Nil) or presence of 10µM LT4 or MN8 for 48h, 72h, or 96h and spheroid size (panel D, area: $µm^2$) calculated as in panel C. At the indicated time points, 100µl of cell suspension were harvested and ATP content (panel E) measured as described in Materials and Methods; results are expressed as luminescence arbitrary units (a.u./10^4cells) and are the mean±SD of quadruplicates from three experiments.

*Suppl. Figure S4. Automated quantification of $CD30^+Ki67^+$ HL cells.*Panel A: 3D culture on a representative Avitene™ sponge repopulated by LN-MSC23274 and L428 cells. Sections (5 µm)

from paraffin-embedded repopulated scaffolds were stained with DAPI for nuclei (blue), anti-CD30 mAb followed by anti-mouse Alexa Fluor594 (red) for HL cells and anti-Ki67 polyclonal antibody followed by anti-rabbit Alexa Fluor488 (green) to identify cycling cells. Images were taken with the Aperio VERSA Digital Pathology Scanner (Leica Biosystems) with a 20x objective. Panel B: application of Aperio IF algorithm (Leica Biosystems) to digitalize images. Final mark-up is shown (enlargement on the right). The algorithm automatically detects cells and classifies single CD30 positive cells (purple spots), negative cells (grey spots) and double positive CD30/Ki67 cells (green spots). Right images in A and B: enlargements of the squares in left images.

Suppl.Figure S5 .Comparison between sequential and mixedLN-MSC/HL cell scaffold repopulation. Avitene^{TM} scaffolds were repopulated either in sequential mode (panel A, LN-MSC16412 cultured for 48h then L428 cells for further 96h) or with a mixture of LN-MSC16412 and L428 cells from time 0 to 96h (panel B). SN were recovered every 24h to measure glucose content (C). A and B images: scaffold sections (5µm) stained with HE and analyzed with the AperioAT2 Digital Pathology Scanner (Leica Biosystems): on the right pictures of the whole scaffold. Bars as indicated. Panel C: Glucose measurement in the SN recovered from A vs B co-cultures, expressed as µg/0.01ml. Panels D andE: Glucose evaluation in the SN recovered from LN-MSC16412+L428 (D) or LN-MSC16412+L540 (E) repopulated scaffolds exposed for the indicated periods of time (24h to 96h) to either 20 or 2µg/ml BtxVed. Panels F and G: Glucose measurement in the SN recovered at 48h or 95h from 2D cultures of either L428 or L540 cells in culture medium containing serial dilutions of DMSO, 1:1000 (10^3) dilution showing no effects. Glucose was measured with the specific kit (Megazyme) in culture SN recovered every 24h and data are expressed as percentage consumpion referred to glucose content in fresh culture medium; mean±SEM from 3 samples/time point. *p<0.05 vs Nil; **p<0.01 vs Nil and vs BtxVed 20µg/ml.

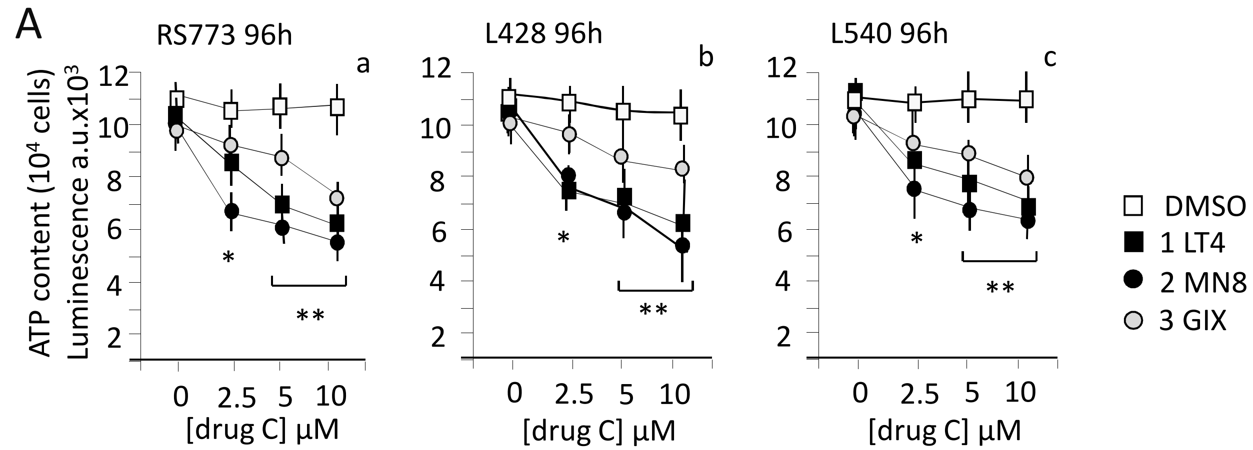
Suppl. Table 1. Effect of different sulfonamido-based hydroxamate compounds on TNFα shedding and ATP content in HL cell lines.

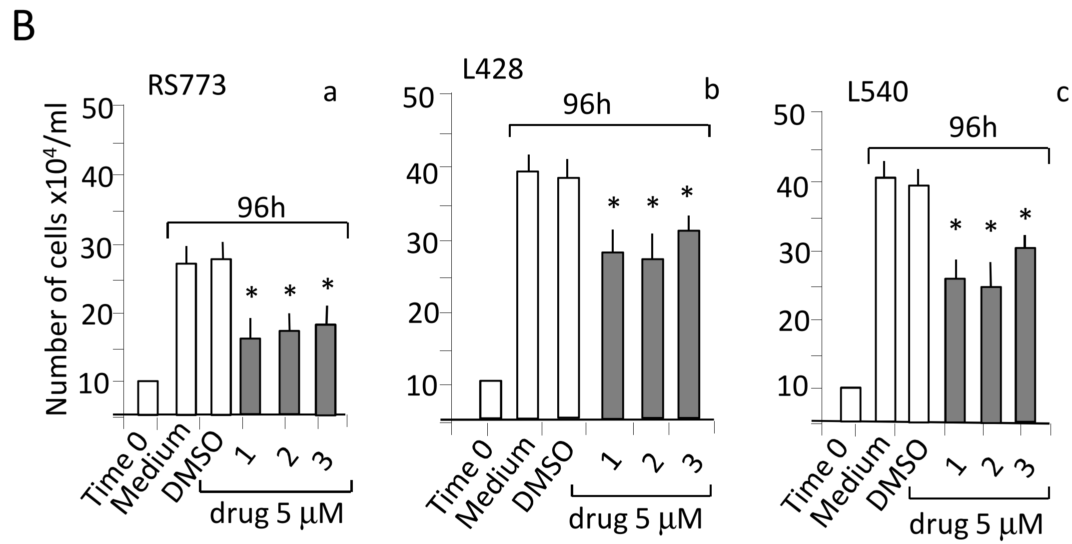
	L428 IC_{50} (μM)		L540 IC_{50} (μM)		In vitro IC_{50} (nM)	
Compound	TNFα	ATP	TNFα	ATP	ADAM10	ADAM17
MN8	7	5	7	7	9.2	90
LT4	10	**7**	10	10	40	1500
FC410 (20)[a]	0.1	15	1	10	300[a]	11[a]
FC143 (17)[b]	0.1	20	0.5	20	240[b]	1.6[b]
FC130 (18)[b]	0.1	50	2.5	50	190[b]	0.70[b]
GIX	5	20	10	15	27	860

IC_{50} (μM) evaluated on the basis of mean values of 3 experiments/cell line, each in triplicate: compound concentration needed to inhibit by 50% either TNFα measured at 48h in the extracellular medium or intracellular ATP content measured at 96h. In vitro enzymatic activity (IC_{50}nM) was evaluated by a fluorometric assay on the purified enzymes as reported and are the average of three determinations with a SD<10%.[17]

[a]Data from compound **20** published in 10.1021/jm4011753.[46]

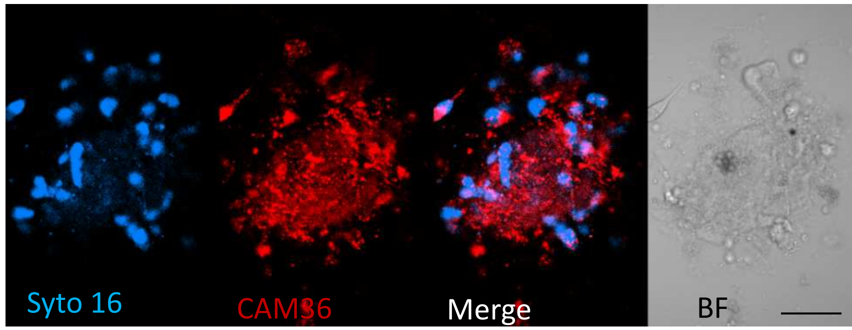
[b]Data from compounds **17** and **18** published in 10.1021/jm901868z.[47]





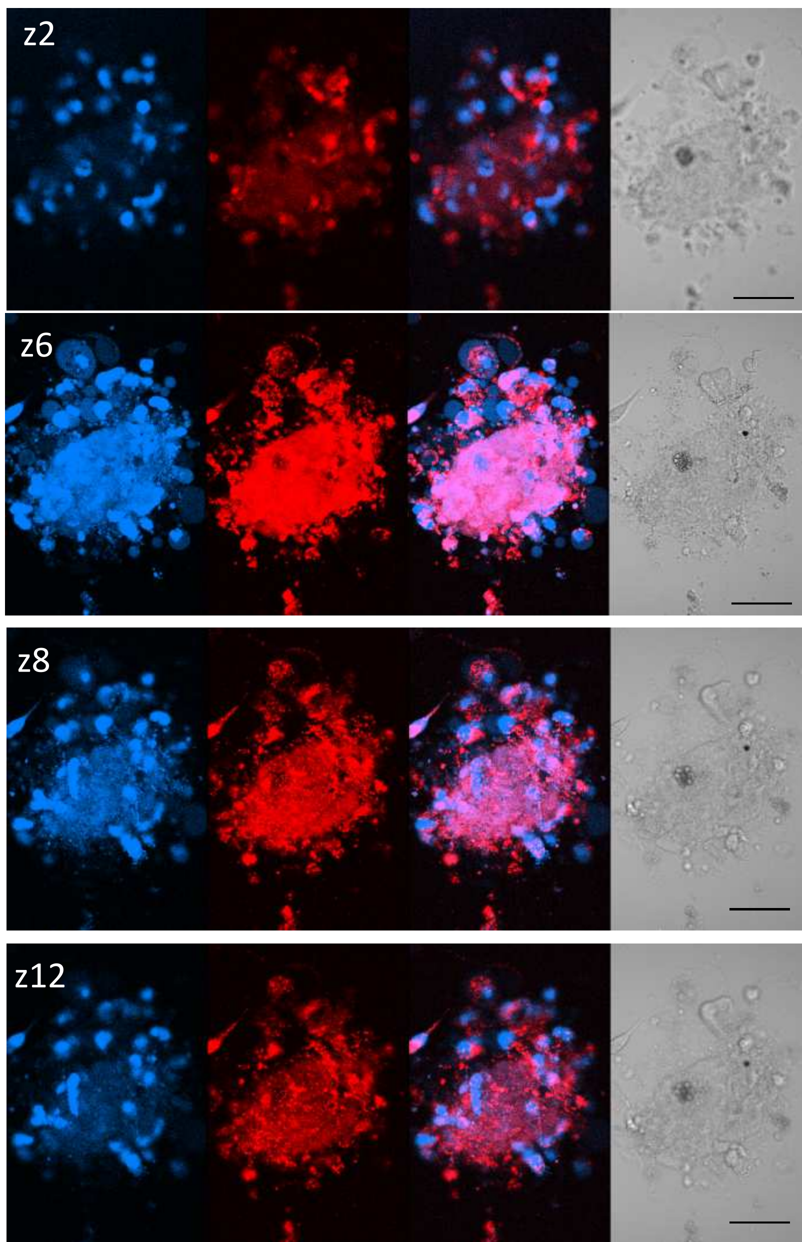
Suppl. Fig. 1

A LN-MSC16412+L540

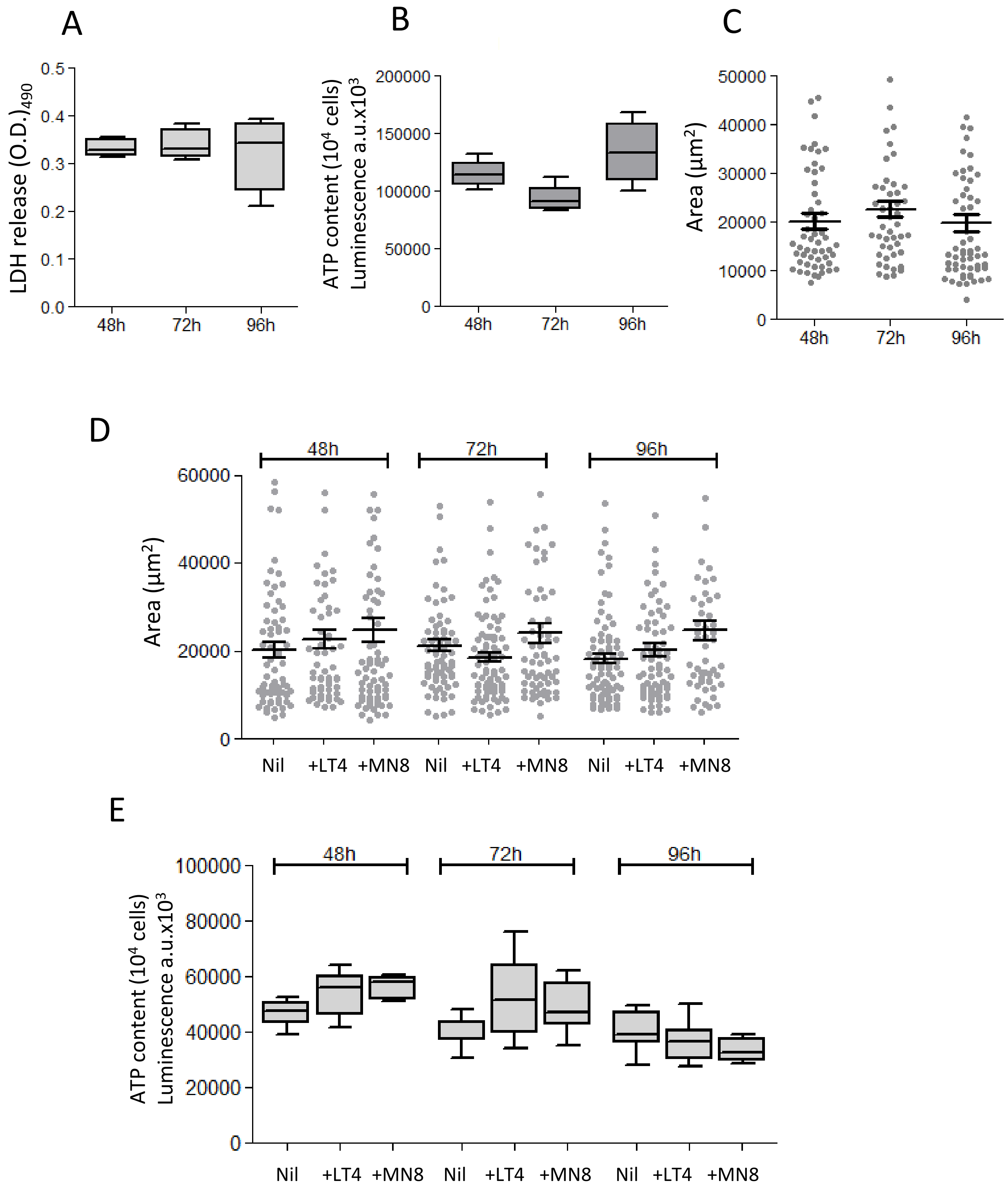


LN-MSC16412+L540 z-stacks

B

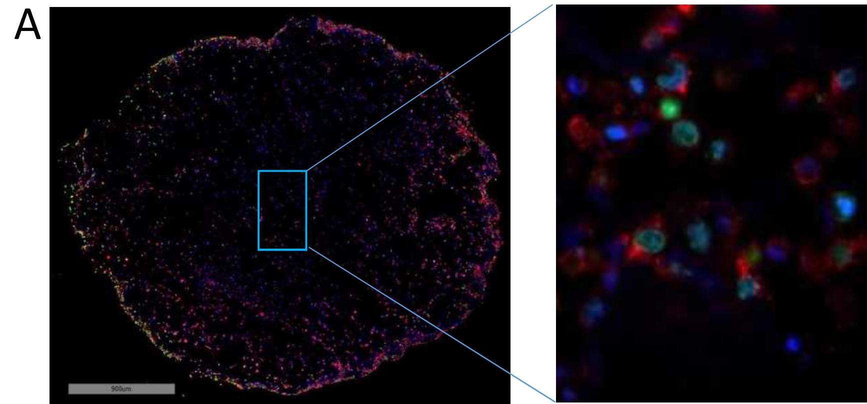


Suppl. Fig.2



Suppl. Fig.3

A



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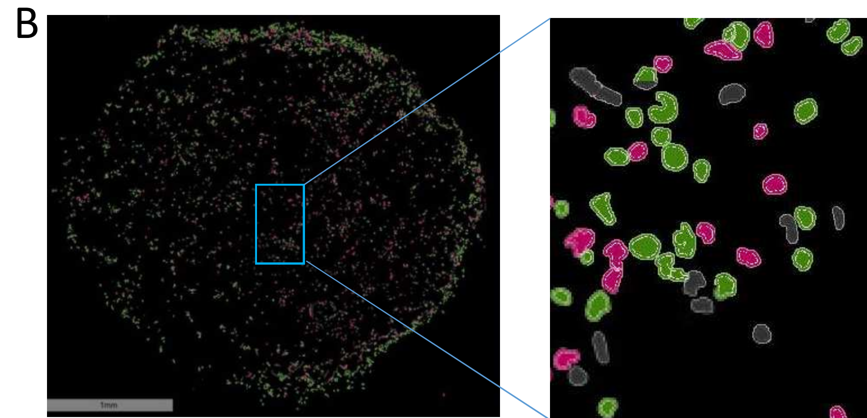
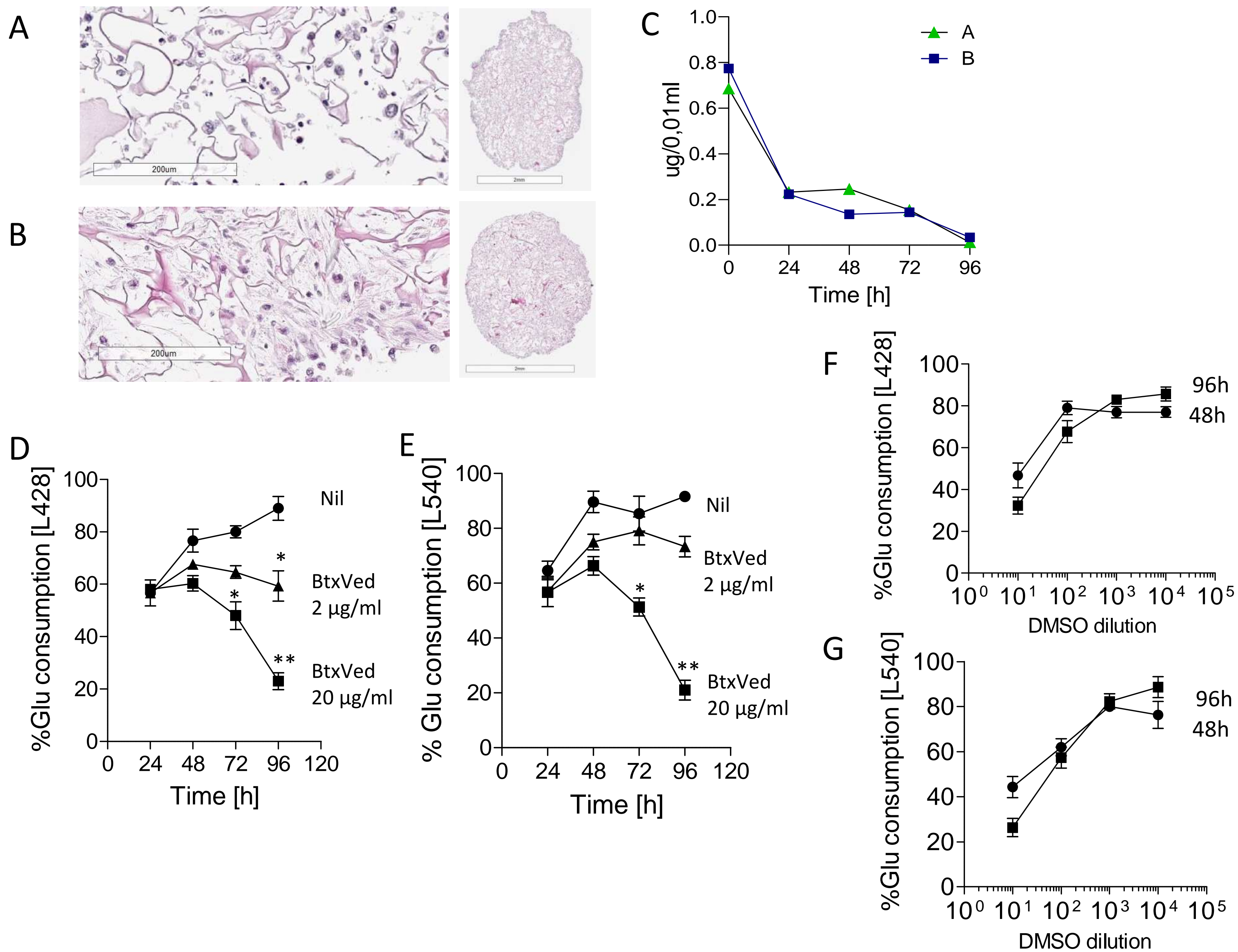


Suppl. Fig.4



Suppl. Fig.5