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Deruxtecan-based antibody-drug-conjugates induce senescence in HER2-positive breast cancer

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ABSTRACT

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Breast cancer (BrCa) represents one of the most common malignancies and the leading cause of cancer-related deaths in women worldwide. Despite the advances in therapeutic treatments, *de novo* and/or acquired resistance still represents a major clinical challenge. Recently, a new class of therapeutic agents has been approved for the treatment of advanced/metastatic BrCa: antibody-drug conjugates (ADCs). Trastuzumab-deruxtecan (T-DXd) has recently become the prevalent treatment in different clinical settings because of its improved efficacy.

Here, we identified two mechanisms of resistance: i. reduction of the payload target (Topoisomerase I) and ii. induction of sustained senescence. This phenotype correlates with increased production of reactive oxygen species (ROS), metabolic rewiring and activation of the p53/p21 axis, and is associated to the senescence-associated secretory phenotype (SASP). Furthermore, dissection of the relative contribution of the antibody (Trastuzumab) vs the payload (DXd) component of the ADC to the action of T-DXd showed that DXd alone is sufficient to promote senescence and its downstream effects. We further corroborated these conclusions exploiting another DXd-based ADC (Datopotamab-DXd) and found that DXd-based drugs promote Topoisomerase I downregulation and senescence.

Altogether, these findings provide the rationale for the treatment of breast cancer patients resistant to DXd-based ADCs with senolytic or senomorphic agents.

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Key words:

HER2, breast cancer, Trastuzumab, Trastuzumab-deruxtecan, deruxtecan, Datopotamab-deruxtecan, senescence, SASP, 3D bioprinting, mitochondria

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BACKGROUND

Breast cancer (BrCa) represents one of the most common malignancies and the leading cause of cancer-related deaths in women worldwide. Despite the advances in therapeutic treatments, *de novo* and/or acquired resistance still represents a major clinical challenge. Different subtypes of BrCa have been classified based on the expression of specific markers, namely estrogen receptor (ER)-positive, progesterone positive (PgR)-positive, Epidermal Growth Factor Receptor type 2 (HER2 or ErbB2)-positive and triple-negative (TNBC). Approximately 15% of BrCa display HER2 gene amplification or protein overexpression and show high risk of relapse and shorter survival[1]. Standard of care for HER2-positive patients comprise a combination of chemotherapy and targeted humanized monoclonal antibodies (Trastuzumab and Pertuzumab), administered in either neoadjuvant or adjuvant setting, regardless of the levels of expression of estrogen and progesterone receptors in the tumour. Recent data from clinical trials have shown that the presence of ER/PgR correlates with residual disease after neoadjuvant treatment and that combination with hormone receptors (HR)-targeted treatments is useful [[2], [3] and [4]], due to the crosstalk between HER2 and ER signalling pathways [reviewed in [5] and [6]]. Furthermore, tumour microenvironment (TME) has been reported to play critical roles in tumorigenesis, progression, invasion and therapy resistance in BrCa[7].

In the last decade, a new class of therapeutic agents has been approved for the treatment of advanced/metastatic BrCa: antibody-drug conjugates (ADCs). In particular, Trastuzumab emtansine (Kadcyla®, T-DM1) that comprises an HER2-targeted antibody, a non-cleavable linker and a microtubule disrupting agent as the payload, and Trastuzumab deruxtecan (Enhertu®, T-DXd), which is composed of a HER2-targeted antibody, a cleavable linker and a Topoisomerase I inhibitor as payload. The advantages of these therapeutic agents are to strengthen the efficacy of the treatment while reducing the adverse effects usually associated to conventional/systemic chemotherapy as well as to allow the release of the payload from targeted to adjacent cells, thus increasing the overall anti-tumour effect [reviewed in [8]].

T-DXd has become the prevalent treatment in different clinical settings because of its improved efficacy as compared to state-of-the-art treatment and/or other ADCs. Indeed, T-DXd has shown anti-tumour activity in patients with HER2-positive metastatic BrCa resistant to T-DM1 in phase 1 and phase 2 trials (DESTINY-Breast01); it has also demonstrated

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superior progression free survival (PFS) as compared to T-DM1 in patients with HER2-positive advanced BrCa in a phase 3 study (DESTINY-Breast03) regardless of HR status, prior treatment and the presence of brain metastases, at all time points of the follow up [reviewed in [9]]. The DESTINY-Breast12 study has proven substantial and durable T-DXd efficacy on brain metastases in previously treated BrCa metastatic patients[10] and the DESTINY-Breast06 trial has shown T-DXd effectiveness in hormone receptor-positive, HER2-low or -ultralow BrCa metastatic patients[11]. On the other hand, T-DM1 has demonstrated poor outcomes in patients showing reduced HER2 expression or heterogeneity in intra-tumour HER2 expression levels, whereas T-DXd displayed benefits in terms of PFS and overall survival on both HER2-expressing and HER2-low tumours. Finally, a trial comparing T-DM1 and T-DXd effectiveness in patients with HER2-positive primary BrCa, showing residual disease after neoadjuvant treatment, is actually ongoing [DESTINY-Breast05[12]].

Considering the results obtained in clinical trials with the use of T-DXd and other ADCs, a new promising strategy for treatment of advanced BrCa is emerging. Nonetheless, primary and acquired resistance can still occur. Preliminary data from non-small cell lung cancer (NSCLC) patients treated with T-DXd indicate that resistance may arise from either the payload (due to the loss of the Topoisomerase I target) or the antibody [due to the appearance of secondary mutations within the HER2 domain IV, which is the binding site for trastuzumab][13]. Also in BrCa patients, either reduction or mutation in HER2 have been reported at resistance [[14] and [15]]. Besides, few *in vitro* studies have investigated the mechanism of action of and/or resistance to T-DXd.

Based on the structure of ADCs, their mechanism of action could depend either on the antibody (Ab) component (maintaining the effects of the naked Ab), on the cytotoxic activity of the payload or on the combined effect of the two components on the targeted tumour cells (intracellular activity). In addition, ADCs can also exert an extracellular action on neighbouring cells via the release of the payload after intracellular cleavage of the linker (bystander effect), which is mediated by the proteases localised in lysosomes. This event represents an important component of the overall mechanism of action of ADCs (especially in tumour displaying low levels or a heterogeneous distribution of the Ab target), but also a potential risk of off-target effects on either stromal or immune cells [reviewed in [9] and [16]]. Thus, we exploited the 3D bioprinting strategy to develop a new 3D matrix-embedded model to generate heterotypic co-cultures comprising tumour and stromal cells (fibroblasts and

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endothelial cells) with the final aim to unravel potential drug effects on the different cell types present in the TME.

Our results suggest that reduction of the levels of the payload target (Topoisomerase I) represents a general response of tumour cells to T-DXd treatment. Moreover, we report that T-DXd triggers senescence and senescence-associated secretory phenotype in HER2-positive breast cancer cells via the activation of the p53/p21 axis. Furthermore, we found that the mechanism of action of T-DXd relies on the payload, and only partially on the Ab component of the ADC. Interestingly, we showed that also Datopotamab-DXd [an ADC exploiting deruxtecan as payload and Datopotamab as antibody, targeting Trophoblast cell surface antigen 2 (TROP2) receptor] triggers: i. induction of p53/p21 axis; ii. senescence and SASP; iii. lysosomal and mitochondrial morphological alterations; iv. metabolic rewiring of HER2- and TROP2-positive BrCa cells. Altogether, these findings: i. support the idea of a shared mechanism of action of DXd-based ADCs; ii. suggest a common unprecedented mechanism of resistance; and iii. provide the rationale for treatment with senolytic or senomorphic agents to by-pass resistance.

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METHODS

Reagents and antibodies

Primary antibodies were purchased from Cell Signalling Technologies (Danvers, MA, USA) or Abcam (Cambridge, UK) unless otherwise stated. LIVE/DEAD™ Fixable Green Dead Cell Stain Kit, AlamarBlue™ Cell Viability Reagent, Alexa 488, Alexa 546, and Alexa 647 secondary antibodies and Hoechst 33342 were obtained from Molecular Probes (Life Technologies, by Thermo Scientific, Waltham, MA, USA). MTT, Glycine, saponin, NH_4Cl, bovine serum albumin (BSA), Triton X-100 were purchased from Sigma Aldrich (by Merck, Kenilworth, NJ, USA). GelXA LAMININK 411 and GelMA Fibrin were obtained from CELLINK (CELLINK AB, Gothenburg, Sweden). All cell culture reagents were from Gibco (by Thermo Scientific, Waltham, MA, USA) unless otherwise specified. All chemical reagents were of analytical grade or higher, and purchased from Sigma Aldrich unless otherwise stated.

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Cell culture

BT474 (HER2- and ER-positive BrCa cells), SKBr3 (HER2-positive BrCa cells) and BJ (fibroblasts) cells were purchased from American Type Culture Collection (ATCC®, Manassas, VA, USA); HuVEC (endothelial) cells were obtained from Lonza (Basel, Switzerland). BT474 cells were grown in the continuous presence of 200 μg/mL TZ for 10 months to generate a model system of TZ resistant cells (BT474-R), accordingly to [17], and tested in viability assays to verify their resistance to TZ treatment [Supplementary Fig.1a]. SKBr3-R were generated as described previously[17].

Tumour cells were grown as monolayers in high-glucose (4500 mg/L) DMEM supplemented with 2 mM GlutaMAX supplement, 50 IU/mL penicillin, 50 mg/mL streptomycin and 10% foetal bovine serum (FBS), whereas they were cultured as mammospheres in MammoCult™ basal medium supplemented with 10% MammoCult™ proliferation supplement (human serum), 4 μg/mL heparin and 0.48 μg/mL hydrocortisone for 10 days (STEMCELL technologies, Vancouver, Canada). BJ fibroblasts were grown in high-glucose (4500 mg/L) DMEM supplemented with 2 mM L-glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin and 10% foetal bovine serum (FBS). HuVEC cells were grown in Endothelial Cell Growth Base Media (EBM-2) supplemented with Endothelial Cell Growth Supplement containing

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optimized concentrations of human FGF basic, human LR3 IGF-1, human VEGF165, human EGF, Heparin, Hydrocortisone, L-Ascorbic Acid, Fetal Bovine Serum (EGM-2 media, Lonza). All cells, mammospheres and scaffolds were grown at 37°C in 5% CO_2 and at 98% humidity.

TZ (at a stock concentration of 21 mg/mL in saline solution), T-DM1 (at a stock concentration of 20 mg/ml), T-DXd (at a stock concentration of 20 mg/mL in saline solution) and Dato-DXd (at a stock concentration of 20 mg/mL in saline solution) were donated by the Pharmacy of the San Raffaele Hospital. DXd was obtained from MedChemExpress (Monmouth Junction, NJ, USA). TZ was used at a working concentration of 10 μg/m[18]. ADCs were used at a working concentration of 10 μg/mL to be compared with the naked antibody TZ. DXd was used at a molar dose equivalent to ADC concentration (considering drug-to-antibody ratio, DAR, equal to 8 for T-DXd). Drug treatment of scaffolds was refreshed every other day.

3D Bioprinted Scaffold Fabrication

We generated 3D models containing tumour cells only (BT474 or BT474-R) or multi-material and multi-cellular scaffolds with tumour cells and microenvironment, represented by HuVEC (endothelial) cells and BJ fibroblasts. Scaffold geometries were designed with Fusion 360 (Autodesk). For tumour-only scaffolds, cells were counted, centrifuged at 1000 RPM for 5 minutes and mixed with the bioink GelXA LAMININK 411 (CELLINK AB, Gothenburg, Sweden) in 1:10 cells:hydrogel ratio at a concentration of 10 and $5x10^6$ cells/mL of hydrogel for BT474 and BT474-R cells, respectively. Bioink mixed with the cells was then loaded in a cartridge and placed in the Bio X 3D bioprinter (CELLINK AB, Gothenburg, Sweden) in the temperature-controlled print-head set at 22°C for 15 minutes before printing. Tumour cells were printed with $5x5x1mm^3$ scaffold geometry, and the slicing process was directly made exploiting the Bio X slicer software, using a rectilinear pattern with 30% infill density. The Bio X was equipped with a 25G (250 μm) nozzle and the layer height was set at 0.25 mm. The pressure applied to the 3D bioprinting process is hydrogel/cells-dependent, a range of values around 12-20 kPa was used. Heterotypic co-cultures containing 3D scaffolds were designed as two concentric cylinders, intended to host microenvironment (fibroblasts) and tumour cells in the external layer ($6x1mm^3$, wall thickness: 1mm) made of GelXA LAMININK 411, and endothelial cells in the inner one ($3x1mm^3$), made of GelMA Fibrin (CELLINK AB, Gothenburg, Sweden). Bioprinting of complex 3D scaffolds was performed directly in 12- or 24-well plates at 5 mm/s deposition speed, with the same pressure range and nozzle size

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cited above. We printed sequentially only the outer layers, then we manually casted the inner compartment (30 µL/drop). The constructs were then crosslinked with 50 mM $CaCl_2$ (CELLINK AB, Gothenburg, Sweden) for 4 minutes at room temperature and UV light for 15 sec (λ = 405 nm)[19]. Scaffolds were washed once with Hank's Balanced Salt Solution (HBSS, EuroClone, Pero, Italy), according to the manufacturer protocol (CELLINK AB, Gothenburg, Sweden). MammoCult™ (STEMCELL Technologies, Vancouver, BC, Canada) medium (tumour-only cultures) or MammoCult™:EGM-2 medium mix (heterotypic co-cultures) was added to scaffolds.

Set up of growth conditions for 3D bioprinted model systems

To generate an embedded 3D model system, BT474 and BT474-R cells were printed in a biocompatible hydrogel to obtain cell-laden scaffolds, which also allow the generation of heterotypic structures comprising the stroma (namely fibroblasts and endothelial cells). Initially, we assessed the compatibility of bioinks with the cell types of interest. To this purpose, tumour BT474 and BT474-R cells, BJ fibroblasts and HuVEC endothelial cells were bio-printed in Cellink GelXA LAMININK 411 bioink and their growth followed for up to a month with regular assessment of their viability by Live/Dead™ staining. Tumour cells and BJ fibroblasts displayed the expected morphology and proliferated when grown in GelXA LAMININK 411 bioink (Supplementary Fig.1b), whereas endothelial cells did not survive (Supplementary Fig.1c). Thus, we tested Cellink GelMA Fibrin hydrogel to bioprint HuVEC cells, and found that it was suitable for endothelial cell growth (Supplementary Fig.1c). Since BT474 and BT474-R tumour cells display distinct growth rates, we tested different bioprinting concentrations of parental or resistant cells, and followed their proliferation in time by regular Live/Dead™ staining. We observed that $5x10^6$ BT474-R cells/mL after 14 days and $10x10^6$ BT474 cells/mL after 21 days of growth in scaffolds form structures of similar dimension and with comparable viability measurements (Supplementary Fig.1d-e). To perform long-term co-culture experiments, we tested three different growth medium for bioprinted scaffolds comprising tumour cells, fibroblasts and endothelial cells: MammoCult™ or MammoCult™ with the addition of growth factors of the endothelial-specific EGM™-2 formulation (BulletKit™) or EGM-2™:MammoCult™ (1:1 ratio). After 21 days, we observed no major differences in terms of tumour cells and fibroblasts growth/morphology, whereas endothelial cells showed better survival in the presence of EGM™-2 (Supplementary Fig.2a). Furthermore, we observed maintenance of viable cells

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for up to 6 weeks of culture, with retention of the identity markers HER2/EpCam for tumour cells, alpha-SMA for fibroblasts and CD31 for endothelial cells (Supplementary Fig.2b-c). We found the optimized ratio for tumour cell:fibroblast:endothelial cell to be 1:3:2 for parental BT474 and 1:6:4 for resistant BT474-R cells.

Survival assays

BJ fibroblasts and HuVEC endothelial cells were seeded in a 96-well plate at a density of 1000 or 2000 cells/well, respectively, in either DMEM (BJ) or EGM-2 (HuVEC) and allowed to grow for 24 h before treatment. Cells were treated with 10 µg/mL TZ, T-DM1 or T-DXd for 7 days, and cell survival was determined by MTT assay.

Samples were incubated with 2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich by Merck, Kenilworth, NJ, USA) for 4 h at 37°C, and lysed with 10% SDS in 0.01 M HCl to extract formazan for 24 h (Roche by Merck, Kenilworth, NJ, USA). The amount of MTT-formazan was determined by reading 570 nm absorbance with 630 nm absorbance as reference[20].

Scaffolds were incubated with AlamarBlue™ Cell Viability Reagent (Thermo Scientific, Waltham, MA, USA) diluted 1:10 with the scaffold culture medium (MammoCult™ or EGM-2: MammoCult™ 1:1 ratio) in a final volume of 1 ml per well for 24h at 37°C. After incubation, 100 µL of mix from each well were collected and transferred to a 96-well white plate for fluorescence quantitation with a Victor spectrophotometer.

BT474 and SKBR3 cells were seeded in a 96-well plate at a density of 10000 (parental and resistant SKBr3) or 5000/2000 (parental and resistant BT474) cells/well, respectively, in DMEM and allowed to grow for 24 h before treatment. Cells were treated with 10 µg/mL TZ, T-DXd, Dato-DXd or molar equivalent DXd for 7 days, and cell survival was determined by MTT assay.

Senescence quantitation

After drug treatment, tumour cells grown as monolayers were treated with Trypsin, counted and processed for senescence quantitation by means of the Senescence Detection kit (Abcam, Cambridge, UK), accordingly to the manufacturer instructions. Briefly, cells were fixed with the Fixative Solution III for 15 min at room temperature, and incubated with the

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Staining Solution Mix overnight at 37°C in a termostated room (with air CO_2 concentration, to avoid pH changes in the samples that would inhibit the enzymatic reaction). On the following day, cells were analysed with the ImageStream^X MKII System (Amnis, part of Cytek Biosciences, San Diego, CA, USA), a multispectral imaging flow cytometer that combines the information content coming from microscopy with the statistical power typical of flow cytometry. Brightfield (BF) and side scatter (SSC) images were collected with the 40X_0.75NA objective for at least 30.000 events for each sample. The IDEAS 6.2 software was used for the analysis.

First of all, cells were gated for single cells using area and aspect ratio features on the brightfield image. Gradient root mean square feature (RMS) on the BF image and intensity of the SSC image were then used to eliminate cells that were strongly out of focus and that had very low or absent scattered light. Gradient RMS was again used together with contrast feature in the BF image to discard still remaining images that were not perfectly in focus. To quantify the levels of β-Gal signal in each cell, and therefore quantify the number of β-Gal positive cells in each sample, Mean Pixel feature in the BF image was used, as previously described[21]. The threshold for the Mean Pixel Intensity that was set for the identification of β-Gal positive cells was below -75 for BT474 cells, and below -98 for SKBr3 cells. Cells without β-Gal staining were used as negative controls.

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VEGF quantitation

VEGF abundance in conditioned medium was determined by human VEGF immunoassay (R&D systems®, Inc. by Biotechne, Minneapolis, MN, USA) accordingly to manufacturer instructions.

Lactate quantitation

Released lactate concentrations were quantitated by Lactate-GLO™ assay (Promega, Madison, WI, USA) following manufacturer instructions.

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ROS quantitation

Released ROS levels were measured by ROS-GLO™ assay (Promega, Madison, WI, USA) accordingly to manufacturer instructions.

Apoptosis evaluation

After drug treatment, tumour cells grown as monolayers were treated with Trypsin, and collected together with those floating in culture medium. After washing with PBS, cells were pelleted and incubated with Annexin V-FITC/Propidium Iodide solutions (eBioscience™ Annexin V Apoptosis Detection Kit, Invitrogen by Thermo Scientific, Waltham, MA, USA), according to the manufacturer instructions. Cells were analysed with the CytoFLEX-S flow cytometer (Beckman Coulter, Brea, CA, USA). The percentage of cells positive for Annexin V and/or Propidium iodide (PI) was determined by the data-analysis software FCS Express 7 (De Novo Software by Dotmatics, Boston, MA, USA) excluding events associated with grouped cells, and displayed as Quadrant Statistics. Low Left (double negative): percentage of live cells; Low Right (Annexin V positive): percentage of cells in early apoptosis; Upper Right (double positive): percentage of cells in late apoptosis; Upper Left (PI positive): percentage of necrotic cells. Total apoptosis was calculated by adding early apoptotic, late apoptotic and necrotic cells.

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Immunofluorescence (IF)

Monolayers grown on glass coverslips were fixed in 4% paraformaldehyde (PFA) for 15 min and permeabilised with blocking solution (PBS supplemented with 0.1% saponin, 0.5% BSA and 50 mM NH_4Cl) for 30 min. Cells were incubated with primary antibodies for 1 hour and with specific Alexa 488, 546 and/or 647-conjugated secondary antibodies for 45 min. All antibodies were diluted in blocking solution. For imaging, samples were examined using a Zeiss (Oberkochen, Germany) Imager A2 microscope, equipped with 49 DAPI (excitation 365, beam splitter FT 395, emission BP 445/50), 43 HE CY3 (excitation BP 550/25, beam splitter FT 570, emission BP 605/70) and 38 HE EGFP (excitation BP 470/40, beam splitter FT 495, emission BP 525/50) filter sets (Zeiss). Images were obtained under a 40x/0.75 EC Plan Neofluar objective (Zeiss), at a definition of 1388 x1040 pixels (0.0059 pixel per micron), by means of a high resolution monochromatic camera (Axiocam MRm Rev3, Zeiss), and analysed with the Axiovision REL 4.8 software (Zeiss).

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Mammospheres grown in suspension in MammoCult™ medium were fixed with 2% PFA and permeabilised with 0.25% Triton X-100 in PBS for 30 min at 4°C. Mammospheres were incubated with primary or secondary antibodies overnight at 4°C. Antibodies were diluted in Antibody dilution solution (comprising 0.5% goat serum, 0.2% Tween 20 and 1% BSA in PBS) and samples washed with Antibody wash solution (comprising 0.2% Tween 20 and 1% BSA in PBS). Nuclear staining was obtained by incubating with Hoechst for 10 min at room temperature. All incubation and washing steps were performed in a laboratory rotating wheel.

Scaffolds were fixed in 4% PFA for 2 hours and permeabilised with the Block-Perm buffer (PBS supplemented with 1 mg/mL BSA, 10% FBS, and 0.3% Triton X-100) for 30 min at room temperature. Scaffolds were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 2 hours at room temperature. Antibodies were diluted in blocking buffer (PBS supplemented with 1 mg/mL BSA and 10% FBS). Nuclear staining was obtained by incubating with Hoechst for 10 min at room temperature. All incubation and washing steps were performed on a laboratory shaker.

For imaging, mammospheres and scaffolds were examined using an Olympus (Tokyo, Japan) FluoVIEW FV3000RS Confocal, equipped with a 405-640 nm laser line (Coherent OBIS solid state lasers, Olympus). Images were obtained under UPLXAPO 4x, 10x or 20x dry objectives (Olympus), at a definition of 1024x1024 pixels, and analysed with the FV31S-SW software (Olympus).

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Antibodies used for IF are listed in Table S1 in the Supplementary Information.

Quantitation of Topoisomerase I and HER2 intensity

Topoisomerase I nuclear mean intensity was quantified via a custom writtem Fiji macro. First, nuclei were segmented from the DAPI channel via the Stardist plugin[22] with a Probability Threshold = 0.5 and an Overlap Threshold = 0.5. Touching edges ROIs and ROIs with an area lower than 0.05 inches were excluded.

For HER2 signal, nuclei were segmented and their mean intensity was measured (I_n). Nuclear ROIs were then expanded by a factor *s* = 1.5 and its HER2 mean intensity were measured (I_{nscale}). Then the mean intensity of the circular crown (I_{cc}) around the nucleus, that we assumed as a good approximation of HER2 mean intensity in the plasma membrane, was computed as follow:

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$$I_{cc} = \frac{s * I_{nscale} - I_n}{s - 1}$$

Analyses were performed on approximately 100 cells (depending on cell types and experimental condition) out of three replicates.

Transmission electron microscopy

After drug treatment, tumour cell monolayers, mammospheres or homotypic scaffolds for transmission electron microscopy analysis were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 hours at 4 °C, and pelleted before the fixation. After primary fixation, samples were post-fixed for 1 hour in a reduced osmium solution (1.5% potassium ferricyanide and 1% aqueous osmium tetroxide in 0.1 M cacodylate buffer). Following the initial incubation with the heavy metal-based solutions, cell pellets were washed with bi-distilled water at room temperature, and then immersed in a 0.5% uranyl acetate solution and left overnight at 4 °C. The samples were dehydrated using a graded ethanol series (70%, 80%, 90%, 100%) and acetone for 10 minutes each, before being embedded in Epon resin. After curing at 60 °C for 48 hours, thin sections were cut using a Leica UC7 ultramicrotome (Leica Microsystems, Vienna, Austria). The sections were mounted onto 300-mesh copper grids and imaged using a Talos L120C G2 transmission electron microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) at an acceleration voltage of 120 kV. Morphometric analyses of the mitochondrial network and the percentage area of degradative organelles relative to cytoplasmic area were performed manually using ImageJ, version 1.54f. For the quantification of the morphology of mitochondrial network, the cross-sectional area derived from each mitochondrion was measured. Analyses were performed on twenty cells, between 250 and 350 organelles for mitochondrial area, perimeter and density evaluation, and between 20 and 30 mitochondria for cristae density determination (depending on cell types and experimental condition).

DNA extraction and c-Myc copy number variation by Droplet Digital PCR

After drug treatment, tumour cells grown as monolayers were treated with Trypsin, pelleted and washed once with PBS before being stored at -80°C.

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Maxwell® RSC Whole blood DNA kit (AS 1520) was used to extract DNA from cell pellets employing the Maxwell® RSC Instrument (Promega, Madison, WI, USA). DNA was eluted in 50 µL of Elution Buffer. The ddPCR mix was prepared in a 20 µL solution containing 1 µL of FAM-labeled CNV c-Myc probe (dHsaCP2500322, target probe), 1 µL of HEX-labeled CNV TERT probe (dHsaCP2500351, reference probe), 1 µL of HindIII Restriction enzyme (2U/reaction), 10 µL of ddPCR™ Supermix for Probes (No dUTP) and 50 ng of DNA. The droplet emulsion was thermally cycled on C1000 Touch Thermal Cycler (Bio-Rad Laboratories, CA, USA) instrument, starting with incubation at 95°C for 10 min, followed by 40 cycles of 94°C for 30s, 60°C for 60s, 10 min incubation at 98°C and a final hold at 4°C. The CNV of c-Myc was calculated automatically by the QuantaSoft™ software version 1.7.4 (Bio-Rad Laboratories, CA, USA).

RNA extraction and Reverse Transcription

Cell pellets were collected as described above, and Maxwell® RSC simplyRNA Tissue Kit was used to extract RNA employing the Maxwell® RSC Instrument (Promega, Fitchburg, WI, USA). Five hundred ng of total RNA were reversed transcribed in a final volume of 20 µL using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA, USA). Target cDNA was amplified by either ddPCR or rtPCR [as described before[23]].

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Droplet Digital PCR (ddPCR) and Real Time PCR (rtPCR)

The QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories, CA, USA) instrument was used for the ddPCR analysis. Two µL of cDNA (equivalent to 25 ng of cDNA for c-Myc or 2.5 ng of cDNA for YWHAZ analysis) were mixed with Droplet Digital PCR Assay probes for c-Myc (fluorophore FAM, dHsaCPE5051056, Bio-Rad Laboratories, Hercules, CA, USA) or YWHAZ (fluorophore FAM, dHsaCPE5051780, Bio-Rad Laboratories, Hercules, CA, USA), respectively. The volume of the final PCR mix was 20 µL including 10 µL of ddPCR™ Supermix for Probes (No dUTP) and 1 µL of the primers/fluorophore probes. ddPCR amplification reagents were purchased from Bio-Rad Laboratories. The droplet emulsion was thermally cycled on C1000 Touch Thermal Cycler (Bio-Rad Laboratories, CA, USA) instrument. Cycling conditions were 95°C for 5 min, followed by 40 cycles of amplification (94°C for 30 s and 55°C for 1 min), ending with 98°C for 10 min. The concentration of the

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target was calculated automatically by the QuantaSoft™ software version 1.7.4 (Bio-Rad Laboratories, CA, USA).

The LightCycler® 480 instrument II (Roche, Basel, Switzerland) was used for the rtPCR analysis. Briefly, 12.5 ng of cDNA were mixed with 10 µL of LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland) and 150 nM final concentration of primers (see table below). rtPCR cycling conditions were: hot start at 95°C for 10 min, 45 cycles of amplification (95°C for 15 s, 60°C for 10 s, and 72°C for 20 s), final extension at 72°C for 20 s, followed by 10 min at 98°C.

Primers used for rtPCR are listed in Table S2 in the Supplementary Information.

Results were expressed as relative expression units (nFold) to the house-keeping reference gene (GAPDH or Actin) calculated by the $2^{-\Delta\Delta Ct}$ method.

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Western blotting

Cell monolayers were washed twice with ice-cold PBS, scraped in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton-X100, 1 mM EGTA, and 1 mM EDTA pH 7.4, complete protease inhibitor (Roche by Merck, Kenilworth, NJ, USA), and phosphatase inhibitors (Sigma Aldrich by Merck, Kenilworth, NJ, USA), incubated for 30 min on a wheel at 4 °C. Supernatant was centrifuged at 16,000 g for 15 min at 4 °C to remove nuclei and membranes. Protein concentrations were determined (BCA protein assay, PIERCE by Thermo Scientific, Waltham, MA, USA), and 15 µg of proteins were used for SDS–PAGE and western blotting. Samples were prepared by adding 2x or 4x sample buffer (Bio-Rad, Laboratories, Hercules, CA, USA) and boiling for 5 min at 95 °C before loading onto the gel. Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Hybond, GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Strips containing the proteins of interest were incubated in 5% (w/v) BSA in TBS containing 0.1% (v/v) Tween-20, pH 7.4 (T-TBS), for 1 h at room temperature and then with fresh blocking buffer containing the primary antibody at its working concentration. After overnight incubation at 4°C, the antibodies were removed and the strips washed with T-TBS for 3x 10 min. Strips were incubated for 1 h with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody and washed 3x 10 min with T-TBS. Western blots were developed using the chemiluminescent method (ECL, GE Healthcare, Chalfont St. Giles, Buckinghamshire,

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UK) and signals acquired by ChemiDoc MP Imaging System (Bio-rad, Laboratories, Hercules, CA, USA). Bands were quantified by densitometric analysis using the National Institutes of Health (NIH) ImageJ program. The quantification of each band was normalized using the signal of house keeping proteins (Vinculin or GAPDH) as loading controls.

Antibodies used for WB are listed in Table S3 in the Supplementary Information.

Statistical information

To compare data sets for a single variable, Student's t-test statistics was performed from at least three independent experiments. Differences were considered statistically significant for $p< 0.05$.

For morphometric analysis, data are presented as means ± s.e.m. One-way ANOVA with Dunn's multiple comparisons test was performed. Differences were considered statistically significant for $p<0.5$ (*); $p<0.01$ (**); $p<0.001$ (***); $p<0.0001$ (****).

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RESULTS

T-DXd is now considered the treatment of choice for patients with advanced HER2-positive BrCa, which have undergone treatment with an anti-HER2 therapy and have developed disease recurrence [in agreement with the ESMO Magnitude of Clinical Benefit Scale scorecards]. Thus, we decided to study its effects on both parental (sensitive) BT474 cells and on derived cells, made resistant to TZ by the continuous exposure to high doses of therapeutic antibody [BT474-R], leveraging several different *in vitro* models, including systems generated with the 3D bioprinting technology to achieve more reliable preclinical models.

ADCs exert off-target effects on the tumour microenvironment

To assess the putative off-target effects of ADCs on stromal cells, we developed 3D model systems of heterotypic co-cultures (comprising tumour cell:fibroblast:endothelial cell), designed as two concentric compartments, intended to host microenvironment (fibroblasts) and tumour cells in the external layer (outer ring), and endothelial cells in the inner one. Fibroblasts and tumour cells were 3D bioprinted in GelXA LAMININK 411 forming an outer hollow cylinder whereas endothelial cells were diluted in GelMA Fibrin and manually casted within the cylinder. We allowed these models to mature for two (BT474-R) or three (BT474) weeks before performing treatment with TZ, T-DM1 or T-DXd for an additional week (Fig.1a).

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Morphological analysis of the self-organization achieved by the different components showed that in BT474 samples endothelial cells remained mainly confined to the inner layer (Fig.1b), whereas in the presence of BT474-R tumour cells, HuVEC cells frequently reached also the outer ring (Fig.1b-c). On the other hand, very few or no BT474 cells migrated from the outer ring (Fig.1c) whereas BT474-R cells colonized also the inner region of the scaffolds (or moved out of the scaffolds, Fig.1b-c). Some fibroblasts moved from the outer to the inner gel in the scaffolds. These observations suggest that resistant cells might have a more invasive phenotype and release attractants/growth factors for endothelial cells promoting their migration to the outer ring as well as for fibroblasts that colonize also the inner region of the scaffolds (Fig.1).

Immunofluorescence analysis of the outer ring showed that fibroblasts tend to re-organize from a sheet-like appearance to a trabecular structure in the presence of tumour

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cells; this rearrangement was partial in parental and almost complete in TZ-resistant tumour cells-containing scaffolds. These results suggest that resistant cells might release growth factors promoting fibroblasts rearrangement. We also observed that trabecular structures were almost preserved upon treatment with TZ and T-DXd, whereas T-DM1 completely disrupted their organization (Fig.1c and Supplementary Fig.3a).

ADCs directly interfere with stromal cells survival

To determine whether ADC-induced death of tumour cells impairs stromal cell survival (bystander effect) or whether therapeutic agents act directly on fibroblasts and/or endothelial cells (off-target effect), we performed a survival assay. We treated monolayers of either BJ fibroblasts or HuVEC endothelial cells with TZ, T-DM1 and T-DXd for seven days and measured survival by MTT assays.

Regarding fibroblasts, we found that while cell survival was unaffected by TZ, it was slightly decreased by T-DXd and significantly impaired by T-DM1 treatment (Fig.2a). As expected, staining of fibroblasts for HER2 (the membrane receptor recognized by TZ) resulted in no specific signal, confirming the absence of the target receptor on these stromal cells (Supplementary Fig.3b). However, incubation with an anti-human antibody recognizing humanized TZ or the Ab component of ADCs, resulted in labelling of the plasma membrane in fibroblasts that had been treated with TZ, T-DM1 and T-DXd. This staining was absent in control untreated fibroblasts (Fig.2d).

Regarding HuVEC cells, all tested therapeutic agents (TZ, T-DM1 and T-DXd) impaired endothelial cell survival, with T-DM1 demonstrating the greatest effect (Fig.2b). Immunofluorescence analysis confirmed that, as expected, endothelial cells do not express HER2 on their surface (Supplementary Fig.3c).

Upon T-DM1 treatment, both fibroblasts and endothelial cells display an alteration of their morphology (Supplementary Fig.3b-c). This morphological change was also evident in the cells within the tri-partite heterotypic scaffolds (Fig.1 and Supplementary Fig.3a) strongly suggesting that T-DM1 might exert direct activity on the stromal component.

To further investigate these direct effects we performed western immunoblotting (WB) analysis on lysates from tumour and stromal cell lines (Fig.2c). Results confirmed that fibroblasts and endothelial cells do not express HER2, whereas BJ fibroblasts displayed reduced basal levels of Topoisomerase I and tubulin, which are the intracellular targets of

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T-DXd and T-DM1 payloads, respectively. This lower expression of the payload targets in fibroblasts (compared to endothelial cells or tumour cells) provides a plausible explanation for the reduced effect of the ADCs observed on fibroblasts compared to endothelial cells (Fig.2a-b).

To directly test the paracrine effect of each drug, we first seeded and treated tumour cells with either naked Ab or ADCs for seven days. Following treatment, tumour cells were subjected to extensive washing to ensure the removal all unbound drugs. Next, we seeded fibroblasts or endothelial cells together with the pre-treated tumour cells (as monolayers). After three days of co-culture, we performed immunofluorescence of the co-cultures using different markers: HER2, to label tumour cells; alpha-SMA, to mark fibroblasts, or CD31, to stain endothelial cells, and an anti-human antibody (recognising TZ) to follow the naked Ab or ADCs. We observed that treated tumour cells exhibited two potential mechanisms to crosstalk with stromal cells (Fig.2e), either releasing extracellular vesicles, some of which were found internalized by adjacent fibroblasts or endothelial cells (arrows, Fig.2e and insets), or extending tiny filaments directly reaching the stromal cells (arrowheads, Fig.2e and insets). These observations support the hypothesis that drug-carrying components (ADC or naked antibody) were transferred from the treated tumour cells to the naïve stromal cells.

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T-DXd reduces Topoisomerase I levels

To dissect the mechanism of action of T-DXd in BrCa cells, we first assessed the levels of expression of both HER2 (target of the antibody component of T-DXd) and Topoisomerase I (target of the payload component of T-DXd), as they have been previously reported to be reduced upon treatment with the ADC in lung cancer patients[13]. By immunofluorescence analysis, we found that both parental and resistant cells express both HER2 receptor and nuclear Topoisomerase I, although BT474-R cells show slightly reduced levels of either marker. T-DXd treated parental cells display redistribution of Topoisomerase I out of the nucleus, whereas T-DXd treated resistant cells show an overall reduction of the labelling (Fig.3a-c). Protein expression analysis by WB showed that HER2 amount was almost not affected by treatment with TZ, T-DM1 and T-DXd in parental BT474 and resistant BT474-R cells. By contrast, Topoisomerase I levels were significantly reduced in T-DXd-treated cells in both cell types [(7.6±3.0)% and (2.6± 0.8)% vs untreated BT474 and BT474-R cells, respectively, Fig.3d-e].

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T-DXd induces senescence

In all model systems (monolayer, mammospheres grown in suspension and 3D bioprinted matrix-embedded scaffolds), we observed an important change in tumour cell morphology (enlargement, and flattening in monolayer growing cells) upon treatment with TZ and ADCs, which is compatible with senescence (Supplementary Fig.4). To test this hypothesis, we treated BT474 or BT474-R cells in monolayer cultures for seven-ten days with TZ, T-DM1 and T-DXd, and assessed the percent of cells positive for senescence-associated (SA) β-galactosidase activity (Fig.4a). Quantitative analysis by ImageStream showed that untreated parental BT474 displayed rare senescent cells [in agreement with previously reported data[24]] whereas both the naked antibody [in agreement with recent observations from our lab[6]] and ADCs triggered senescence (Fig.4a-c).

Furthermore, we observed that resistant BT474-R already show an increased percentage of senescent cells at steady state. This percentage was slightly augmented upon treatment with T-DXd (Fig.4a-c). This finding was further supported by the assessment of the levels of p15 mRNA, which demonstrated an increase upon treatment with T-DXd (Fig.8a).

To assess whether the senescent phenotype induced by T-DXd was reversible or sustained, we performed washout experiments at different time points: in particular, cells treated with the ADC were allowed to recover in normal growth medium for either 3 or 7 days after the end of treatment. Results show that, even after a week of washout, surviving tumour cells display a senescent phenotype and an increased activity of SA-β-galactosidase (Fig.4a-c).

To dissect the mechanism underlying T-DXd triggered senescence, we performed WB. Protein expression analysis showed that treatment with T-DXd of either parental or resistant tumour cells triggers an increase in the levels of p53 and p21 (Fig.4d-e). Since inhibition of Topoisomerase I might lead to DNA damage, we assessed the levels of p53 phosphorylation on Ser15 [a residue that can be phosphorylated by ATM/ATR[25]] and found that indeed parental and resistant cells treated with T-DXd display this post-translational modification (Fig.4d). Furthermore, since p53/p21 pathway activation might also lead to apoptosis, we investigated the levels of Bcl2 and observed a decrease in its amount upon T-DXd treatment (Fig.4d-e). We also performed Annexin/PI staining, but flow cytometry

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analysis showed no major changes in the frequency of apoptotic events upon treatment with naked antibody or ADCs, suggesting that the activation of p53 by T-DXd triggers mainly senescence instead of apoptosis in BT474 cells (Supplementary Fig.5).

Senescence is a complex process that may involve or affect key organelles, namely lysosomes [the compartment responsible for macromolecules and organelles degradation [26], [27] and [28]], and mitochondria [the energy- and ROS-generating compartment [29], [30] and [31]]. To investigate potential morphological changes related to senescence, we performed ultrastructural analysis of these organelles in cells treated with T-DXd across three different culture formats: monolayer, mammospheres and matrix-embedded scaffolds. Our observations revealed that the area occupied by the degradative compartment increased upon T-DXd treatment mainly in parental BT474 (Fig.5, quantitation of the monolayer is shown in Fig.8a). This augment was less pronounced in resistant BT474-R cells, possibly due to the already high prevalence of senescent cells in untreated cells (Fig.4c). Furthermore, the degradative compartment appeared more engulfed of undigested material in T-DXd treated cells (Fig.5). In addition, in BT474 parental cells T-DXd promoted a measurable increase in mitochondrial number (density), and a reduction of their dimension (area and perimeter) without significantly affecting their overall morphology. Moreover, T-DXd treatment triggered a decrease in cristae density within the mitochondria of parental cells (Fig.5 and Fig.7a, quantitation of the monolayer is shown in Fig.8a).

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T-DXd mechanism of action depends mainly on the payload and is common to other DXd-based ADCs

To assess whether the mechanism of action of T-DXd relies on the Ab or the payload component, or on their combined effect, we investigated the effect of TZ or DXd or another DXd-based ADC, Datopotamab-deruxtecan (Dato-DXd, Datroway®, where Datopotamab antibody targets the TROP2 receptor). Moreover, we tested all the therapeutic drugs on an additional HER2-positive BrCa cell line (SKBr3) and its TZ-resistant derivative (SKBr3-R), showing similar HER2 amounts and even higher levels of expression of TROP2 compared to BT474 cells (Fig.6a).

We found that DXd alone and Dato-DXd also trigger senescence in both parental and resistant BT474 cells via the activation of the p53/p21 pathway (Fig.6b-c). However, Dato-DXd was less efficient than T-DXd possibly because of its lower drug-antibody-ratio (DAR, 4 vs 8) and/or the lower expression levels of the antibody target (TROP2 vs HER2, whose

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gene expression is amplified in both cell lines). Indeed, both TROP2 and Topoisomerase I protein amounts were only partially reduced by ADC treatment in parental and resistant cells (Fig.6b-c). When we investigated the effects of DXd-based ADCs on SKBr3 cells, we found that the three drugs dampen Topoisomerase I levels and trigger senescence via activation of the p53/p21 pathway upon induction of DNA damage (Supplementary Fig.6).

We also observed that SKBr3 were more sensitive to DXd-based drugs than BT474 cells, as they displayed a higher frequency of cells undergoing apoptosis at earlier time points of drug treatment (after 2 days vs 7 days in BT474 cells, Supplementary Fig.7a-c). Recently, it has been shown that Topoisomerase I inhibitors demonstrated increased efficacy in c-Myc driven cancers [[32] and [33]] and that contemporary targeting of p53 and c-Myc triggers a synthetic lethality effect in leukemic cells[34]. Therefore, since both SKBr3 and BT474 cells express mutant p53, we quantified the number of c-Myc gene copies (copy number variation, CNV) in each cell line at steady state. We found that parental and resistant SKBr3 display a higher CNV compared to BT474 and BT474-R cells, and that both resistant cells display a higher c-Myc CNV than parental cells (Supplementary Fig.7d). Furthermore, to estimate the impact of Topoisomerase I inhibition on c-Myc activity, we also evaluated the levels of c-Myc transcripts upon treatment with ADC-based drugs in SKBr3 and BT474 cell types. As expected, we observed that SKBr3 were more sensitive to Topoisomerase I inhibition compared to BT474 cells (Supplementary Fig.7e). Of course, we cannot exclude that the co-expression of hormone receptors in BT474 cells would contribute to their ability to resist to apoptosis after long-term treatment with DXd-based ADCs.

We next analysed the ultrastructure of BT474 cells treated with either the payload alone or Dato-DXd and found that they both promote an expansion of the degradative compartment in BT474 cells as T-DXd (Fig.7a). DXd was sufficient to induce fragmentation (reduction of area and perimeter) of mitochondria in both BT474 and BT474-R cells (Fig.8a). Also parental and TZ-resistant SKBr3 cells display an increase of the area occupied by the degradative compartment and fragmentation of mitochondria upon treatment with either ADCs and DXd alone (Fig.7b and Fig.8b).

Unlike the mitochondrial hyper-fused phenotype described in oncogene-induced and ageing-associated senescence models[30], we found that senescence induced by DXd-based ADCs (therapy-induced-senescence, TIS) promotes fragmentation of the mitochondrial network as well as an alteration of the organization of their cristae (Fig.7 and Fig.8). Morphological alteration of mitochondria is usually associated to impaired organelle

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function, among which oxidative phosphorylation [35 and 36]. Therefore, we investigated whether treatment with DXd-based ADCs induces tumour cells to rely more on aerobic glycolysis. To this end, we measured the amount of lactate released in the conditioned medium of parental or resistant BT474 or SKBr3 cells grown as monolayers: indeed, treated cells tend to rewire towards glycolysis (Fig.8). Since Topoisomerase inhibitors promote DNA damage and oxidative stress, we evaluated the abundance of reactive oxygen species (ROS) in the conditioned media from cell monolayers treated with DXd-based ADCs: indeed, we observed an increase of the amount of ROS released (Fig.8).

DXd-based ADCs trigger Senescence-Associated Secretory Phenotype (SASP)

To corroborate further the senescent phenotype triggered by treatment with DXd-based ADCs, we investigated whether treated cells also exhibit a secretory phenotype by analysing the levels of different SASP-specific cytokines/chemokine/growth factors at the mRNA and/or protein levels. To this purpose, RNA extracts from monolayers of parental and resistant BT474 or SKBr3 cells treated with T-DXd, TZ, DXd or Dato-DXd were analysed for the evaluation of cytokine/chemokine transcript levels. We found that treatment with DXd-based ADCs of both BT474 and SKBr3 cells (either parental or TZ-resistant) induced an increase of the amount of RNA coding for IL-1β, IL-6, and IL-8 cytokines (Fig.8). Moreover, we quantitated the levels of vascular endothelial growth factor (VEGF) in the conditioned media from monolayers of parental and resistant cells, and found that DXd-based ADCs triggered the release of the growth factor (Fig.8).

Altogether, these findings support a general mechanism by which DXd-based drugs reduce the payload target amount and trigger senescence as a survival mechanism in BrCa cells.

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DISCUSSION

In the last decade, ADCs have secured regulatory approval for the treatment of advanced BrCa and, more recently, T-DXd has become the prevalent treatment in different clinical settings. Despite its efficacy, primary and acquired resistance are expected to occur. Relatively few *in vitro* studies, however, have investigated the mechanism of action of and/or resistance specifically associated to T-DXd. Recent research has highlighted some potential mechanisms, including the reduction in the expression levels of either the antibody target (HER2) or the payload (DXd) target in HER2-positive cells [[13], [14] and [15]] and the activation of the extracellular protease Cathepsin L within the TME, which leads to payload release and internalization in surrounding HER2-low/negative cells[37].

In this paper, we demonstrate that T-DXd treatment of HER2-positive BrCa cells induces a reduction of Topoisomerase I levels, suggesting that the downregulation of the payload target may represent a general response and mechanism of resistance to the ADC. Furthermore, we report for the first time, to our knowledge, that T-DXd triggers cellular senescence in HER2-positive BrCa. We corroborated this finding through multiple lines of evidence, including the assessment of SA-β-galactosidase activity levels, and expression of SASP (cytokine/chemokine levels and growth factors amount in conditioned media). We identified the underlying molecular mechanism: T-DXd treatment triggers an increase in the levels of p53 and p21. These results are in line with previous findings obtained using Camptothecin and SN-38 [Topoisomerase I inhibitor and its metabolic derivative, respectively[38]]. However, the ultimate balance between senescence and apoptosis induced by T-DXd might be influenced by the activity of other specific cancer drivers, such as c-Myc in SKBr3 cells.

Moreover, the ultrastructural analysis of T-DXd treated cells showed an expansion of the degradative compartment associated to accumulation of undigested material, possibly because of an impaired enzymatic activity of lysosomal enzymes [due to increased lysosomal pH[39]] and deposition of lipofuscin. Thus, the possibility exists that cleavage of the linker between antibody and payload is impaired, leading to a decreased effectiveness of the internalized drug in senescent cells. Furthermore, the ultrastructural analysis of ADC-treated cells highlighted a morphological alteration of mitochondria. In aging or in oncogene-induced senescent cells, mitochondria usually form a hyper-fused network [[30] and [31]], whereas here we report organelle fragmentation and alteration of their cristae density, which

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is associated with dysfunctional mitochondria[35]. Indeed, we show that this phenotype is associated to increased lactate levels in the extracellular media. Altogether, these results suggest that DXd-triggered TIS activates different pathways as compared to other types of senescence.

SASP factors may exert both autocrine and/or paracrine effects, thus spreading the senescent phenotype to surrounding cells, the TME. The crosstalk between senescent and bystander cells may occur via ROS release, extracellular vesicles secretion and/or formation of bridges allowing direct cargo transfer [reviewed in [40]]. Indeed, we observed the release of extracellular vesicles and the formation of tiny filaments contacting tumour and stromal cells (Fig.2e). Furthermore, SASP might exert both anti- as well as pro-tumoral effects, as it may affect matrix organization, vascularization, immune cells recruitment and activation, stemness, and EMT transition [[29], [41], [42] and [43]]. In particular, senescent cells might upregulate the expression of surface MHC I or II class molecules (thus directly positively modulating the immune response), or of immunosuppressive receptors, such as CD47, CD24, PD-L1 or PD-L2[43]. Thus, SASP might trigger a dichotomous effect by acting on innate and adaptive immune responses [reviewed in [43]]. Senescent cells may also induce paracrine senescence in neighbouring cells by means of SASP factors release or via direct cell-cell transfer of ROS through gap junctions, and their increase in both tumour and stromal senescent cells might underlay resistance to therapy [[43]].

In addition to SASP factors, DNA damage and metabolic rewiring of senescent cells might contribute other signalling molecules that alter bystander cells behaviour. Indeed, increased levels of lactate in the TME trigger several pro-tumoral events: i. activation of endothelial cells and VEGF production; ii. proliferation, migration and synthesis of collagen type I by cancer-associated fibroblasts; iii. expression of matrix metalloproteases leading to invasion; iv. induction of epithelial-mesenchymal transition via either TGFβ/SMAD or Wnt/β catenin pathway; v. education of immune cells towards an immunosuppressive microenvironment [reviewed in [44]]. Indeed, lactate favours the differentiation of Th17 $CD4^+$ T lymphocytes, reduces the inflammatory function of T_{regs} and the proliferation, degranulation and cytolytic activity of $CD8^+$ T cells, and promotes the timely conversion of M1- into M2-like macrophages [[44] and references therein]. Macrophage rewiring towards M2-like phenotype is also mediated by lactylation; thus, lactate exerts its function both as a metabolite and as an epigenetic modifier, mainly promoting the post-translational modification of lysine residues of histones and thus inducing the expression of genes associated with an immunosuppressive function of macrophages [[44] and references therein].

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More, the release of ROS in the TME has been reported to promote: i. recruitment and polarization of macrophages towards an M2-like phenotype; ii. NK cells exhaustion; iii. reduction of effector T cells signalling, activation, proliferation and viability; iv. increase of regulatory T cells [reviewed in [45]]. Altogether, these observations suggest that ADC-triggered lactate and ROS release would favour the establishment of an overall immunosuppressive TME that could contribute to tumour resistance to DXd-based ADC treatment.

One of the main limitations of this study is the lack of information on immune cells contribution to T-DXd mechanism of action and to ADC effect on tumour/stroma crosstalk. Indeed, since TZ-derived ADCs comprise the same immunoglobulin G1 backbone of the naked antibody, they should maintain the ability of TZ to induce ADCC *in vivo* [reviewed in [9], [16] and references therein]. Indeed, the ability of T-DXd to promote ADCP has been reported in co-culture model systems comprising BrCa cell lines and macrophages[37]. Moreover, the involvement of cGAS-STING pathway in the response to T-DXd has been reported both in breast[37] and gastric[46] cancer. Thus, adding the immune component to the stroma in the 3D bioprinted model would help clarify the ultimate effect of lactate, ROS and SASP factors released by tumour cells, upon treatment with ADCs, on the TME. Testing the efficacy of ADCs in 3D bioprinted heterotypic model systems comprising also immune cells represents an important challenge for future studies. Indeed, the 3D model system developed for this study has proven useful in assessing the off-target effect of the ADCs on stromal cells, and this capability would be very informative in the context of the preclinical study of new drugs.

Since HER2 overexpression and mutations work as pathogenic drivers in other types of cancer, although less frequently than in BrCa, the dissection of the mechanism of this ADC could be of more general interest since T-DXd has been approved for patients with locally advanced or metastatic HER2-positive gastric cancer or gastroesophageal junction adenocarcinoma [reviewed in [47]]. Furthermore, T-DXd showed activity not only on high-HER2 expressing but also on HER2-low or HER2 non-expressing cancers (phase II DAISY trial) and HR- and HER2-positive tumours (post hormone-targeting treatments, DESTINY-Breast06), and Dato-DXd has proven effective in HR-positive BrCa and TNBC [[48] and [49]]. Thus, the identification of putative mechanisms of resistance to DXd-based ADCs might be relevant for a wider cohort of patients.

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Finally, several DXd-based ADCs are being tested in clinical trials nowadays[50]; therefore, our findings that T-DXd and Dato-DXd promote senescence would provide the rationale for treatment with senolytic or senomorphic agents to by-pass resistance to DXd-based ADCs.

Moreover, our finding obtained with T-DXd and Dato-DXd would suggest that reduction of Topoisomerase I target represents a general response to treatment with DXd-based ADCs, thus implying that the ADC post-ADC regimen might not be effective if both the drugs exploit DXd as payload [as recently reported by [51]].

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ADDITIONAL INFORMATION

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AUTHOR CONTRIBUTIONS:

Conceived and designed the experiments: GB, CT, TD.
Performed the experiments: EV, RP, SG, DZ, CDA, AL, LV, TD.
Analysed and interpreted the data: EV, RP, SG, DZ, EC, BG, CS, CDP, GB, CT, TD.
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DATA AVAILABILITY:

Data are available in the article itself and its supplementary information.

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FIGURE LEGENDS

Figure 1: Tumour and stromal cells organization in matrix-embedded scaffolds. Parental BT474 and resistant BT474-R cells were printed in GelXA LAMININK 411 together with BJ fibroblasts (outer ring) while endothelial cells were casted in GelMA Fibrin (inner region), grown for two (BT474-R) or three (BT474) weeks, and treated with 10 µg/mL drug (as indicated) for 7 days. **a)** Scheme showing cell disposition upon 3D bioprinting and at treatment. After treatment scaffolds were processed for immunofluorescence analysis. Green: EpCam (epithelial cells); Red: alpha-SMA (fibroblasts); White: CD31 (endothelial cells). **b)** inner region (pink); **c)** outer ring (light blue). Scale bar: 500 µm. Images from one experiment are shown, as representative of two independent experiments.

Figure 2: Analysis of drug off-target and paracrine effect on stromal cells. a-b) Fibroblasts (**a**) or endothelial cells (**b**) were seeded, and the following day treated with 10 µg/mL drug (as indicated) for 7 days. Cell viability was assessed by MTT assay. Graphs show mean ± s.e.m. out of three independent experiments. *, $p<0.05$; ***, $p<0.001$ Student's t test.**c)** Cells were lysed and processed for western blot analysis. The labelling of HER2 (Trastuzumab target), Topoisomerase I (deruxtecan target, Topo I) and tubulin (emtansine target) are shown. GAPDH was used as the loading control. Original blots are presented in Supplementary Figure 8. Numbers indicate densitometry of protein bands, out of a single experiment. **d)** Fibroblasts cells were seeded, and the following day treated with 10 µg/mL drug (as indicated) for 7 days. Cells were processed for immunofluorescence. Blue: Hoechst (nuclei); Red: anti-human (TZ and ADCs); Green: Ki67 (proliferation marker). Scale bar: 120 µm. **e)** Parental and resistant tumour cells were seeded, and the following day treated with 10 µg/mL drug (as indicated) for 7 days. After extensive washing, fibroblasts or endothelial cells were seeded. After three days, samples were processed for immunofluorescence. Green: HER2 (tumour cells); Red: alpha-SMA (fibroblasts) or CD31 (endothelial cells); Blue: Hoechst (nuclei); White: anti-human (TZ and ADCs). Scale bar: 50 µm. Inset: 30 µm. Arrows: extracellular vesicles; arrowheads: filaments.

Figure 3: T-DXd treatment reduces Topoisomerase I levels and activates p53/p21 pathway. Parental and resistant tumour cells were seeded, and the following day treated with 10 µg/mL drug (as indicated) for 7 days. **a-b)** Cells were processed for immunofluorescence. **a)** Red for HER2 (antibody target receptor) and Blue for Hoechst (nuclei). **b)** Red for Topoisomerase I (payload target enzyme) and Blue for Hoechst (nuclei). Scale bar: 100 µm. **c)** Graphs show mean ± s.e.m, out of three replicates. *, $p<0.05$; Student's t-test vs each untreated control. Analysis was performed on approximately 100 cells (depending on cell types and experimental condition). **d-e)** Cells were lysed and processed for western blotting. **d)** Shown data are representative of three independent experiments. Vinculin was used as the loading control. Original blots are presented in Supplementary Figure 9. **e)** Graphs show mean ± s.e.m, out of three biological replicates. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, Student's t-test vs each untreated control.

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Figure 4: T-DXd treatment induces senescence in tumour cells. Parental and resistant tumour cells were seeded, and the following day treated with 10 µg/mL drug (as indicated) for 7 days. **a-c)** Cells treated with T-DXd were washed three times with PBS to remove the drug, and grown for additional 7 days in normal medium (washout). **a)** Cells were fixed and processed for SA-β-gal activity detection by colorimetric assay. Brightfield images from one experiment are shown, as representative of three independent experiments. Scale bar: 500 µm. **b-c)** Cells were detached, counted, fixed and processed for SA-β-gal activity detection by colorimetric assay. The amount of senescent cells was assessed by analysis at CytekBio ImageStream^X MKII. **b)** Representative brightfield images of SA-β-gal negative and positive cells from one experiment are shown as representative of two independent experiments. Scale bar: 15 µm. **c)** Table shows the percentage of SA-β-gal positive cells out of total analysed cells in each sample or as fold change, vs each untreated sample. Data represent mean ± standard deviation, out of two independent experiments. **d-e)** Cells were lysed and processed for western blotting. **d)** Shown data are representative of three independent experiments. Vinculin was used as the loading control. P-p53 stands for phospho-Ser^{15} p53. Original blots are presented in Supplementary Figure 9. **e)** Graphs show mean ± s.e.m, out of three biological replicates. *, p<0.05; **, p<0.01; Student's t-test vs each untreated control.

Figure 5: Ultrastructural analysis of control and T-DXd-treated tumour cells grown as monolayers, mammospheres or scaffolds. Parental BT474 **(a,c,e)** and resistant BT474-R **(b,d,f)** cells were grown as monolayer (2D, **a and b**), mammospheres (3D, suspension, **c and d**), or scaffolds (3D, matrix-embedded, **e and f**) and treated with 10 µg/mL T-DXd for 7 days. Cells were processed for electron microscopy for the morphological analysis of the degradative compartment (degr.comp., upper row) and mitochondria (lower row). N: nucleus; m: mitochondria; ly: lysosomes (degradative compartment). Scale bar: 1 µm.

Figure 6: DXd payload and Dato-Dxd induce senescence in tumour cells. a) Cells were lysed and processed for western immunoblotting to evaluate the relative abundance of antibody and payload targets (HER2 for TZ, TROP2 for Dato and Topo I for DXd). Histone 3 (H3) was used as the loading control. Original blots are presented in Supplementary Figure 10. Numbers indicate densitometry of protein bands, out of a single experiment. **b-d)** Parental BT474 and resistant BT474-R cells were grown as monolayer and treated with 10 µg/mL TZ, T-DXd, Dato-DXd or molar equivalent DXd (as indicated) for 7 days. **b)** Cells were lysed and processed for western immunoblotting. Vinculin/GAPDH were used as loading controls. P-p53 stands for phospho-Ser^{15} p53. Acet. p53 indicated Acetyl-Lys^{382} p53. Original blots are presented in Supplementary Figure 11. Numbers indicate densitometry of protein bands after normalization vs housekeeping. Data from one experiment are shown, as representative of two independent experiments. **c)** Cells were fixed and processed for SA-β-gal activity detection by colorimetric assay. Brightfield images from one experiment are shown, as representative of three independent experiments. Scale bar: 200 µm.

Figure 7: Ultrastructural analysis of tumour cells treated with DXd-based ADCs grown as monolayers. Parental (upper row) and resistant (lower row) BT474 **(a)** or SKBr3 **(b)** cells were grown as monolayer and treated with 10 µg/mL TZ, T-DXd, Dato-DXd or molar equivalent DXd (as indicated) for 7 or 2 days, respectively. Cells were processed for electron

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microscopy for the ultrastructural analysis of both the degradative compartment and mitochondria. Representative images of mitochondrial morphology are shown. N: nucleus; m: mitochondria. Scale bar: 1 μm.

Figure 8: Senescence and related features of tumour cells treated with DXd-based ADCs. Parental and resistant BT474 **(a)** or SKBr3 **(b)** cells were grown as monolayer and treated with 10 μg/mL TZ, T-DXd, Dato-DXd or molar equivalent DXd (as indicated) for 7 or 2 days, respectively. Tables show: 1. the percentage of senescent cells (senescence), 2. the morphometric analyses of the percentage area of degradative organelles relative to cytoplasmic area and of mitochondrial parameters and density (ultrastructural alterations), 3. the levels of lactate and ROS detected in conditioned media (metabolic rewiring) and 4. the amount of cytokines/chemokines/growth factors (SASP), as indicated by the headings.
For morphometric analysis of either cell type, data are presented as means ± s.e.m. One-way ANOVA with Dunn's multiple comparisons test was performed. Differences were considered statistically significant for $p<0.5$ (*); $p<0.01$ (**); $p<0.001$ (***); $p<0.0001$ (****). Data derive from one experiment. For the quantitation of ultrastructural alterations, analyses were performed on twenty cells, between 250 and 350 organelles for mitochondrial area, perimeter and density evaluation, and between 20 and 30 mitochondria for cristae density determination (depending on cell types and experimental condition).
For lactate, ROS and SASP factors quantitation in either cell type, data from one experiment are shown, as representative of two, each performed in two technical replicates.
Regarding senescence evaluation, data from one experiment are shown, as representative of two, for BT474 cells **(a)** and data derive from a single experiment performed on SKBr3 cells **(b)**. For the analysis, at least 30.000 events for each condition were analysed at ImageStream.

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