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SD-1008 a Novel JAK2 Inhibitor, Increases Chemotherapy Sensitivity in Human Ovarian Cancer Cells

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Running title: Effect of SD-1008 on STAT3 Signaling in Human Cancer

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Abreviations: STAT3, signal transducer and activator of transcription; IL-6, interleukin 6; JAK, janus-activated kinase; NCI, National Cancer Institute;

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Abstract

Interleukin 6 and the Signal transducer and activator of transcription (STAT) 3 proteins have important roles in cancer cell survival and proliferation. Recent studies demonstrate that abnormal STAT3 activation promotes tumor growth and supports survival of many human cancers and thus this protein or the pathway responsible for its activation are potential targets for the new anti-cancer therapy. STAT3 is a DNA-binding transcription factor and therefore its function depends on nuclear translocation. To discover inhibitors of the STAT3 pathway we have designed a cell-based screening assay capable of identifying small molecules which inhibit nuclear translocation. Among the 2000-compound NCI Diversity set we have identified SD-1008 as a micromolar inhibitor of IL-6 or oncostatin-induced STAT3 nuclear translocation. Additionally, SD-1008 inhibits tyrosyl phosphorylation of STAT3, JAK2 and Src. SD-1008 also reduces STAT3-dependent luciferase activity. Biochemical studies with recombinant JAK2 proteins demonstrate that high concentrations of SD-1008 directly inhibit JAK2 kinase autophosphorylation. Exposure of various cell lines to SD-1008 decreases levels of the STAT3 dependent proteins, Bcl-X_L and survivin, inducing apoptosis. SD-1008 also enhances apoptosis induced by paclitaxel in ovarian cancer cells. These results demonstrated that SD-1008 directly blocks the JAK-STAT3 signaling pathway in human cancer cells that express constitutively active Stat and add to the growing literature that identifies this pathway as a viable target for drug development. Finally, SD-1008 may be a suitable prototype for further chemical modification and exploration as a therapeutic agent.

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Many recent studies have demonstrated expression and activation of the interleukin and signal transducer and activator of transcription 3 (IL-STAT3) pathway in a variety of human cancers (Barre et al., 2007; Diaz et al., 2006; Gamero et al., 2004; Gritsko et al., 2006; Yu and Jove, 2004). Specifically, this pathway appears to be active in numerous solid tumors, including tumors of the ovary, breast and prostate, as well as in hematologic malignancies such as leukemia, multiple myeloma, and lymphoma (Bromberg and Darnell, 2000; Catlett-Falcone et al., 1999; Silver et al., 2004; Syed et al., 2002; Turkson and Jove, 2000). In these diseases, STAT3 activation is evidenced by increased phosphorylation of Tyr ^{705} followed by nuclear translocation and DNA binding. Both *in vitro* models and clinical studies of a variety of tumors demonstrate that STAT3 pathway activation is associated with high-grade tumors, drug resistance, and induction of anti-apoptotic proteins such as survivin and Bcl-X_L (Diaz et al., 2006; Duan et al., 2006b; Gritsko et al., 2006; Ikuta et al., 2005; Silver et al., 2004; Vigneron et al., 2005). A pivotal role of this pathway in promoting oncogenesis has been demonstrated through the identification of activating Janus kinase (JAK) point mutations in a variety of myeloproliferative disorders, providing genetic evidence for observed, constitutive Stat activation (Levine et al., 2005a; Tefferi and Gilliland, 2005). Mechanisms including cytokine-mediated autocrine or paracrine stimulation of upstream activators such as Src have also been described and may provide alternative mechanistic explanations of pathway activation. Studies involving transgenic animals also support the direct role of STAT3 in the malignant phenotype (Bromberg, 2002; Bromberg et al., 1998; Cao et al.,

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1996). Inhibition of the STAT3 signaling pathway via the known tool compound and JAK2 inhibitor, AG490, and transfection of cells with a dominant-negative STAT3 expression construct significantly suppress the growth of ovarian and breast cancer cell lines with constitutively active STAT3 (Huang et al., 2000; Mora et al., 2002). Likewise inhibition of the STAT3 pathway in several models of human malignancies induces growth arrest and apoptosis (Nam et al., 2005; Turkson et al., 2005; Yu and Jove, 2004). More recent experiments also support the role of IL-6 in tumor angiogenesis and the drug resistant phenotype (Bellone et al., 2005; Nilsson et al., 2005).

IL-6 associated activation of the JAK-STAT3 pathway signals through a heterodimeric receptor (IL-6R) consisting of two membrane-bound glycoproteins: an 80-kD IL-6-binding subunit, IL-6Rα, and gp130. The gp130 component of the receptor is responsible for stabilization of the α-chain ligand complex and subsequent downstream signal transduction. The binding of IL-6 to its receptor triggers the association of the α chain with gp130 and subsequent phosphorylation of gp130 by Janus activated kinase (JAK1, JAK2, JAK3 or Tyk2), leading to activation of STAT3 (Schindler and Strehlow, 2000). STAT3 binds activated receptors through an interaction between its Src homology 2 (SH2) domain and phosphotyrosine docking sites on the intracellular domains of activated receptors. JAK or gp130 dependent STAT3 phosphorylation (pSTAT3) at a single tyrosine residue (Tyr^{705}) induces homodimerization of STAT3 *via* paired SH2 domains. Dimeric pSTAT3 then translocates from the cytoplasm to the nucleus where it binds DNA as a transcription factor. STAT3 dimers bind a canonical 8-10 base pair inverted repeat element with a consensus sequence 5'-TT(N_{4-6})AA-3', commonly referred to as an interferon (IFN)-gamma activated sequence (GAS) element.

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Engagement of pSTAT3 with DNA then initiates an increase in transcription of a number of genes including the anti-apoptotic regulatory genes Bcl-X_L, survivin, MCL-1, c-myc (Alvarez et al., 2005; Darnell, 1997).

The credentialing of JAK-Stat as a target pathway in human cancers has led to a search for novel pathway antagonists. Previously, our group and others have described STAT3 specific siRNA or small molecules that inhibit cell growth and increase chemotherapy drug induced apoptosis in cancer cell lines that expressing constitutively active STAT3 (Duan et al., 2006b; Faderl et al., 2005; Nam et al., 2005; Song et al., 2005). Several small molecules have been identified to inhibit STAT3 signaling by cell-free systems (Faderl et al., 2005; Nam et al., 2005; Pedranzini et al., 2006; Schust et al., 2006; Song et al., 2005; Turkson et al., 2005). None of the prior approaches have used cell-based systems targeting STAT3 nuclear translocation. Our recently published study was the first to use a cell-based system targeting STAT3 nuclear translocation (Duan et al., 2006a). Cell-based screening techniques have the advantage of identifying small molecule hits that are accessible to intracellular targets, active with intact signaling pathways and in the presence of serum. This cell-based, STAT3 nuclear translocation screen has identified a low micromolar inhibitor of STAT3 nuclear translocation, and has proven amenable to high-throughput screening (Duan et al., 2006a). Here we describe the identification of a JAK kinase inhibitor, a polycyclic dicarboxylic acid belongs to tropidine compound class, referred to here as SD-1008. This compound has been characterized as a potent inhibitor of IL-6, oncostatin and

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STAT3 signaling in human breast and ovarian cancer cell lines. SD-1008 also enhances apoptosis induced by paclitaxel in human cancer cells.

Materials and Methods

Plasmids, Cell Lines, Antibodies and Small Molecule Library. The STAT3 and EGFP fusion protein expression vector pCORON1000 EGFP-STAT3 (Abbreviated as pEGFP-STAT3) was purchased from Amersham Biosciences (Buckinghamshire, UK). This pEGFP-STAT3 vector was generated by fusing STAT3 to the C-terminus of enhanced green fluorescent protein (EGFP). The expressed EGFP fusion protein comprises a full-length functional STAT3. A hamster kidney cell line (BHK-21), human osteosarcoma cell line (U2-OS), and human ovarian cell line (OVCAR8) were stably transfected with pEGFP-STAT3 through selection with G418. STAT3, pSTAT3, pJAK1, pSrc, Bcl-X_L, MCL-1, pAKT and survivin antibodies were obtained from Cell Signaling Technologies (Cambridge, MA). JAK2 and pJAK2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody to human actin was obtained from Sigma (St. Louis, Missouri). AG490 was purchased from Calbiochem (La Jolla, CA). The human ovarian cancer cell lines SKOV-3; SW626; CaOV-3, human breast cancer cell lines MDA-MB-468; MDA-MB-435; MDA-MB-231; MCF-7 and human osteosarcoma cell line U-2OS were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the OV1063; IGROV-1 and OVCAR8 human ovarian cancer cell lines. The paclitaxel resistant cell lines were established in this laboratory as

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described previously (Duan et al., 1999; Duan et al., 2005; Lamendola et al., 2003). The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100-units/ml penicillin and 100-µg/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA). The Structural Diversity Set is a library of 1,990 small molecules derived from the almost 140,000 compounds available on plates through the NCI. Detailed information on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program web site (http://dtp.nci.nih.gov).

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Screening for Inhibition of Phospho-STAT3 Nuclear Translocation Assays**. T**he **pEGFP-STAT3 vector was stably introduced into hamster kidney BHK-21 cells, human osteosarcoma U-2OS cells or human ovarian cell OVCAR8. BHK-21, U-2OS or OVCAR8-derived EGFP-STAT3 expressing cells were seeded at a density of 4000 cells per well in 96-well plates followed by an overnight incubation at 37˚C. Cells were then incubated for an additional four hours in the presence of either vehicle (DMSO) alone or a 10 μM concentration of a single compound from the NCI Diversity Set. After incubation, human recombinant IL-6 (R&D Systems, Minneapolis, MN) was added to the wells to a final concentration of 30ng/ml for** an additional hour. In separate experiments human recombinant oncostatin (R&D Systems) was added to the wells of OVCAR8- EGFP-STAT3 cells to a final concentration of 60 ng/ml for an additional hour. IL-6 or oncostatin-dependent nuclear translocation of EGFP-STAT3 was analyzed using an Olympus 1X71 fluorescence microscope and the data were captured as digital images using IPLab Software from Scanalytics (Rockville, MD). AG490 (20μM) was used in all assays as a positive control.

Quantitative Measurement of EGFP-STAT3 Nuclear Translocation in 384 Well Plate Format. Detailed description of the quantitative measurement of EGFP-STAT3 nuclear translocation is described elsewhere (Duan et al., 2006b). Briefly, stably transfected EGFP-STAT3 BHK-21 cells were seeded in black, clear-bottom 384-well plates from Costar (Cambridge, MA). Following incubation, cells were fixed, washed, counterstained with Hoechst and visualized by epifluorescent microscopy using automated image acquisition. Analysis was performed using a modified script within the MetaXPress software suite (Molecular Devices, Sunnyvale CA). Individual cells were

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identified using a primary mask established by fluorescence intensity due to EGFP. Nuclei were then identified using a secondary mask established by fluorescence intensity due to Hoechst staining within identified EGFP-rich loci. Average EGFP intensity within both regions is captured, along with , the cytoplasmic and nuclear area. An average cytoplasmic:nuclear (C:N) EGFP intensity ratio for each cell was calculated, and the mean C:N ratio across all cells was reported as a summary statistic for an individual well.

Western Blotting. Protein lysates from cells were generated through lysis with 1X RIPA buffer (Upstate Biotechnology, Charlottesville, VA) with protein concentration determined by Dc Protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of total protein was resolved on NuPage^{TM} 4-12% Bis-Tris Gel (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (PH7.4) with 0.1% Tween-20 and 5% nonfat milk (Bio-Rad) and overnight at 4°C as described in the antibodies supplier's instructions. Signal was generated through incubation with horseradish peroxidase-conjugated secondary antibodies incubated in TBS (pH 7.4) with 5% nonfat milk and 0.1% Tween-20, at 1:2000 dilution for 1 hour at room temperature. Positive immunoreactions were detected by using SuperSignal® West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL).

JAK2 Kinase *in vitro* Autophosphorylation Assay. The JAK2 autophosphorylation kinase assay was performed using full-length recombinant JAK2 protein (Upstate Biotechnology) as described in the manufacturer's protocol. Briefly, 15 μL of JAK2 agarose was washed with kinase assay buffer, resuspended and mixed with either DMSO control, SD-1008 or AG490 for 1 hour at room temperature with constant,

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gentle agitation. Following the addition of 1 mM ATP and an additional 60 minutes of incubation at room temperature, the reaction was concluded with a termination buffer. Relative autophosphorylation of JAK2 kinase was evaluated by Western blot as described above using anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology).

Establishment of STAT3-Dependent Luciferase Reporter in Ovarian Cancer Cells. The pLucTKS3 vector (provided by Dr. Richard Jove, Lee Moffitt Cancer Center & Research Institute) contains seven copies of STAT3-binding sites and its activation specifically depends on STAT3 status in the cell (Turkson et al., 1998). The pLucTKS3 and pIRESneo (Clontech, for G418 resistance coselection) plasmids were cotransfected into $OVCAR8_{TR}$ cell line, by using Lipofectamine 2000 reagent and selected with G418. Clones were selected for high luciferase activity and used for evaluation. The selected clones were exposed to SD-1008 at varying concentrations, and luciferase activity was measured after 24 hours of exposure by using a Promega Bright-Glo^{TM} luciferase kit, following the manufacturer's instructions.

Apoptosis Assays. Whole-cell lysates were immunoblotted with specific antibodies to PARP (Cell Signaling Technologies) and its cleavage products. Positive immunoreactions were detected by using Super Signal® West Pico Chemiluminescent Substrate. Apoptosis was also evaluated using The M30-Apoptosense Elisa assay Kit (Peviva AB, Bromma, Sweden) per the manufacturer's instructions. For drug exposure experiments $OVCAR8_{TR}$ cells were seeded at 8000 cells/per well in a 96-well plate for 24 h before treatment. The cells were then treated with 0.01 μM paclitaxel, 30 μM AG490, 5

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μM or 10 μM SD-1008 or combinations of paclitaxel with either AG490 or SD-1008 drugs for additional 24 h.

Results

Analysis of STAT3 Nuclear Translocation and Identification of SD-1008. To identify novel compounds that interrupt IL-6 dependent STAT3 nuclear translocation, a novel real-time cell-based method was developed to image the EGFP-STAT3 chimera in the nucleus and cytoplasm in the hamster kidney cell line BHK-21, human osteosarcoma cell line U-2OS as well as human ovarian cancer cell line OVCAR8. Resting cells demonstrated that the majority of EGFP-STAT3 was cytoplasmic (Fig. 1A a and Fig. 1B a) until the addition of IL-6, which then promptly induced translocation of fluorescent STAT3 to the nucleus in BHK-21 and U-2OS cells (Fig. 1A b and Fig. 1B b). Similar results were also seen in the human ovarian cancer cell line, OVCAR8, transfected with pEGFP-STAT3 and treated with oncostatin (data not shown). Pre-treatment of the cells with the JAK2 inhibitor AG490 (20 μM) blocked IL-6 dependent translocation of EGFP-STAT3 (Fig. 1A c and Fig. 1B c). A screen of a 1992-member library of diverse bioactive small molecules identified several compounds that inhibited STAT3 nuclear translocation. One of the most potent of these compounds, SD-1008 (NCI identifier: NSC 360215, 8-benzyl-4-oxo-8-azabicyclo[3.2.1]oct-2-ene-6,7-dicarboxylic acid, Fig. 1C) suppressed EGFP-STAT3 nuclear translocation at a concentration of 10 μM in both BHK-21 and U2-OS cells (Fig. 1A d, Fig. 1B d). Compound SD-1008 was analyzed by LC/MS in order to verify identity and purity. Surprisingly, the analysis yielded a 28 mass

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units greater molecular mass (329.04 amu) than predicted based on the reported structure (301.10 amu). For further structure elucidation, we analyzed the SD-1008 compound by 1D and 2D 1H-NMR. Based on this analysis we determined the structure to correspond to the bis methyl ester of the reported structure. The vicinal coupling constants predict a relative stereochemistry of SD-1008 (Fig. 1C). In addition, we further analyzed and compared the SD-1008 structure with SD-1029 and AG490. The results demonstrated that there is no plausible structural similarity between the three classes of compounds; the only common motif that shared by SD-1008 and SD-1029 is the aromatic substituent and the basic nitrogen. SD-1008 belongs to tropidine compound class and SD-1029 belongs to xanthene compound class.

Quantitative Assessment of STAT3 Nuclear Translocation and Confirmation of SD-1008 Inhibition. In attempt to generate a quantitative technique for scoring translocation we developed and have recently described an unbiased, semi-automated, high-throughput approach, to measure relative cytoplasmic to nuclear signal following IL-6 treatment. Maximum inhibition of nuclear translocation was seen at a concentration of 20 μM of AG490 and 10 μM for SD-1008 (Fig. 2). Notably, SD-1008 shows more robust inhibition of IL-6 dependent nuclear translocation than AG490.

SD-1008 Suppresses Phosphotyrosine STAT3 (pSTAT3) Levels in Human Breast and Ovarian Cancer Cell Lines. After identification of SD-1008 as an inhibitor of STAT3 nuclear translocation in BHK-21 cells, the effect of SD-1008 on STAT3 phosphorylation was examined in human cancer cell lines. Studies focused on the breast cancer lines MDA-MB-468 and MDA435, the ovarian cancer cell line OV1063 and the paclitaxel resistant ovarian cancer lines SKOV-3_{TR} and $OVCAR8_{TR}$ all of which express

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high levels of pSTAT3. In each of these cell lines, incubation in 10 μM SD-1008 for 24 hours led to reduced levels of pSTAT3 (Fig. 3A). To evaluate the time and dose-dependent inhibition of STAT3 activation, the MDA-MB-468 and $OVCAR8_{TR}$ cell lines were treated with SD-1008 (10 μM) for varying time periods or alternatively with varying doses (1 μM, 5 μM and 10 μM) for 24 hours. The pSTAT3 level decreased as early as 30 min after the addition of 10 μM SD-1008 treatment. Lower concentrations of SD-1008 (1 μM) inhibited STAT3 phosphorylation with longer 24 incubations (Fig. 3B), consistent with the derived phenotypes and quantitative methods of the high content imaging protocol. Importantly, SD-1008 had no effect on the total amount of cellular STAT3 protein, as determined qualitatively by immunoblot (Fig. 3A and Fig. 3B).

SD-1008 Inhibits STAT3-dependent Luciferase Reporter Activity in Ovarian Cancer Cells with Constitutive STAT3 Activation. The effect of SD-1008 on STAT3-dependent transcription was assessed using a luciferase reporter system. Starting with the $OVCAR8_{TR}$ cell line, a cloned and stably transfected daughter line was isolated after transfection with the STAT3-dependent luciferase reporter, pLucTK3. Exposure of this line to SD-1008 or AG490 for 24 hours significantly inhibited STAT3-dependent luciferase activity (Fig. 4). The STAT3-dependent luciferase activity was decreased by > 3-fold after the exposure to 50 μM of AG490 and by > 8-fold after exposure to 50 μM of SD-1008. Similar inhibition was also observed in human osteosarcoma cell line $U\text{-}2OS_{TR}$ transfected with pLucTK3 (Data not shown).

SD-1008 Inhibits STAT3 –Mediated Expression of Anti-Apoptotic Proteins. We next examined whether exposure of cells lines to SD-1008 resulted in decreased expression of the anti-apoptotic proteins Bcl-X_L, MCL-1and survivin. Incubation in SD-

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1008 for 24 hours significantly down-regulated Bcl-X_L and survivin expression in both OVCAR 8_{TR} and MDA-MB-468 cells (Fig. 5). Following 24 h incubation, the expression of MCL-1 protein expression also decreased in OVCAR 8_{TR} with minimal change in MDA-MB-468 cells (Fig. 5).

SD-1008 Suppresses Phosphotyrosine Levels of JAK2 and Src. SD-1008 suppresses pSTAT3 levels suggesting that this compound may interfere with the function of one or more of the upstream tyrosine kinases such as JAK or Src. Evaluation of SD-1008 on the phosphotyrosine levels of pJAK1, pJAK2 and pSrc in the OVCAR8_{TR} and MDA-MB-468 cell lines *in vitro* demonstrated suppression of levels of pJAK2 and pSrc with much less inhibition of pJAK1 (Fig. 6A). To more directly evaluate the effects of SD-1008 on pJAK2, a cell-free *in vitro* assay was performed incubating recombinant JAK2 with SD-1008. Western blot demonstrated that SD-1008 inhibits autophosphorylation of JAK2 more effectively than AG490 (Fig. 6 B), though evidenced at relatively high concentrations of the inhibitors. In separate control experiments SD 1008 did not alter the level of phosphorylation of AKT (data not shown).

SD-1008 Induces Apoptosis in Human Cancer Cells. The effect of SD-1008 on induction of apoptosis was investigated by immunoblotting for PARP cleavage. PARP cleavage was detected following incubation of OVCAR8_{TR} or MDA-MB-468 cells with 10 μM SD-1008. A dose-response analysis revealed the appearance of PARP cleavage products with a 1 μM SD-1008 concentration when cells were allowed to incubate for 24 h (Fig. 7).

SD-1008 Enhances Apoptosis Induced by Paclitaxel in Human Cancer Cells. Constitutively activated STAT3 may contribute to the survival advantage of human

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cancer cells in part through induction of anti-apoptotic regulatory proteins. We hypothesized that inhibition of this pathway and its dependent apoptotic proteins would lower the apoptotic threshold and increase chemotherapy sensitivity. To investigate this hypothesis, several pSTAT3 activated cell lines were treated with either 5 μM or 10 μM SD-1008 for 24h, paclitaxel, or the combination of paclitaxel and SD-1008. Apoptosis was scored using the M30-Apoptosense ELISA assay. SD-1008 treatment resulted in a marked, 20-fold induction of apoptosis in the $OVCAR8_{TR}$ cells that express constitutively activated STAT3 (Fig. 8). Additionally, the combination of the paclitaxel with SD-1008 resulted in significantly greater cell death as compared to paclitaxel or SD-1008 alone.

Discussion

Nuclear translocation of activated STAT3 is required for its function as a transcription factor (Liu et al., 2005; Schindler and Strehlow, 2000). Compounds capable of inhibiting STAT3 phosphorylation and translocation represent a desirable approach for the inhibition of the STAT3 signaling pathway in human cancer. In this study, we report that SD-1008 is a novel JAK/STAT3 signaling pathway inhibitor whose mechanism of action includes the inhibition of STAT3 phosphorylation and nuclear translocation. SD-1008 is the second inhibitor of STAT3 signaling identified through a cell-based assay of STAT3 nuclear translocation. The inhibition of JAK2 and Src phosphorylation by SD-1008 in cell culture and inhibition of pJAK2 in cell free autophosphorylation *in vitro* assays suggests that the principal mechanism of SD-1008 activity is kinase inhibition. Specifically, incubation with SD-1008 downregulates STAT3-dependent protein expression, cell proliferation, and induces apoptotic cell death. Lowering of the apoptotic

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threshold also increases the sensitivity of these cells to the cytotoxic effects of paclitaxel. Potentially, through downstream effects of STAT3 inhibition, including decreased expression of several anti-apoptotic genes including Bcl-X_L, survivin and MCL-1. Many studies have found these growth and survival-promoting genes are highly expressed in human cancer, especially in high-grade tumors (Gritsko et al., 2006; Silver et al., 2004). For example, we recently demonstrated that STAT3 and more notably pSTAT3 expression levels are increased in recurrent tumors collected post chemotherapy as compared to matched primary tumors collected prior to chemotherapy (Duan et al., 2006b). The Src induced STAT3 activation also increases survival of fibrosarcoma cells in response to doxorubicin (Vigneron et al., 2005). Constitutive activation of STAT3 could be a predictive marker of drug resistance (Barre et al., 2007). The finding that SD-1008 induces apoptosis in cells that express constitutively activated STAT3, both as a single agent and when combined with paclitaxel, suggests this agent or other agents that target the STAT3 pathway may be useful in the clinic. The expression of anti-apoptotic proteins Bcl-X_L and survivin is significantly decreased in cancer cells treated with SD-1008 is consistent with a hypothesis that the decrease in Bcl-X_L and survivin expression contributes to the induction of apoptosis(Diaz et al., 2006; Gritsko et al., 2006). This hypothesis is also supported by a study indicating that a novel Bcl-X_L (STAT3 target protein) inhibitor, ABT-737, significantly enhanced activities of paclitaxel in lung cancer cells(Oltersdorf et al., 2005).

Previously we identified SD-1029, a halogenated xanthene compound as the first inhibitor of STAT3 signaling selected by a cell-based assay of STAT3 nuclear translocation (Duan et al., 2006a). Maximum inhibition of nuclear translocation was seen

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at a concentration of 20 μM of AG490 and 5μM for SD-1029 as compared to 10μM for SD-1008. While the inhibition of nuclear transport could be explained by inhibition of JAK2 it is notable that SD-1008 significantly inhibits the level of phosphorylation of Src, which distinguish it from SD-1029 and may provide an alternate mechanism for STAT3 inhibition. In addition, SD-1008 is structurally distinct from SD-1029 and AG490. This is important as it could define a new scaffold for JAK inhibitors or suggest a non-nucleoside pocket-binding motif.

This study provides evidence that phenotypic screening using a cell-based assay is an effective approach for identifying inhibitors of the JAK-Stat pathway. Similar to SD-1029, SD-1008 distinguishes itself from other inhibitors of the JAK-Stat pathway as it was identified through an ability to inhibit nuclear translocation. More importantly, this biologically relevant cell-based biomarker of pathway activation is compatible with miniaturization and antibody-independent high-content, high-throughput screening. Further screening studies are underway using both biased and large, diverse chemical libraries.

In conclusion, we have identified SD-1008 as a novel inhibitor of STAT3 activation. Biochemical and cellular assays support the hypothesis that this molecule inhibits JAK and Src activity with resultant inhibition of Stat phosphorylation, nuclear transport and a decrease in STAT3-dependent transcription leading to apoptosis and enhanced chemosensitivity (Alas and Bonavida, 2003; George et al., 2005). Importantly, the superior potency of SD-1008 compared to AG490 enables experimentation in animal models of human malignancies where the JAK-Stat pathway contributes to the malignant phenotype. Furthermore, wealth of evidence supporting JAK2 inhibition has a treatment

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potential in a variety of myeloproliferative disorders (Levine et al., 2005b; Tefferi and Gilliland, 2005). These results add further evidence that the JAK-Stat pathway is a potential therapeutic pathway in a number of human malignancies.

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Footnotes:

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Figure Legends:

Fig. 1. SD-1008 inhibits EGFP-Stat3 nuclear translocation in BHK-21 and U-2OS cells. BHK-21 or U-2OS cells which stably express the EGFP-Stat3 fusion protein were incubated for 4 hours with DMSO (control), SD-1008 (10 μM) or AG490 (20 μM) followed immediately thereafter with the addition of IL-6 to a final concentration of 30 ng/ml rIL-6. Cells were photographed one hour later. Subcellular localization of the fusion protein was assessed by fluorescence microscopy. **A**, BHK-21 derived pEGFP-Stat3 expression cells. **B**, U-2OS derived pEGFP-Stat3 expression cells. **C**, SD-1008 Structure

Fig. 2. Quantitative measurement of IL-6 induced Stat3 nuclear translocation. Stably transfected EGFP- Stat3 BHK-21 cells were incubated with AG490 followed by IL-6. After formaldehyde fixation and counterstaining with Hoechst, cells were visualized by epifluorescent microscopy with automated image acquisition. Average cytoplasmic:nuclear EGFP-Stat3 ratios are displayed in a dose-ranging experiment using SD-1008 and AG490. All experiments were performed in triplicate with error bars representing one standard deviation from the mean cytoplasmic:nuclear ratio.

Fig. 3. SD-1008 inhibits phosphorylation of Stat3 levels in different cell lines. **A**, SD-1008 reduces pStat3 levels in different human ovarian and breast cancer cell lines. The cells were treated for 24 h with SD-1008 (10 μM) and then harvested and processed for pStat3 Western blotting as described in Material and Methods. **B**, dose and time dependent inhibition of Stat3 phosphorylation by SD-1008. $OVCAR8_{TR}$ cells and MDA-MB-468 cells were treated with 10 μM SD-1008 in a time-dependent manner or with SD-

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1008 for 24 h in a dose-response manner. For Western blot analysis, 25 μg of cellular proteins was subjected to immunoblotting with specific antibodies to pStat3 and Stat3 as described in "Materials and Methods".

Fig. 4. SD-1008 inhibits Stat3-dependent luciferase activity in $OVCAR8_{TR}$ cells. $OVCAR8_{TR}$ cells stably cotransfected with pLucTKS3 Stat3-dependent luciferase reporter vector and pIRESneo vector. The cells were treated with 0, 5, 10, 30 or 50μM concentration of SD-1008 or AG490 for 24 h and with quantitation of luciferase activity as described in the methods. The results were based on the means and standard deviations from three experiments performed in triplicate.

Fig. 5. SD-1008 down-regulated Stat3 targeted anti-apoptotic proteins Bcl-X_L and survivin. $OVCAR8_{TR}$ or MDA-MB-468 cells were treated with SD-1008 in a time- or dose-dependent manner. For Western blot analysis, 25 μg of total cellular proteins was subjected to immunoblotting with specific antibodies to Bcl-X_L, MCL-1, survivin or β-actin. The results were detected by a chemiluminescence detection system as described in " Materials and Methods".

Fig. 6. SD-1008 blocks Jak2 and Src tyrosine phosphorylation. **A**, SD-1008 inhibits phoosphorylation of Jak2 and Src in cell lines. $OVCAR8_{TR}$ cells and MDA-MB-468 cells were treated with 10 μM SD-1008 in a time-dependent manner or with SD-1008 for 24 h in a dose-response manner. For Western blot analysis, 25 μg of cell-free extracts was subjected to immunoblotting with specific antibodies to pJak1,pJak2 and pSrc as described in "Materials and Methods". **B**, SD-1008 directly inhibits Jak2 kinase autophosphorylation *in vitro*. Equal amounts of Jak2 recombinant protein were divided and preincubated in the presence or absence of various concentration of SD-1008,

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AG490, or DMSO control for 1 hour. Following the addition of 1 mM ATP and an additional 60 minutes of incubation at room temperature, the reaction was halted using stop buffer. Jak2 autophosphorylation was visualized as described in "Materials and Methods".

Fig. 7. SD-1008 induces apoptosis in cancer cells. $OVCAR8_{TR}$ or MDA-MB-468 cells were treated with SD-1008 in a time- or dose-dependent manner. Total cellular proteins were subjected to immunoblotting with specific antibodies to PARP and β-actin as described in " Materials and Methods".

Fig. 8. SD-1008 inhibits cell growth and induces apoptosis in $OVCAR8_{TR}$ ovarian cancer cells. $OVCAR8_{TR}$ cells were seeded at a density of 8000 cells per well in a 96-well plate for 24 h. Cells were then treated with different drugs for 24 h. The cells were lysed with 10% NP-40 and the M30-Apoptosense Elisa assay was performed as described in "Materials and Methods".

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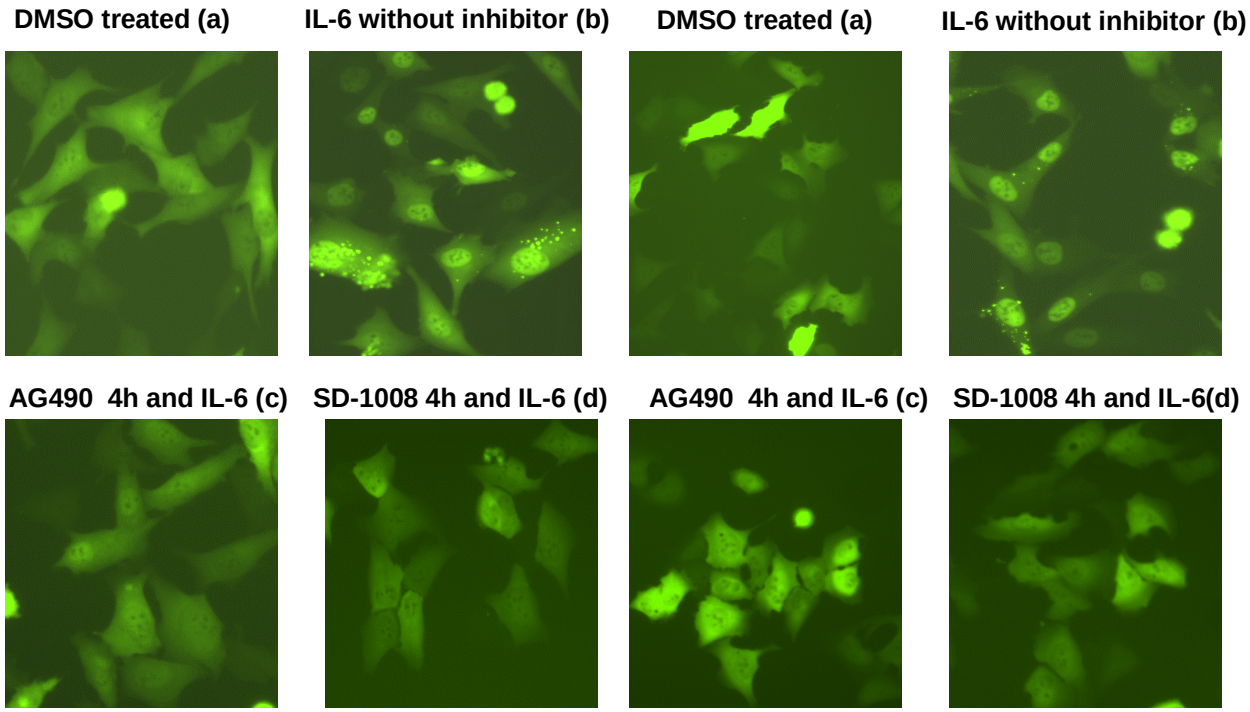

A. BHK-21 cells

B. U-2OS cells

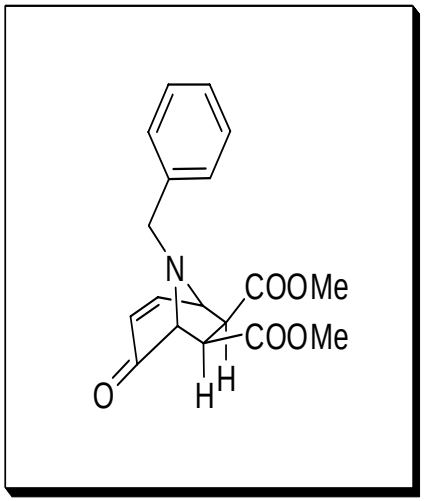

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Fig. 1

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Fig. 2

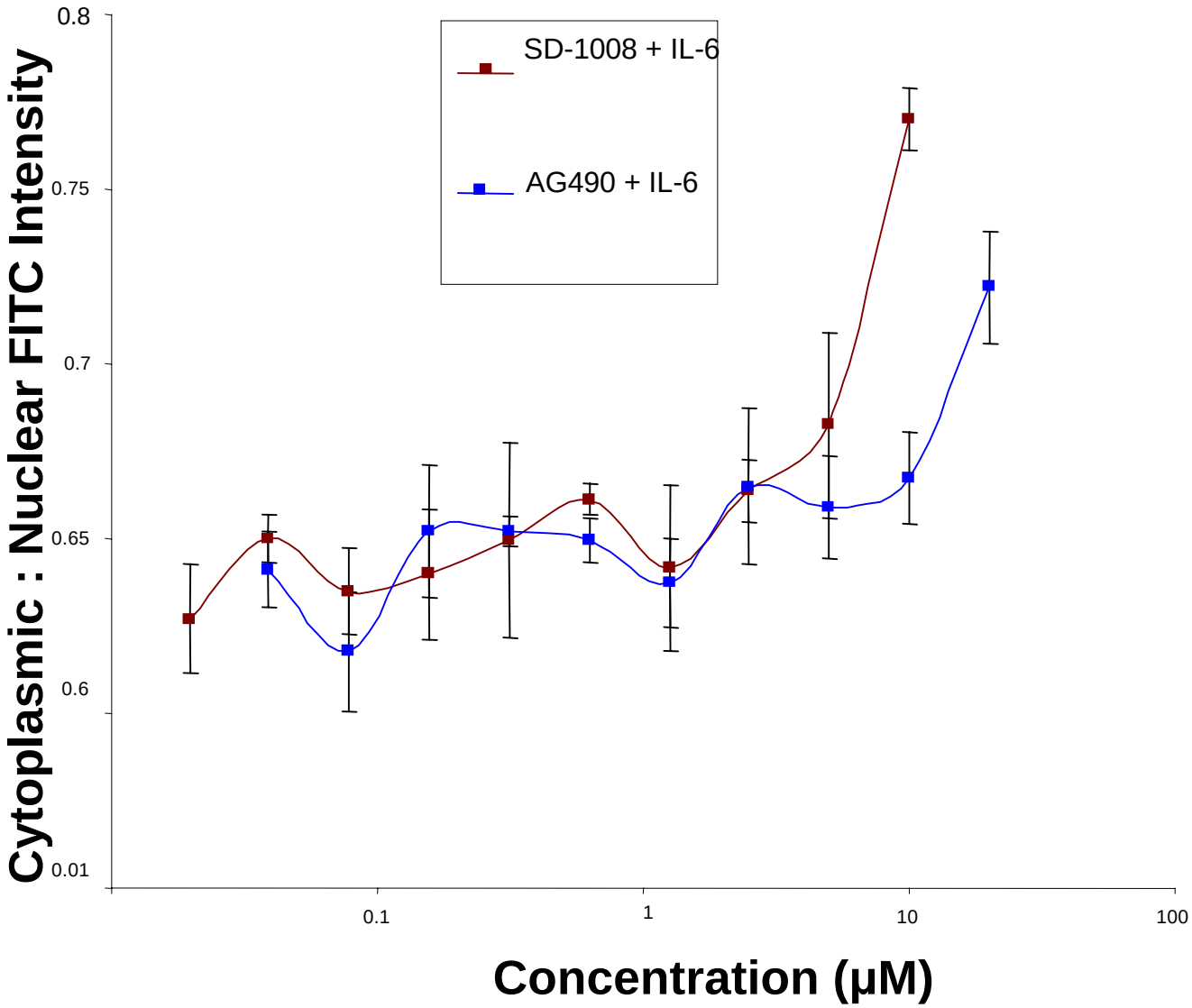

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Fig. 3

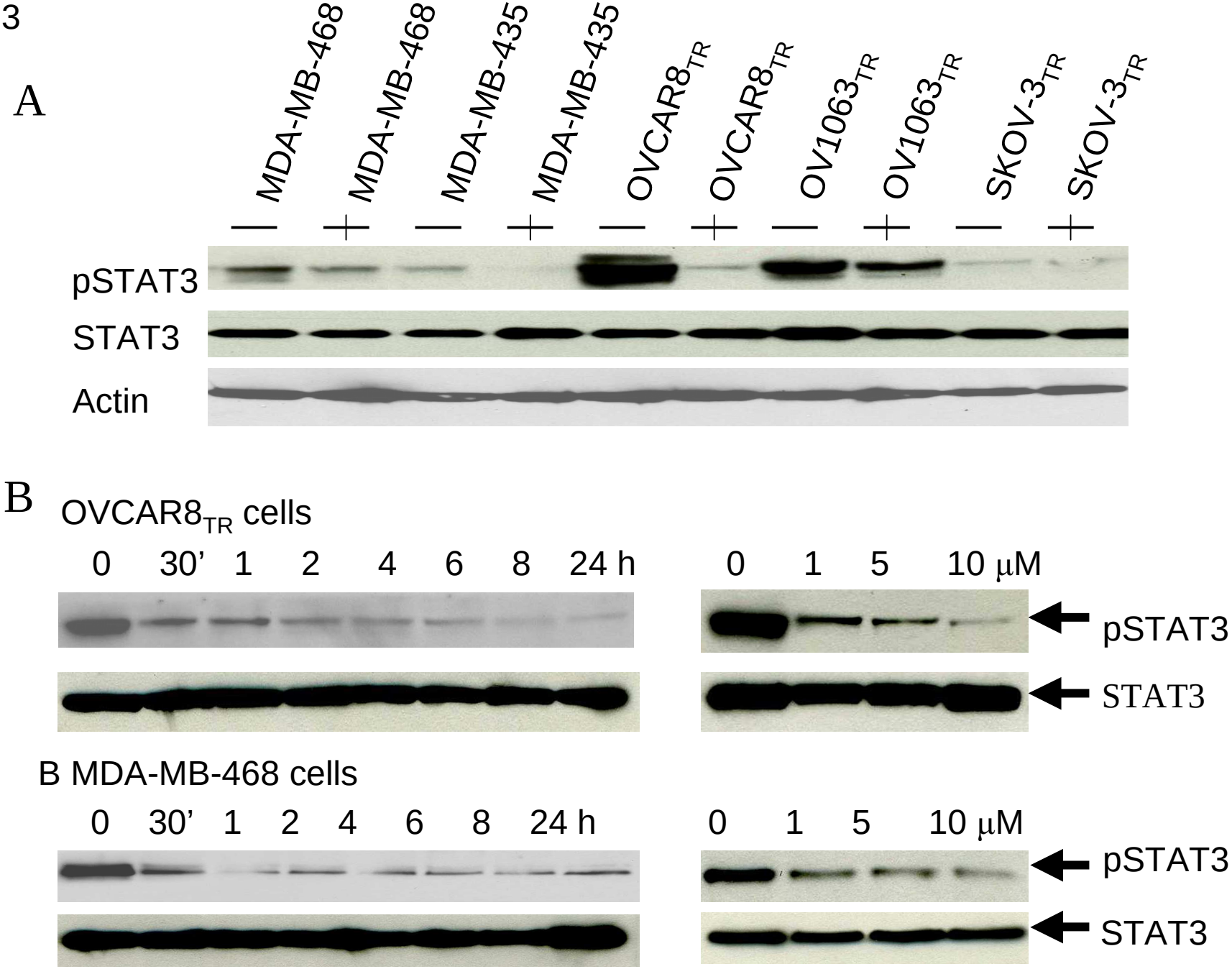

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Fig. 4

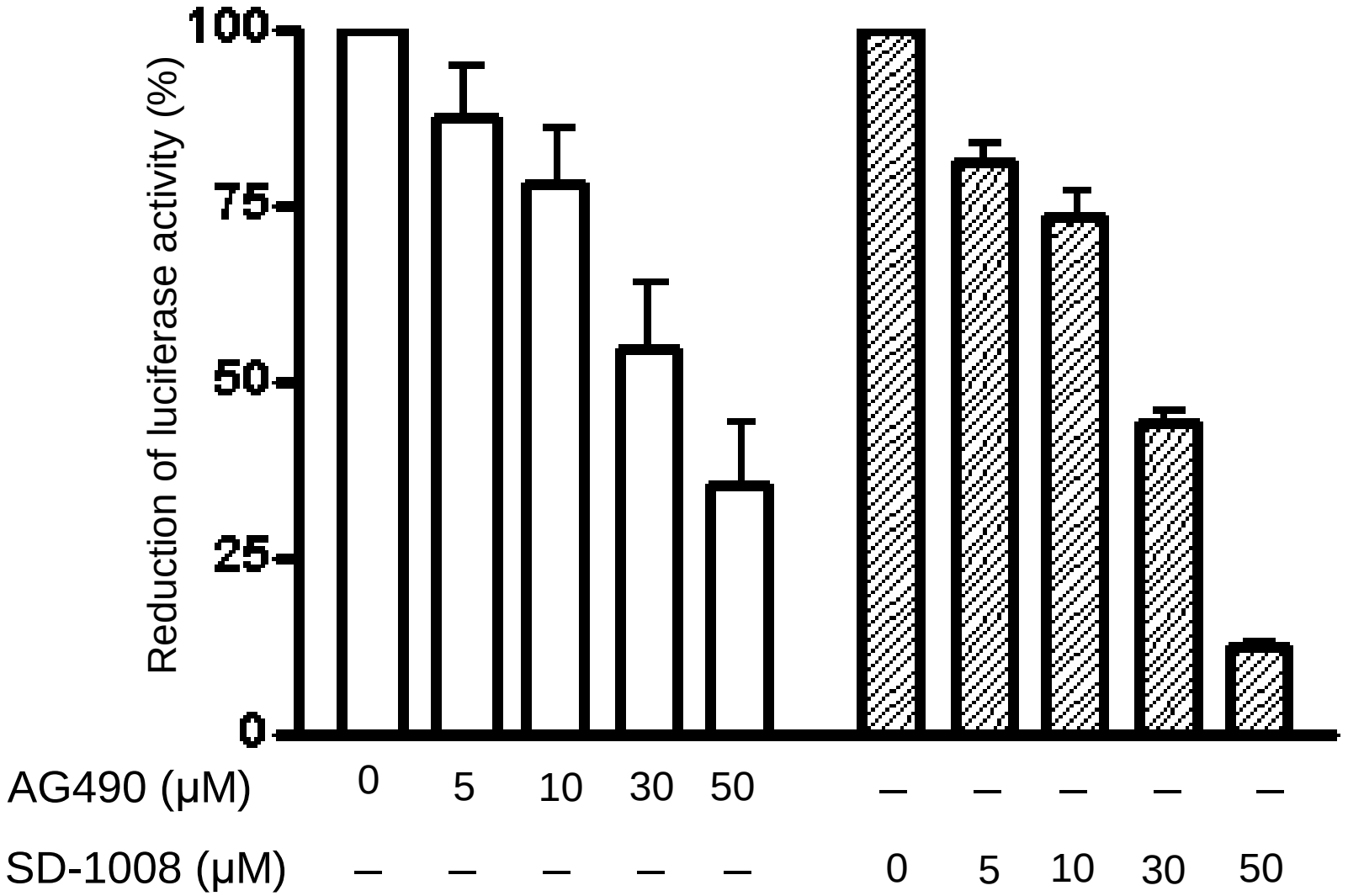

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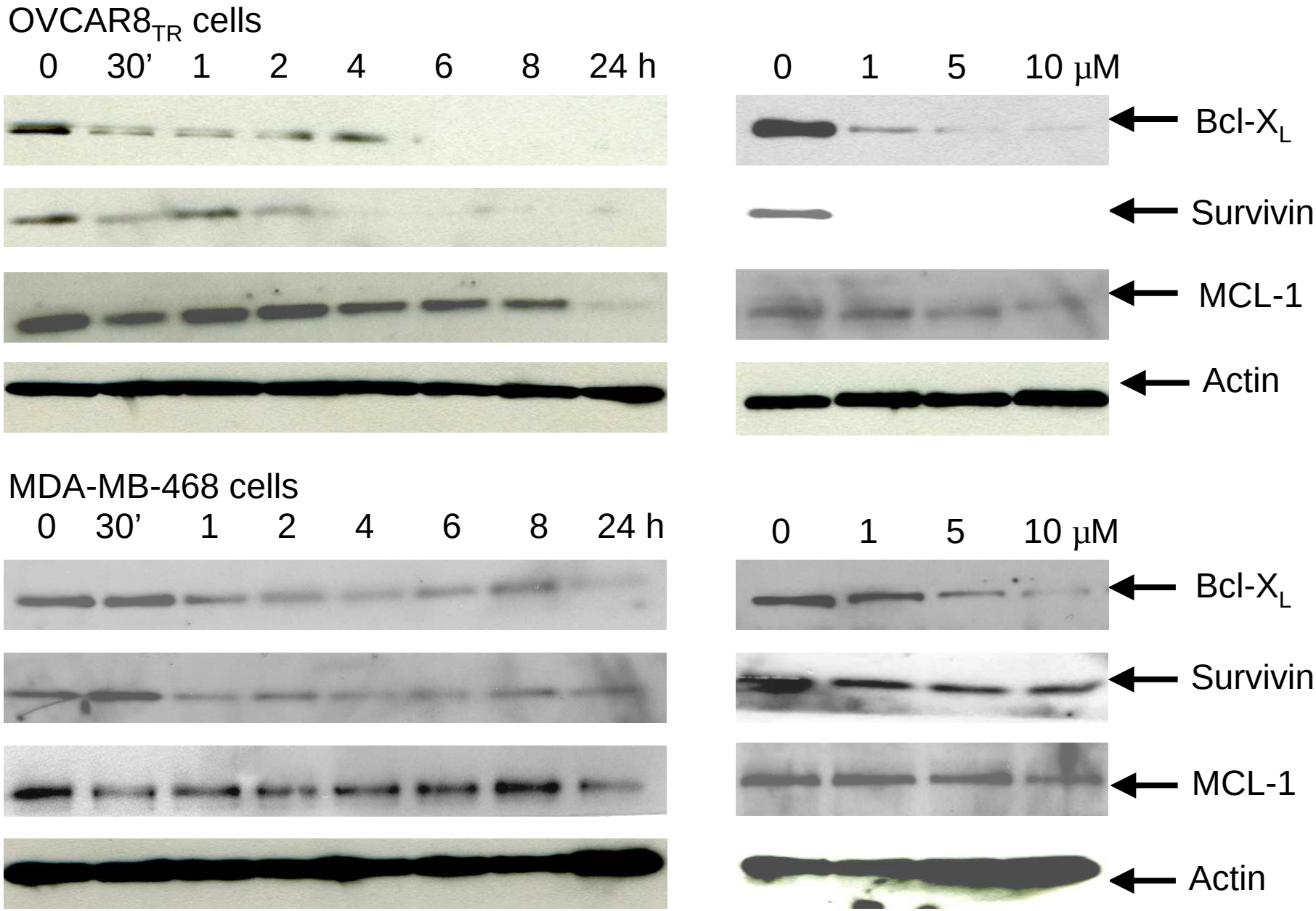
Fig. 5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Actin

Fig. 6

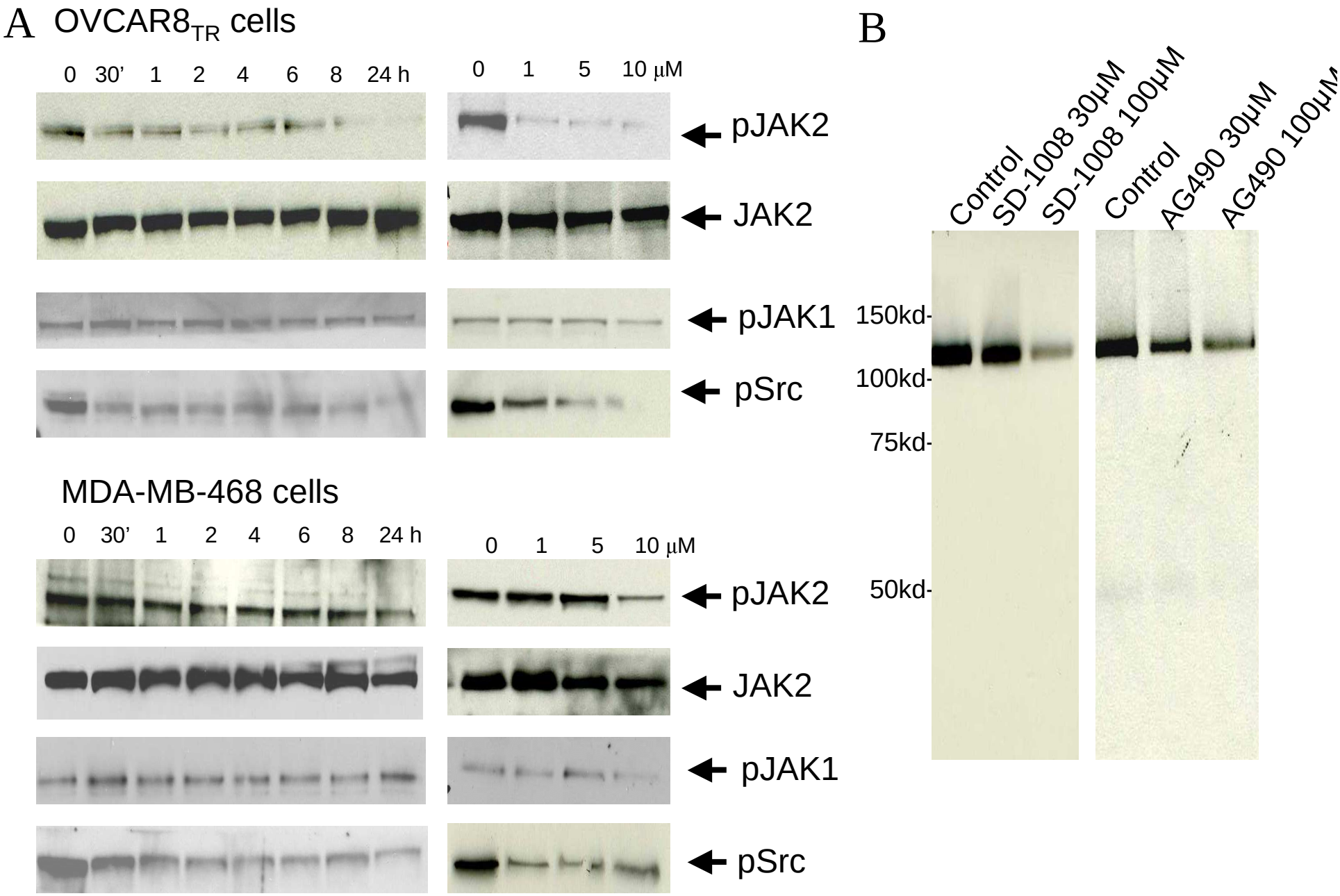

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Fig. 7

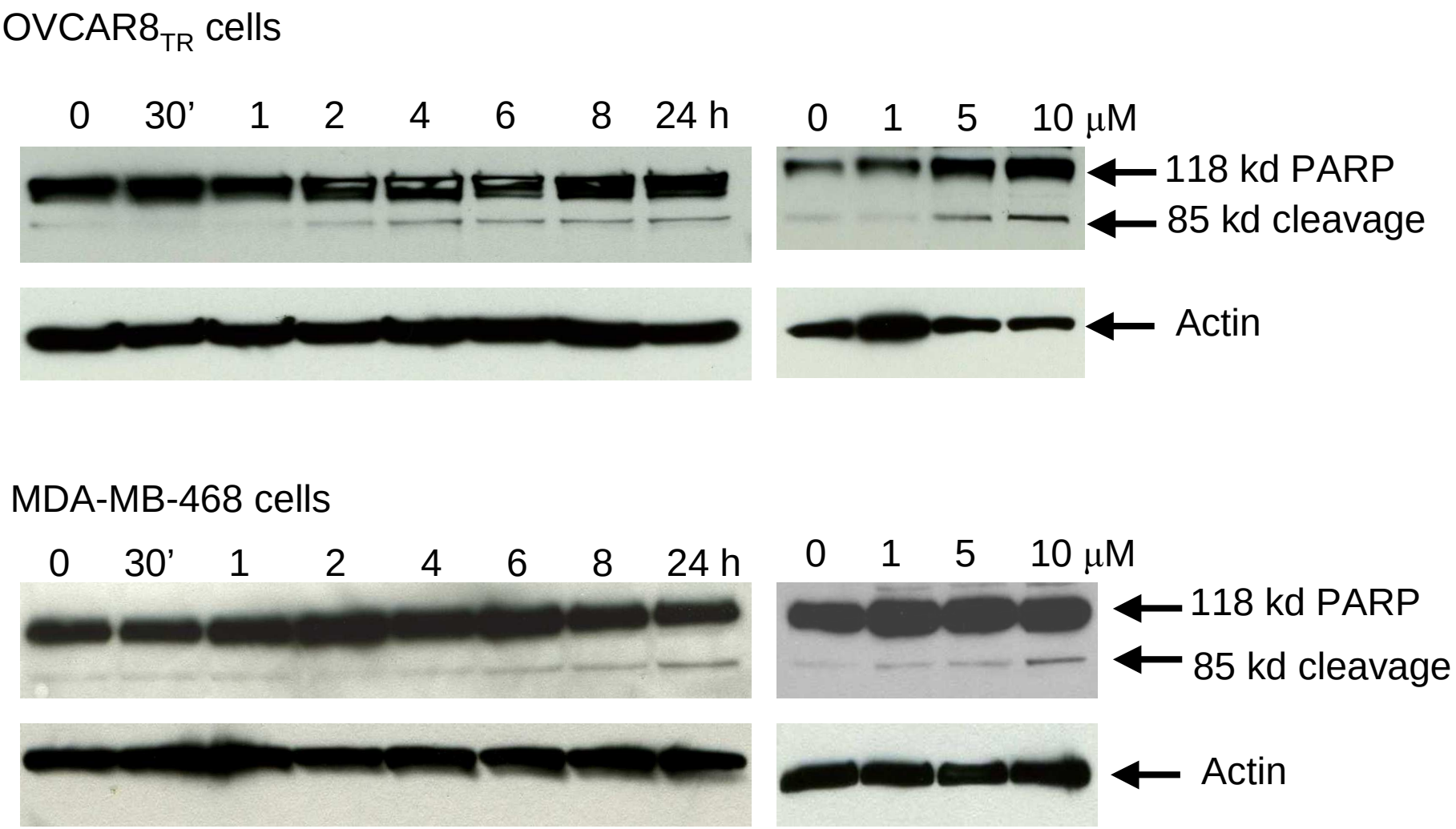

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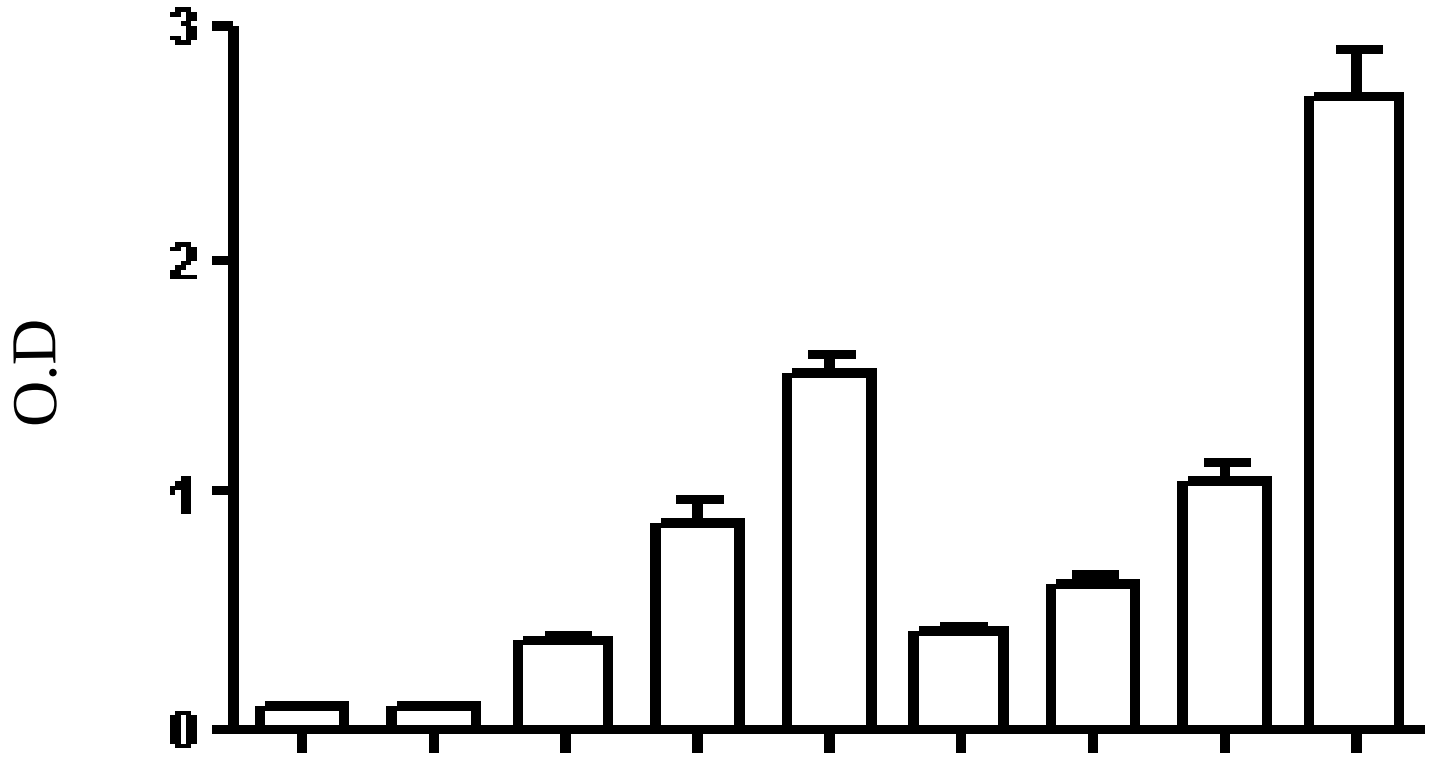
Fig. 8



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