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The phosphoinositide-dependent kinase-1 inhibitor, OSU-03012, prevents Y-box binding protein-1 (YB-1) from inducing epidermal growth factor receptor (EGFR).

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Running title:

OSU-03012 disrupts YB-1 from binding to the EGFR promoter.

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basal-like breast cancer (BLBC), chromatin immunoprecipitation (ChIP), epidermal growth factor receptor (EGFR), electrophoretic mobility shift assay (EMSA), estrogen receptor (ER), human epidermal growth factor receptor-2 (Her-2), phosphoinositide-dependent protein kinase-1 (PDK-1), progesterone receptor (PR), proliferating nuclear antigen (PCNA), S6 ribosomal protein (RP), topoisomerase II (TOPO-II), Y-box binding protein-1 (YB-1).

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Abstract

The epidermal growth factor receptor (EGFR) is integral to basal-like and Her-2 over-expressing breast cancers. Such tumors are associated with poor prognosis, the majority of which express high levels EGFR. We reported that EGFR expression is induced by the oncogenic transcription factor Y-box binding protein-1 (YB-1) that occurs in a manner dependent upon phosphorylation by Akt. Herein, we questioned whether blocking Akt with OSU-03012, a phosphoinositide-dependent protein kinase-1 (PDK-1) small molecule inhibitor, could prevent YB-1 from binding to the EGFR promoter. MDA-MB-468 and SUM 149 are basal-like breast cancer (BLBC) cells that were used for our studies because they express high levels of activated PDK-1, YB-1 and EGFR compared to the immortalized breast epithelial cell line 184htrt. In these cell lines, YB-1 preferentially bound to the -1kB of the EGFR promoter whereas this did not occur in the 184htrt cells based on chromatin immunoprecipitation. When the cells were exposed to OSU-03012 for 6 hours, YB-1/EGFR promoter binding was significantly attenuated. To further confirm this observation, gel shift assays showed that the drug inhibits YB-1/EGFR promoter binding. The inhibitory effect of OSU-03012 on EGFR was also observed at the mRNA and protein levels. OSU-03012 ultimately inhibited the growth of BLBC in monolayer and soft agar coordinate with the induction of apoptosis. Furthermore, OSU-03012 inhibited the expression of EGFR by 48% in tumor xenografts derived from MDA-MB-435/Her-2 cells. This correlated with loss of YB-1 binding to the EGFR promoter. Hence, we find that OSU-03012 inhibits YB-1 resulting in a loss of EGFR expression in vitro and in vivo.

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Introduction

Breast tumors express characteristic molecular markers that allow for the design and development of targeted drug therapies to control the malignant, deregulated pathways, leaving normal cell systems unscathed. There are five subtypes of breast cancer, luminal A, luminal B, normal-like, basal-like and Her-2 (Perou et al., 2000). There is now substantial evidence that patients with the basal-like and Her-2 subtypes have significantly poorer survival times compared to the other types (Perou et al., 2000; Sorlie et al., 2001; Van 't Veer et al., 2002). The basal-like breast cancers (BLBC) are particularly challenging clinically because they do not express the estrogen receptor (ER), progesterone receptor (PR), nor have amplified human epidermal growth factor receptor-2 (Her-2). Thus, patients with these so-called "triple negative" breast tumors are ineligible for targeted therapies such as tamoxifen or Herceptin. In addition, this breast cancer subtype is associated with shorter overall survival, shorter disease-free interval, and is more aggressive than other breast carcinomas such as those of the luminal subtype (Sorlie et al., 2001). This alludes to two challenges: to further understand the pathways that contribute to tumorigenesis in the BLBC and to find targeted therapies for those pathways.

The epidermal growth factor receptor (EGFR, or Her-1) is a molecular marker for BLBC; where it is highly expressed in over half of the tumors in this subtype (Nielsen et al., 2004). EGFR belongs to a family of tyrosine kinase receptors (Her-1, Her-2, Her-3 and Her-4) that are involved in tumor cell growth. It is noteworthy that EGFR is not only important in BLBC but also plays an essential role in mediating Her-2 driven breast cancer. In this breast cancer subtype, the significance of EGFR lies in the fact that Her-2 does not have its own ligand, which

is required for its activation. Rather Her-2 relies on forming a heterodimer with EGFR to trigger signal transduction (Yarden, 2001). Thus, EGFR is integral for promoting the growth of BLBC and Her-2 expressing breast cancers.

YB-1 is over-expressed in various human cancers and has been consistently associated with their increased growth potential (Kuwano et al., 2003). In the context of breast cancer, YB-1 is preferentially expressed in breast tumors over normal ductal epithelial cells (Bargou et al., 1997). It stimulates the proliferation of pre-neoplastic breast cancer cells, and mice that express YB-1 in their mammary glands all develop invasive tumors (Bergmann et al., 2005). We recently determined that YB-1 is associated with EGFR by screening primary breast tumor tissue microarrays (Wu et al., 2006). YB-1 is also expressed in approximately 73% of BLBC (submitted). This builds on our previous work in breast cancer cell lines demonstrating that YB-1 must be phosphorylated in order to induce EGFR. Furthermore, we showed that YB-1 is a direct substrate of the serine/threonine kinase Akt. Upon growth factor stimulation, phosphoinositide dependent kinase-1 (PDK-1) activates Akt, thereby phosphorylating YB-1 at S102 within the DNA binding domain (Sutherland et al., 2005). The loss of the S102 by site directed mutagenesis attenuates YB-1's ability to bind to the EGFR promoter (Wu et al., 2006). Collectively, these data indicate that the Akt pathway is important for regulating the nuclear functions of YB-1. This prompted us to examine the ability to regulate BLBC EGFR over-expression through the disruption of YB-1 activation by the Akt kinase.

We previously reported that OSU-03012 inhibits the Akt pathway (Crowder and Ellis, 2005; Kucab et al., 2005). The OSU-03012 compound was derived from the cyclooxygenase-2

inhibitor, Celecoxib, and structurally optimized in PDK-1 inhibition (Zhu et al., 2004). This compound blocks Akt but not the MAP kinase or p38 pathways (Kucab et al., 2005). An attractive feature of the inhibitor is that it has demonstrated activity against a wide-range of cancer cell lines. In a screen of 60 cancer cell lines conducted at the National Cancer Institute, OSU-03012 inhibited tumor cell growth with an IC_{50}=2 μM (Zhu et al., 2004). OSU-03012 can also over-come Herceptin (Tseng et al., 2006) and Gleevec (Tseng et al., 2005) resistance, at least in part, by decreasing the Akt signaling pathway (Tseng et al., 2006). We therefore embarked on the possibility that OSU-03012 could be used to control YB-1 action by addressing whether it could inhibit the expression of the YB-1 responsive gene EGFR.

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Material and Methods

Cell lines and reagents. 184-htrt cells were a gift from Dr. J. Carl Barrett (National Institute of Health, Bethesda, MA) and were maintained in MEBM (Clonetics, Walkersville, MD) supplemented with Single Quots (Clonetics, San Diego, CA), and 400 g/mL G418. Breast cancer cell lines, MDA-MB-468, MDA-MB-231 and MCF-7, were obtained from the Amercian Type Culture Collection (Manassas, VA) and were cultured in 10% fetal bovine serum DMEM (Gibco, Carlsbad, CA). SUM 149 cells were purchased from Astrand (Ann Arbor, MI) and were cultured in F-12 (Ham's) media (Gibco/Invitrogen, Burlington, ON), supplemented with 5 µg/mL of insulin (Sigma, Oakville, ON), 1 µg/mL of hydrocortisone (Sigma), 10 mM Hepes (Sigma), 5% fetal bovine serum (Gibco/Invitrogen). PDK-1 inhibitor OSU-03012 was a generous gift from Dr. Ching Shih-Chen, The Ohio State University. The small molecule inhibitor chemical structure and method of synthesis have been previously described (Zhu et al., 2004).

Western blotting. Cells were harvested at 80% confluency by trypsin-EDTA (Gibco/Invitrogen), lysed in an ELB buffer as previous described (Wu et al., 2006) and then lysates were sheared through a 21-gauge needle and quantified using the Bradford assay. Proteins extracts (50 µg/lane) were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane at 100V for 2 hours at room temperature. The membranes were blocked with 5% milk in PBS-0.1% Tween (PBS-T) for one hour at room temperature before they were probed with primary antibodies overnight at 4°C. Primary antibodies were used at the following concentrations (all diluted in 5% BSA in PBST): anti-Her-2 (1:1000, Cell Signaling

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Technologies, Beverly, MA), anti-ER (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-PR (1:1000, Santa Cruz Technology), anti-CK5/6 (1:1000, Chemicon, Temecula, CA), rabbit polyclonal anti-phospho-YB-1(S102) (1:2000, designed against the serine 102 phosphorylation site of YB-1 protein (KYLRpSVGDGE-C) **,** a generous gift from Dr. Peter Mertens, University Hospital Aachen, RWTH Aachen, Aachen, Germany), anti-total YB-1 (1:10000, a C-terminal polyclonal antibody, a generous gift from Dr. Colleen Nelson, University of British Columbia), anti-EGFR (1:275, StressGen, San Diego, CA), anti-phospho-PDK-1 (1:1000, Cell Signaling Technologies), anti-phospho-Akt_{Thr308} (1:200, Cell Signaling Technologies), anti-phospho-Akt_{Ser473} (1:1000, Cell Signaling Technologies), anti-total-Akt (1:1000, Cell Signaling Technologies), anti-phospho-S6 kinase, anti-phospho-S6 ribosomal protein (1:1000, Cell Signaling Technologies), anti-total-S6 ribosomal protein (1:500, Cell Signaling Technologies), and anti-vinculin (1:1000, Sigma). After washing in PBST, the membranes were incubated in either a horseradish-peroxidase-conjugated mouse (1:5000) or rabbit (1:2000) IgG antibody (Amersham Biosciences, Piscataway, NJ) in 5% milk in PBST for 1 hour at room temperature. Images were visualized using the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences). Vinculin was used as the loading control.

Chromatin immunoprecipitation. Cell lines MDA-MB-468 and SUM 149 were plated at a density of 1 x 10^7 per 150 mm dish and allowed to attach overnight. They were then incubated in the presence of OSU-03012 at 10 μM or DMSO vehicle control for 5 – 9 hours and cross-linked with 1% formaldehyde for chromatin immunoprecipitation (ChIP). The YB-1 promoter complexes were isolated as previously described (Wu et al., 2006). The primers to the "1b" and "2a" YB-1 binding sites in the EGFR promoter were also previously described (Wu et al., 2006).

Purified immunoprecipitated DNA was eluted in 40 µL of sterile, DNase/RNase-free deionized water and 6 µL was used as template for PCR reaction as previously described (Wu et al., 2006). Input DNA was diluted 10 times prior to PCR amplification. GAPDH forward 5'-ATGACATCAAGAAGGTGGTG-3' and reverse 5' CATACCAGGAAATGAGCTTG-3' primers were used to amplify a 177 bp fragment spanning exons 8 and 9 of the GAPDH gene in the input DNA from the three different cell lines, 184-htrt, MDA-MB-468, and SUM 149 to determine that equal cell numbers had been used in the ChIP experiments.

Electrophoretic mobility shift assay (EMSA). Nuclear and cytoplasmic protein was extracted from log-growing SUM 149 cells using the NE-PER nuclear and cytoplasmic extraction reagents (Product #78833, Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. Briefly, cells were centrifuged to obtain a packed cell volume and lysed in ice cold CER I with protease inhibitors. Following incubation on ice for 5 minutes, ice-cold CER II was added and samples centrifuged at 13,000 x g for 10 minutes. Cytoplasmic protein was retained and the pellet resuspended in ice-cold NER with protease inhibitors. The sample was incubated on ice for 40 minutes with frequent mixes and then centrifuged at 13000 x g for 10 minutes. The supernatant containing nuclear protein was stored. Proteins were quantified using the Bradford Assay. EMSAs were carried out using the Lightshift Chemiluminescent EMSA kit (Product #20148, Pierce Biotechnology), following the manufacturer's protocol. 5' Biotin-labelled complementary oligonucleotides with the following sequence: TTCACACATTGGCTTCAAAGTACCCATGGCTGGTTGCAATAAACAT (-979 to -937), corresponding to the EGFR "2a" sequence, were annealed to form double stranded DNA. Binding reactions consisted of 1 x binding buffer, 50 ng/µL poly dIdC, 20 fmol Biotin-labeled

DNA and 10 µg nuclear protein in a 20 µL reaction. In the competition reaction, the protein was first incubated with 16 pmol of the unlabelled oligonucleotide for 20 minutes. Samples were subsequently incubated in the binding reaction mix for 20 minutes at room temperature. For the super-shift controls, anti-YB-1 (20 µg, anti-chicken antibody from Dr. Isabella Berquin, Wake Forest University, Winston-Salem NC) or Creb (20 µg, Cell Signaling) were incubated with 10 µg nuclear extracts from DMSO treated cells. The reaction mixture was run on a 6% non-denaturing polyacrylamide gel and transferred to positively charged nylon membrane (Amersham Biosciences). DNA was cross-linked to the membrane at 120 mJ/cm^2 using the Stratalinker UV-light cross-linker (Stratagene, La Jolla, CA) and detected using chemiluminescence (Pierce Biotechnology).

Real-time quantitative reverse transcription-PCR. RNA was isolated from SUM 149 cells grown in log phase using the Qiagen RNease Mini kit (Qiagen (Canada), Inc., Mississauga, ON). The RNA was reverse transcribed and amplified using EGFR, PCNA, or TOPO-II-specific primers and probes (Applied Biosystems, Foster City, CA). TATA box binding protein mRNA was quantified as a housekeeping gene (Applied Biosystems). Each sample was analyzed in triplicate on three separate occasions.

PDK-1 siRNA gene knockdown. The siRNA transfection protocol closely followed the Qiagen HiPerFect Transfection Reagent Handbook Protocol for Reverse Transfection of Adherent Cells with siRNA in 6-well Plates (Qiagen (Canada) Inc., Missisauga, ON). We used the Qiagen Negative Control siRNA (Catalog No. 1022076, Qiagen) and the Qiagen Hs_PDPK1_8 HP and Hs_PDPK1_9 HP Validated siRNAs (Catalog Nos. SI00605780 and SI00605787, Qiagen) for PDK-1 gene knockdown. SUM 149 cells were seeded shortly before transfection at 6 x 10^5 cells

per well of a 6-well plate in 2.3 mL of SUM 149 cell media and incubated under normal growth conditions. PDK-1 and negative control siRNA (20 nM) were diluted in 100 µL of medium without serum and 12 µL of HiPerFect Transfection Reagent was added subsequently. siRNA and the transfection reagent were mixed by vortexing and samples were incubated at room temperature for 10 minutes. The siRNA complexes were then added drop-wise to the cells and incubated under normal growth conditions for 96 hours. Cells were harvested for protein extraction 96 hours later.

MTS *in vitro* cytotoxicity and soft agar assays. MDA-MB-468 and SUM 149 cells were plated in 96-well plates at 1 x 10^4 cells/well and 5 x 10^3 cells/well respectively and incubated for 24 hours at 37ºC in their normal culture conditions. Cells were treated with OSU-03012 for 24 hours at 0, 2.5, 5 and 10 µM in 96-well plate with 6 replicates per dose. DMSO was used as vehicle control. After 24 hours, culture media was removed from the cells and replaced with 120 µL of MTS complex solution (Promega, Madison, WI) and incubated at 37ºC for 60 minutes. Plates were shaken and absorbance read at 490 nm for 0.1 seconds. For the soft agar assays, the MDA-MB-468 and SUM 149 cells were plated at a density of 3x10^5 and 1x10^5 respectively in 0.6% agar containing either DMSO, or OSU-03012 (2.5, 5 or 10 µM) as previously described (Sutherland et al., 2005). Colonies developed over 21 or 30 days for MDA-MB-468 and SUM 149 respectively, at which time they were measured using Image Quant software. We only considered colonies larger than 150 µm in diameter. Each experiment was performed in replicates of three and repeated twice.

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Analysis of apoptotic induction in the SUM 149 cells using the ArrayScan® Reader. To understand the fate of the SUM 149 cells following drug treatment we established a multi-channel high content screening protocol to simultaneously measure different aspects of apoptosis. Using the ArrayScan VTI (Cellomics, Pittsburg PA), we first established that PDK-1 was activated by developing a simple immunofluorescence method to detect phosphorylated PDK-1. SUM149 cells were seeded in 96 well plates in Ham's F12 medium (Gibco/Invitrogen) supplemented with 5% FBS and incubated (37ºC, 5% CO_2) for 48 hours. Immediately after aspirating the medium, 100 µL of 2% paraformaldehyde in PBS was added and the cells were kept at room temperature (RT) for 20 minutes. After washing with PBS three times, the cells were permeabilized in 0.1% Triton-100 in PBS for 15 minutes followed by 1% BSA in PBS for 30 minutes. The cells were then incubated with rabbit anti-phospho-PDK1 antibody diluted 100X (Cell Signaling) overnight at 4 ºC followed by the secondary goat anti-rabbit antibody conjugated with Fluro®488 diluted 200X (Invitrogen) for 1 hour at room temperature. The nuclei of the cells were stained in Hoechst dye (100 µL at 1 µg/ml) for 5 minutes. The cells were washed with PBS 3 times after each of the steps mentioned above. The images were taken on an ArrayScan® Reader (Cellomics, Pittsurgh, PA). The control cells were treated as above however there was no primary antibody added. Once we established that PDK-1 was indeed activated under the culture conditions used, we established a screen for apoptosis indices following exposure to OSU-03012. SUM 149 cells were seeded in 96-well plates (Collagen I coated, BD, Franklin Lakes, NJ) at 5,000 cells/well in 100µl of Ham's F12 medium (Gibco/Invitrogen) supplemented with 5% FBS. We found that the Collagen I coated plates provides two advantages. The first advantage was this two-dimensional culture system was more representative of the epithelial-stromal environment that breast cancer cells would normally

encounter. Collagen I was selected because it constitutes one of the most abundant extracellular matrix proteins in the breast (Provenzano et al., 2006). Secondly, the cells attached to the plates better when plated on collagen I which was a particular benefit because there are several wash steps in the protocol which otherwise caused significant variability due to cell detachment. The plates were incubated at 37ºC with 5% CO_2 for 24h. OSU-03012, dissolved in DMSO, was diluted in the medium and added accordingly (100 µL/well) to each well to give final concentrations of 0 (DMSO only), 2.5 and 5 uM of the drug (total volume = 200 µL/well). There were three replicate wells for each treatment. Thirty minutes before each endpoint (2, 6 or 24 hours), 20 µL of Hoechst and propidium iodide (PI) solutions were added to each well to give a final concentration of 1 µg/ml of each dye in the medium. Following 30-minute incubation, the medium was aspirated and the cells were gently washed with PBS three times. The cells were then fixed in 2% paraformaldehyde in PBS for 20 minutes and permeabilized in 0.1% Triton-100 in PBS for 15 minutes. After blocking in 1% BSA in PBS for 30 minutes, the cells were incubated with primary mouse anti-phospho-H2AX antibody (AbCAM, Cambridge, MA) diluted 100X for 1 hour at RT followed by secondary donkey anti-mouse antibody (FITC) (Jackson ImmunoResearch, West Grove, PA) diluted 200X for 1 hour at RT. The cells were washed with PBS three times after each step mentioned above and were finally kept in PBS (100 µL/well) at 4 ºC. The plates were analyzed and the images taken on the ArrayScan® Reader. Six hundred cells were analyzed for each replicate well and the results were presented as a mean ± SD. Repeated tests (n=3) showed identical results.

OSU-03012 dosing *in vivo.* SCID/Rag2m mice (6-8 weeks old, female) were subcutaneously injected with $1x10^7$ MDA-MB-435/LCC6 cells (gift from Dr. Robert Clark, Georgetown

University, Washington, DC), previously stably transfected with HER-2/*neu* in our laboratories (Dragowska et al., 2004). Each mouse was inoculated with the cells on the right and left sides of the lower back. A total of eight mice were injected, each harboring two tumors. After 6 weeks, the mice were randomly assigned into groups (vehicle: 0.5% methyl cellulose/ 0.1% Tween 80 or OSU-03012 at 200 mg/kg/day). Mice were dosed daily for three days with either the vehicle or OSU-03012 by oral gavage. On the fourth day, the study was terminated, mice were sacrificed, and the tumors were collected for ChIP and protein isolations. The proteins were isolated in ELB buffer using a Dounce homogenizer and then analyzed for EGFR. ChIP was performed on the tumors (25% of total tumor mass) by cross-linking the tissue in 1% formaldehyde for 20 minutes. The tissues were minced and homogenized using a Dounce homogenizer and subsequently processed for ChIP as described above using the chicken anti-human YB-1 antibody. Input DNA was normalized for differences in extraction. The relative amounts of DNA were determined by spectrophotometry. P-Akt was evaluated by immunohistochemistry as previously described by us (Sutherland et al., 2005) using a contract service (Wax-it, Vancouver BC). The tumors were considered positive if greater than or equal to 50% of the cells stained for P-Akt. The levels of glucose in mouse plasma was determined using the Ultrasoft® blood glucose monitoring system (OneTouch); 5 µL of blood from the mouse was obtained from the tail vein of the animal (tail nick) and spotted onto test strips; glucose levels were determined by the meter. Prior to each set of readings, the meter was calibrated with control solutions provided by the manufacturer. The meter provides glucose levels as mg glucose/dL and is calibrated for glucose concentrations within 20 – 600 mg/dL of blood plasma.

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Results

Activated PDK-1/Akt correlates with YB-1's ability to bind to the EGFR promoter.

MDA-MB-468 and SUM 149 are both aggressive, breast cancer cell lines that we initially characterized for features of the basal-like breast cancer subtype and comparisons were made to non-basal-like breast cancer cell lines, MDA-MB-453, a human epidermal growth factor receptor-2 (Her-2) over-expressing cell line, and MCF-7, an estrogen receptor (ER)-positive breast cancer cell line. We show for the first time that MDA-MB-468 and SUM 149 cell lines exhibit the hallmark features of BLBC. They are Her-2, ER, and progesterone receptor (PR)-negative (Figure 1). We were unable to confirm the expression of CK5/6 because the antibodies that are commercially available did not yield interpretable data. Importantly, the MDA-MB-468 and SUM 149 cell lines expressed the new BLBC marker, EGFR. It should be noted that the MDA-MB-468 cells have reported amplified EGFR gene copy numbers, which contributes to its apparent EGFR protein over-expression (Filmus et al., 1985). YB-1 is invariably expressed in breast cancer cell lines thus it was used as an internal control. Given that EGFR is highly expressed in these cells, we anticipated that PDK-1 would also be active. The PDK-1 substrates Akt and S6 kinase-1 were both phosphorylated and therefore highly activated in the BLBC cell lines compared to the 184htrt cells (Figure 2A). The S6 ribosomal protein, a downstream substrate of the Akt pathway, was also phosphorylated in BLBC but not the 184htrt cells.

Our lab has recently published that YB-1 regulates EGFR expression by binding to the first -1kB of the EGFR promoter using primers that we refer to as 1b and 2a. Consistent with the observation that the PDK-1/Akt pathway is highly active in the BLBC, we also find that YB-1 binds to the first -1kB of the EGFR promoter in these cells (Figure 2B). However, the 184htrt

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cells have virtually undetectable levels of YB-1/EGFR promoter binding (Figure 2B). Thus, YB-1 binds to the EGFR promoter only in cells with activated PDK-1/Akt.

PDK-1 inhibitor, OSU-03012, disrupts Akt signaling and prevents YB-1 from binding to the EGFR promoter.

After incubation with the compound, at 10 μM for 6 hours, both MDA-MB-468 and SUM 149 cell lines were exposed to OSU-0302 (10 uM) for 6 hours and as expected the drug decrease levels of phosphorylated Akt at threonine-308 (the PDK-1 phosphorylation site), and the serine-473 residue, while total Akt protein levels remained unchanged (Figure 3A). Similarly, S6 ribosomal protein was inhibited. Importantly, we find that OSU-03012 inhibited phosphorylation of YB-1 at S102 using a newly developed antibody (Figure 3B). The specificity of the P-YB-1(S102) antibody was confirmed by showing an induction of phosphorylation in MDA-MB-231 cells following IGF-1 stimulation (Figure 3B). This is consistent with our previous studies showing that IGF-1 stimulation in those cells caused Akt to bind to and phosphorylate YB-1 (Sutherland et al., 2005). To further demonstrate the specificity of the antibody, MCF-7 Flag:YB-1 cell lysates were compare to MCF-7 Flag:YB-1(A102) where the serine phosphorylation site was destroyed by site directed mutagenesis (Wu et al., 2006). As expected, the P-YB-1(S102) was detected in the MCF-7 Flag:YB-1 cells. Alternatively, the P-YB-1(S102) signal was attenuated in cells expressing the mutant Flag:YB-1(A102) (Figure 3B). Subsequently, we demonstrated that YB-1 is phosphorylated at S102 in SUM 149 cells when it binds to the EGFR promoter using chromatin immunoprecipitation again using the YB-1(S102) antibody (submitted). Thus, we concluded that OSU-03012 attenuated signaling through the PDK-1/Akt/YB-1 pathway.

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Consistent with these observations, inhibiting signaling through PDK-1 prevented YB-1 from binding to the EGFR promoter in the MDA-MB-468 and SUM 149 cells (Figure 3C). More specifically, in the MDA-MB-468 cells OSU-03012 reduced YB-1 binding to the EGFR promoter at the "1b" site by 40% of the control (p-value = 0.36) whereas at the "2a" site, binding was completely abolished (p-value = 0.024) (Figure 3C). The immunoprecipitation with chicken IgY antibody negative controls and the sheared input DNA controls exclude the possibilities of non-specific antibody binding and uneven DNA loading. In SUM 149 cells, OSU-03012 also inhibited YB-1 from binding to the "2a" loci on the EGFR promoter by 60% (Figure 3D) however there was no effect on the 1 b site (data not shown). To further investigate the binding interactions between YB-1 and the regulatory region of EGFR flanked by primer set "2a", an electrophoretic mobility shift assay was performed employing biotin-labeled EGFR "2a" probes, designed to coincide with the primer-flanked region detected in our ChIP assays (Figure 3D, bottom right). Nuclear extracts of the SUM 149 cells after OSU-03012 treatment were incubated with the biotin-labeled oligonucleotides and the binding interaction between YB-1 and the oligonucleotides was significantly hindered (Figure 3D, lane 3) when compared to the DMSO control (Figure 3C, lane 1). To demonstrate specificity, an unlabelled EGFR "2a" probe competed for binding (Figure 3D, lane 2). An antibody to YB-1 was used to demonstrate the specificity of binding. In this case, the addition of YB-1 caused a supershift in binding thereby demonstrating its involvement in EGFR promoter binding to the "2a" site (Figure 3D, lane 5). Conversely, using a Creb antibody did not cause a supershift (Figure 3D, lane 4). To conclude, OSU-03012 disrupts the Akt signaling pathway in BLBC cells such that the YB-1 binding pattern at the EGFR promoter sequence mirrored that of a normal cell line with low Akt signaling activity and minimal EGFR expression.

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Targeted PDK-1 gene knockdown disrupts the Akt signaling pathway and down-regulates the expression of EGFR.

To determine the specificity of the OSU-03012 compound, we attempted PDK-1 inhibition by targeted gene knockdown with siRNA in the SUM 149 cells. We were able to achieve greater than 90% knock-down of PDK-1 when the cells were treated with either of the siRNAs for 96 hours (Figure 4). Moreover, both of the siRNAs targeting PDK-1 caused a substantial loss of EGFR protein expression. This effect echoes that of the OSU-03012 on the YB-1/EGFR axis in the same cell line.

OSU-03012 deceased EGFR protein expression by reducing its mRNA levels in SUM 149 cells.

As OSU-03012 treatment affected YB-1 binding to the EGFR promoter, the capacity of differential binding patterns to subsequently affect gene transcription was the next question. Using quantitative real-time PCR, we examined EGFR transcript levels in SUM 149 cells after 6 hours of OSU-03012 10μM treatment where we had seen differential binding effects. We found OSU-03012 decreased EGFR mRNA by 26% (p-value = 0.016) (Figure 5A). Furthermore, YB-1 responsive genes such as the proliferating nuclear antigen (PCNA) and topoisomerase II (TOPO-II) mRNA levels were also inhibited by OSU-03012 to a similar degree (Figure 5A). Exposing the SUM 149 cells to OSU-03012 for 8 hours resulted in a decrease in EGFR protein expression (Figure 5B). In addition, we confirmed that signaling through the Akt pathway was suppressed at that time (Figure 5B). We therefore concluded that the PDK-1/YB-1 pathway

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leads to the induction of EGFR and that OSU-03012 has the ability to block this growth-promoting network.

OSU-03012 induces apoptosis and can be monitored using a newly developed High Content Screening method

We noted that the longer the cells were treated with the drug, the less viable they were, suggesting that this drug could be used as an anticancer agent against BLBC. For example, if we exposed the cells to OSU-03012 for 9 hours the cells lifted and appeared to be dying. This prompted us to study the effect of the drug on cell viability in monolayer and in soft agar. We determined that OSU-03012 inhibited the growth of SUM 149 and MDA-MB-468 cells in a dose dependent manner (Figure 6A). The 184htrt cells however were not sensitive to the drug (Figure 6A). OSU-03012 (2.5, 5.0 or 10 μM) also had a profound inhibitory effect on the ability of the SUM 149 cells to grow in soft agar (Figure 6B). Likewise, the drug inhibited the anchorage-independent growth of MDA-MB-468 cells (Figure 6B).

Subsequent to discovering OSU-03012's effects on the growth of BLBC cells in monolayer and soft agar, we were curious to determine if OSU-03012 could induce apoptosis in these cells. We therefore developed high content screening (HCS) protocols first to measure levels of activated PDK-1 and secondly to simultaneously monitor distinct aspects of apoptosis. The apoptosis suite included chromatin condensation, phosphorylation of histone 2AX and finally propidium iodide uptake all of which can be done quantitatively using HCS. Initially, we confirmed that PDK-1 was activated in our screen by staining for P-PDK-1 by immunofluorescence (Figure 7A). As one would expect, P-PDK-1 localized to the plasma membrane as indicated by the chicken-wire staining pattern observed (Figure 7A). SUM 149

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cells were then treated with OSU-03012 (5 μM) for 24 hours and examined for evidence of apoptosis. The cells exhibited condensed chromosome based on an increase in the intensity of Hoechst staining relative to nuclear size (Figure 7B, panel i). H2AX also became phosphorylated and was evident in the nucleus of drug treated cells (Figure 7B, panel ii). Finally the cells took up propidium iodide (Figure 7B, panel iii) and this was coordinate with the other markers of apoptosis in the same cells (overlay, Figure 7B, panel iv). The induction of apoptosis was monitored over a time-course. It appeared that OSU-03012 (2.5 or 5 μM) induced cell death in a time and concentration-dependent manner in the SUM 149 cells. Quantification of the analyses is represented in the bottom panel of Figure 7C. We therefore concluded that that ultimate fate of SUM 149 cells treated with OSU-03012 was death, providing even more optimism for the use of the drug to treat aggressive forms of breast cancer.

OSU-03012 disrupts YB-1 function and down-regulates the expression of EGFR in mice.

In cancer cells, Her-2 is not able to signal unless it partners with other members of its family such as EGFR. We therefore utilized a system where Her-2 is over-expressed in MDA-MB-435/LCC6 cells. The MDA-MB-435/LCC6/Her-2 cells were used for in vivo studies in part because they are known to express EGFR (Warburton et al., 2004) and they readily form tumors in mice (Dragowska et al., 2004; Warburton et al., 2004). We therefore questioned whether OSU-03012 could inhibit YB-1 function and therefore down-regulate EGFR in this pre-clinical model of breast cancer. To achieve this, we performed bilateral subcutaneous injections of the MDA-MB-435/LCC6/Her-2 cells into the rear flank of SCID/Rag2 mice (n=8) and established tumors over 6 weeks. All of the mice developed two tumors and were therefore assigned to either the vehicle control or OSU-03012 (200 mg/kg) treatment group, which was given orally

for three days. OSU-03012 remarkably decreased EGFR protein expression in the tumors by ~48% compared to expression levels found in the tumors taken from mice that received the vehicle control (Figure 8A&B). OSU-03012 also prevented YB-1 from binding to the EGFR promoter at the "1b" and "2a" sites (Figure 8C). Following this, we immuno-stained the tumors from the mice for P-Akt. Overall, there was a reduction in the intensity of P-Akt staining in the OSU-03012 treated tumors compared to the vehicle control. There tended to be less P-Akt staining in the tumors taken from mice that were given OSU-03012 (Figure 8A). Tumors were considered positive if at least 50% of the cells stained for P-Akt. What is being shown is the data from four tumors taken from each treatment group. We did however exam all of the tumors for P-Akt. There were 6/7 (86%) tumors in the vehicle control group that expressed P-Akt where as only 3/8 (37.5%) were positive in the OSU-03012 treated group. One of the tumors from the vehicle control had to be omitted because of poor overall staining. Thus, OSU-03012 decreased P-Akt staining by approximately 44%, which is consistent with the degree to which EGFR was inhibited. Finally, we wanted to make sure that the mice were not adversely reacting to the drug because Akt was being inhibited. Therefore we measured serum glucose levels after they received the drug for 72 hours. Importantly, the drug was well -tolerated by the mice with no observed changes in serum glucose (Figure 8D).

Discussion

In this study, we demonstrate that the nuclear function of YB-1 can be inhibited by OSU-03012. This is the first time that a pharmacologically-relevant inhibitor has been shown to control the action of this oncogene. The inhibition of YB-1 with OSU-03012 resulted in a decrease in EGFR that is important not only to BLBC but also to Her-2 over-expressing breast cancers. Using ChIP and gel shift assays, we demonstrated that OSU-03012 inhibits YB-1 from binding to the –1kB of the EGFR promoter. Having illustrated this effect in a number of models suggests that using OSU-03012 to inhibit the growth of YB-1 over-expressing cancers could have broader reaching implications particularly as this transcription factor is over-expressed in cancers of the prostate (Gimenez-Bonafe et al., 2004), lung (Shibahara et al., 2001), colon (Shibao et al., 1999), ovary (Yahata et al., 2002) and more recently childhood brain tumors (Faury et al., 2007). Importantly the aforementioned tumor types are also known to express activated Akt (Vivanco and Sawyers, 2002) thus inhibitors such as OSU-03012 could be used a means to perturb YB-1 action.

It should be pointed out that while we (Wu et al., 2006), and others (Berquin et al., 2005) find that YB-1 regulates EGFR at the level of transcription it is entirely possible that it could also influence the translation and/or turnover of the receptor through endocytosis. For example, YB-1 has previously been characterized for its ability to stabilize interleukin-2 mRNA by binding to AU-rich elements (ARE) on the 3' untranslated region of IL-2 (Chen et al., 2000). This was a rather novel finding because YB-1 is more commonly thought to regulate translation through cap-dependent binding (Soop et al., 2003). While YB-1's ability to regulate ARE's is a relatively

new concept, it is reminiscent of the way in which the widely characterized translation factor HuR stabilizes the mRNA of proto-oncogenes such as urokinase plasminogen activator (Tran et al., 2003). Interestingly, EGFR has two ARE regions which are occupied by a protein that could potentially be YB-1 given that it is described as a cytoplasmic protein that is 50-80 kDa in size which was initially isolated from the MDA-MB-468 cells (Balmer et al., 2001). This excludes HuR because it is 36 kDa. Furthermore the uncharacterized protein is similar to YB-1 in that it is expressed in a wide range of breast cancer cell lines (Balmer et al., 2001). While this is speculative it does raise the possibility of other mechanisms whereby YB-1 could control the expression of EGFR. A similar argument can be mounted for YB-1 at the level of EGFR turnover. It is conceivable that YB-1 inhibits EGFR turnover by down-regulating the c-able proto-oncogene (CBL), which is known to mediate receptor cycling (Ettenberg et al., 1999). This possibility is raised because we recently performed ChIP on Chip (COC) to profile the global promoter occupancy of YB-1 in the SUM 149 cells. Our preliminary data indicates that YB-1 potentially binds to CBL based upon two different COC hybridizations (data not shown) presenting yet another potential mode of regulation. Such avenues are additionally rationalized given that our data indicates that the over-expression of EGFR is not due to gene amplification (submitted for publication) suggesting that transcription/translation and receptor turnover events are likely the cause of enhanced expression supports these possibilities.

The expression of YB-1 in aggressive types of cancer calls into question its potential as a therapeutic target for treatment. We took an indirect approach to inhibiting YB-1 but alternatively, one could consider inhibiting YB-1 directly. By knocking down YB-1 we find that the anchorage-independent growth of breast cancer cells is inhibited by approximately 50-70%

as a single agent (unpublished). This is a promising result that could translate into the clinic by silencing YB-1 using either antisense or small interfering RNA's. Clinical trials are underway using antisense to inhibit BCL-2 (Chi et al., 2001; Tolcher et al., 2005) and clusterin (OGX-011) (Chi et al., 2005; Miyake et al., 2000; So et al., 2005). By the same token, there are now many examples of how small interfering RNA's can be used to slow the growth of cancer cells in pre-clinical models although the clinical development of this technology is still emerging. Recently, short hairpin RNA's targeting survivin were expressed in a lentiviral vector (Jiang et al., 2006) and tested in a model of oral squamous cell carcinoma where this target is highly expressed. The loss of survivin sensitized the cells to chemotherapy in vitro and inhibited tumor growth in mice (Jiang et al., 2006). The first clinical trials using siRNA have begun; however, they are not yet being applied in the field of oncology, but to silence the vascular endothelial growth factor (VEGF) in age-related macular degeneration (Grunweller and Hartmann, 2005). It is therefore within reach that shRNA could be used to treat other diseases such as cancer. Taking a completely different approach, one might consider using the expression of YB-1 to drive the replication of oncolytic viruses as a way of treating cancer. It has been known for some time that YB-1 facilitates the replication of adenoviruses (Holm et al., 2002), which can then used to kill tumor cells (Glockzin et al., 2006; Holm et al., 2004). The expression of YB-1 in basal-like and Her-2 over-expressing breast cancers provides an excellent opportunity for using oncolytic viruses for therapy. While these gene-based approaches are promising they are still limited by bioavailability, formulations, safety and the expense of making the products. A small molecule inhibitor would perhaps circumvent some of these issues. OSU-03012 is an indirect YB-1 inhibitor that is promising because it is orally available, well tolerated in mice and kills a wide range of cancer cells types.

While we have concentrated on the effect of OSU-03012 on the interface between YB-1 and EGFR we are aware that blocking this transcription factor will inevitably inhibit other target genes that are important to the growth and survival of cancer cells. YB-1 is classically known to regulate genes such as the multi-drug resistance-1, TOPO-II, cyclin A, cyclin B, DNA polymerase and PCNA to mention a few (Kohno et al., 2003; Kuwano et al., 2003). Taking this into consideration, we demonstrated that OSU-03012 also inhibited the expression of the YB-1 target genes PCNA and TOPO-II. It is noteworthy that PCNA and topoisomerase II alpha are expressed in BLBC (Perreard et al., 2006) both of which are regulated by YB-1 (Kohno et al., 2003). In colorectal carcinomas, YB-1 and topoisomerase II alpha are coordinately expressed (Shibao et al., 1999). Likewise, similar expression patterns are reported in lung cancer (Gu et al., 2001) and synovial sarcomas (Oda et al., 2003). More direct evidence for the association is supported by Shibao et al who reported that knocking down YB-1 with antisense attenuates topoisomerase II reporter activity (Shibao et al., 1999). These YB-1 target genes have now been confirmed in BLBC. These data could now begin to explain why the expression of this transcription factor is clearly associated with poor survival based on the work done previously by our laboratory (Wu et al., 2006) and by others (Bargou et al., 1997). Arguably, inhibiting a protein such as YB-1 that has pleotrophic effects is desirable particular in treating cancer, a disease that manifests from multiple cellular defects. Particularly as inhibiting just EGFR for example has proven to be surprisingly ineffective in the clinic. Disappointingly, the results of two clinical trials report that EGFR inhibitors do not have activity against breast cancer (Baselga et al., 2005; von Minckwitz et al., 2005). We therefore suggest that inhibiting YB-1 could be a better molecular target because it regulates so many genes involved in tumor cell growth and

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importantly drug resistance (Kuwano et al., 2003). In closing, we are nominating OSU-03012 as a potent small molecule inhibitor that has the potential to block the function of YB-1 and therefore the expression of EGFR. These data provide novel mechanistic implications for the use of OSU-03012 to inhibit the growth of BLBC and Her-2 breast cancer subtypes.

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Legends for Figures

Figure 1. MDA-MB-468 and SUM 149 cell lines both exhibit the hallmarks of the basal-like breast cancer subtype. MDA-MB-468 and SUM 149 cell lines lack Her-2, ER**,** and PR expression**,** making them classic "triple negative" breast cancer cell lines that would be categorized as basal-like by immunoblotting. They also both express the new basal-like marker, EGFR. The MDA-MB-453 and MCF-7 cells are established non-basal-like breast cancer cell lines known to express high Her-2 and ER respectively.

Figure 2. The PDK-1 pathway is activated in basal-like breast cancer cell lines and correlates with YB-1's ability to bind to the EGFR promoter. A) The PDK-1 substrates, Akt and S6 kinase, are highly activated in MDA-MB-468 and SUM 149 cells but not human mammary epithelial cell line, 184htrt based on immunoblotting**.** In addition, S6 ribosomal protein (RP) is highly activated in the basal-like breast cancer cells but not the 184htrt cells. These data correlate the preferential expression of EGFR in the basal-like cell lines where the receptor is only expressed in cell lines that have activated Akt/S6kinase/S6RP and YB-1. **B)** YB-1 does not bind to the EGFR promoter in 184htrt cells whereas it does in MDA-MB-468 and SUM 149 cells by ChIP. YB-1/EGFR binding was observed using two sets of primers (1b and 2a) designed to flank YB-1 responsive elements in the first -1kb of the EGFR promoter region. To control for non-specific binding, species matched IgY was immunoprecipitated with DNA from the respective cell lines and then amplified with each of the EGFR primer sets. The amount of input was controlled for by amplifying with GAPDH primers. There were no differences in the amount of input DNA based on GAPDH amplification.

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Figure 3. OSU-03012 inhibits PDK-1/Akt signaling and YB-1 from binding to the EGFR promoter. A) OSU-03012 inhibits the phosphorylation of Akt at thr-308 residue and consequently phosphorylation of Akt at ser-473 when cells are exposed to OSU-03012 (10 μM) for 6 hours. Phosphorylation of S6 ribosomal protein was also inhibited, giving evidence for disrupted Akt signaling in both cell lines as a result of drug treatment. However, total Akt and S6 ribosomal protein levels remained unchanged. Vinculin served as a loading control. **B)** Phosphorylated levels of YB-1 were significantly down-regulated with OSU-03012 treatment in both cell lines, while total YB-1 protein levels were also unaffected. The specificity of the P-YB-1(S102) antibody was confirmed by serum starving MDA-MB-231 cells and then stimulating them with IGF-1 (100 ng/ml for 30 minutes) (bottom left). In addition, lysates were analyzed from MCF-7:Flag YB-1 and MCF-7 Flag YB-1(A102) by immunoblotting (bottom right). **C)** OSU-03012 inhibits YB-1 occupancy at the EGFR promoter in MDA-MB-468 and SUM 149 BLBC cells**.** ChIP of YB-1 in the BLBC cell line, MDA-MB-468. YB-1 binding at the EGFR promoter was suppressed at the "1b" site and abolished at the "2a" site as detected by DNA agarose gel following ChIP assay after 6-hour OSU-03012 (10 μM). Bottom panel: Quantification of YB-1 binding to the EGFR promoter by densitometry. OSU-03012 reduced YB-1 binding at the "1b" site to 40% of the DMSO solvent control treatment and there was no detectable signal at the "2a" site after treatment with the compound. **D)** ChIP of YB-1 in the BLBC cell line, SUM 149. The cells exhibit decreased YB-1 binding at the "2a" site but not the "1b" site after being exposed to the OSU-03012 (10 μM) for 6 hours. **Bottom left panel:** Quantification of YB-1 binding to the EGFR promoter in the presence of OSU-03012. In the SUM 149 cells at the "2a" sequence shows OSU-03012 decreased YB-1 binding to 60% of the

DMSO vehicle control. **Bottom right panel:** SUM 149 cells were treated as above and then nuclear extracts used an electrophoretic mobility shift assay where the biotin-labeled oligonucleotide was designed against the "2a" binding site of the EGFR promoter. EGFR binding occurred from nuclear extracts taken from cells treated with DMSO (lane 1) which could be reversibly inhibited with competitive unlabeled oligonucleotide (lane 2). Nuclear extracts from SUM 149 cells treated with OSU-03012 did not bind to the EGFR "2a" oligonucleotide (lane 3). To confirm that binding was specific, DMSO nuclear extracts were treated with 2 µg of anti-CREB which did not cause a super-shift in binding (lane 4). Conversely, the YB-1 antibody did cause a super-shift (lane 5).

Figure 4. PDK-1 targeted gene knockdown achieves inhibition of the Akt pathway and decreases YB-1 responsive gene EGFR protein levels. SUM 149 BLBC cells were grown in monolayer at an optimized density for siRNA transfection efficiency just prior to the addition of siRNA and transfect reagents. Two unique sequences of the PDK-1 gene were employed in the design of siRNA. PDK-1 siRNAs and a control siRNA (20 nM) were incubated with the SUM 149 cells for 96 hours and cells were harvested for western blot analyses.

Figure 5. OSU-03012 decreased EGFR protein expression through reduced transcript levels in SUM 149 BLBC cells. A) EGFR mRNA transcript levels were measured in qRT-PCR reactions by harvesting total RNA from SUM 149 cells after 6 hours incubation of OSU-03012 (10 µM). EGFR transcript levels were reduced by 26%. The drug had a similar effect on the YB-1 responsive genes PCNA ($p<0.001$) and TOPO-II ($p<0.003$). **B)** EGFR protein levels

exhibit a marked decrease in parallel with a down-regulated Akt signaling pathway after being exposed OSU-03012 (10 µM) for 8 hours.

Figure 6. OSU-03012 suppresses the growth of BLBC cells. A) 184htrt, MDA-MB-468 and SUM 149 cells were treated with OSU-03012 (2.5, 5 and 10 µM) for 24 hours and cell viability was assessed. The drug inhibited the growth of BLBC cell lines in a dose dependent manner whereas the 184htrt cells were relatively insensitive. At the highest concentration (10 µM), only ~25% of the BLBC cells were viable. Each experiment was performed in replicates of six on two separate occasions. OSU-03012 (5 and 10 µM) significantly inhibited the growth of MDA-MB-468 and SUM 149 cells, $p<0.0001$. **B)** SUM 149 and MDA-MB-468 cells were exposed to OSU-03012 (2.5, 5 and 10 µM) for 21 and 30 days respectively in soft agar and number of colonies were then measured. The treatments were performed in replicates of three on two separate occasions. OSU-03012 significantly inhibited anchorage-independent growth in MDA-MB-468 cells at the two highest concentrations ($p<0.01$). The SUM 149 cells were even more sensitive to inhibition given that compared to the DMSO control the drug inhibited anchorage independent growth at all of the concentrations (2.5 µM $p<0.02$, 5 µM $p<0.002$, 10 µM $p<0.002$).

Figure 7. OSU03012 induces apoptosis in SUM 149 cells. A) SUM 149 cells were immuno-stained for phosphorylated PDK-1 and visualized using a FITC secondary antibody on the ArrayScan VTI. This was done to ensure that PDK-1 was activated prior to screening for the potential apoptotic effects of OSU-03012. **B)** Fluorescent microscopic images of SUM 149 cell nuclei treated with DMSO (control) or OSU-03012 (5 µM) for 24 hours and subsequently

stained with Hoechst, phospho-H2AX antibody (conjugated to FITC), and propidium iodide (PI). SUM 149 cells exhibit characteristics of apoptotic cells after OSU-03012 treatment. **C)** Quantification of fluorescent intensity of Hoechst, P-H2AX (FITC), and PI (TRITC) in an OSU-03012 time course treatment of the SUM 149 BLBC cells at 0 (Control, C), 2.5, and 5 μM for 2, 6, and 24 hours. Data are represented by means ± standard deviation, n = 3.

Figure 8. OSU-03012 suppresses the expression of EGFR and prevents YB-1 promoter occupancy in vivo. A) EGFR protein was evaluated from mice given either the vehicle or OSU-03012 (200 mg/kg/day) for 72 hours. The drug caused a decrease in EGFR expression in 8/8 tumors. An asterisk denotes which tumors were selected for chromatin immunoprecipitation. **B)** The levels of EGFR were on average 48% lower in the tumors taken from the OSU-03012 treated mice compared to those given the vehicle control. Relative levels were determined by densitometry. **C)** ChIP was performed on tumors from the vehicle control and OSU-03012 treated mice. The DNA was amplified for EGFR using EGFR "1b", and EGFR "2a" primers. YB-1 bound to each of these sites when the mouse was given the vehicle but not when OSU-03012 was administered. **D)** Serum glucose levels were comparable between mice treated with either the vehicle control or OSU-03012. Measurements were taken after 72 hours of treatment. The data represents the average serum levels from four mice per treatment group.

Figure 1

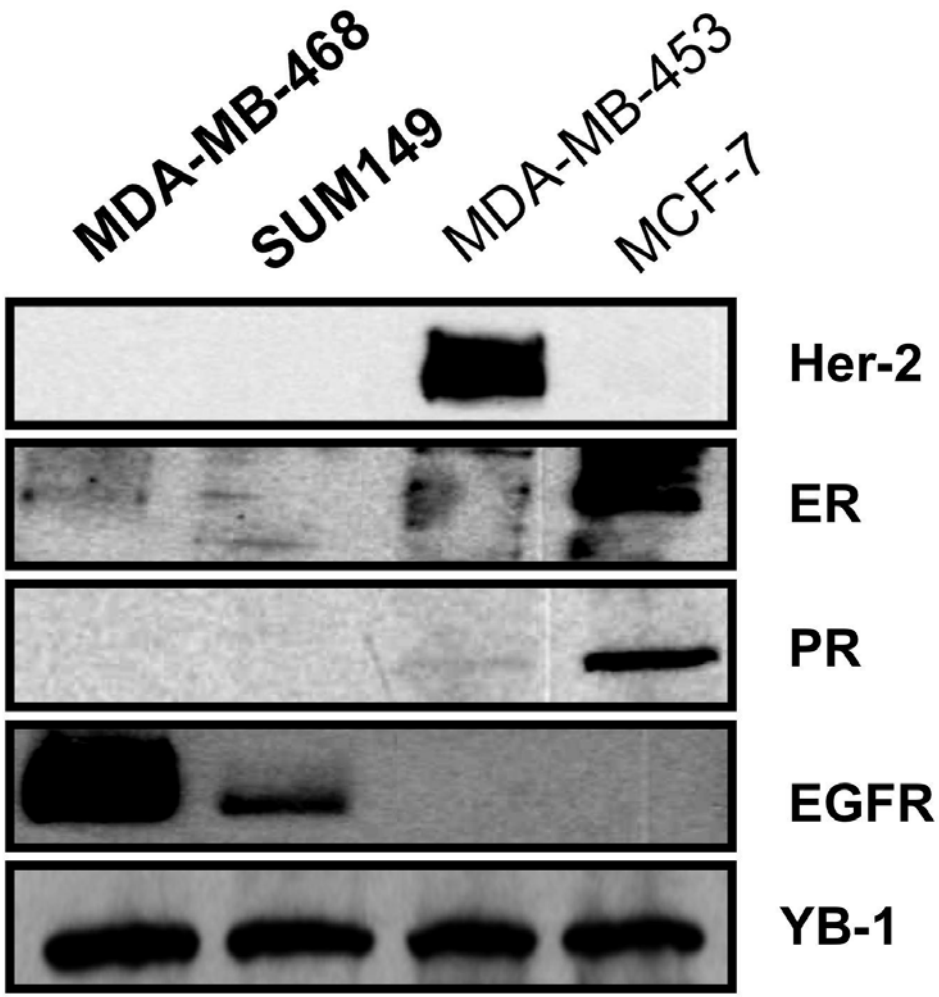
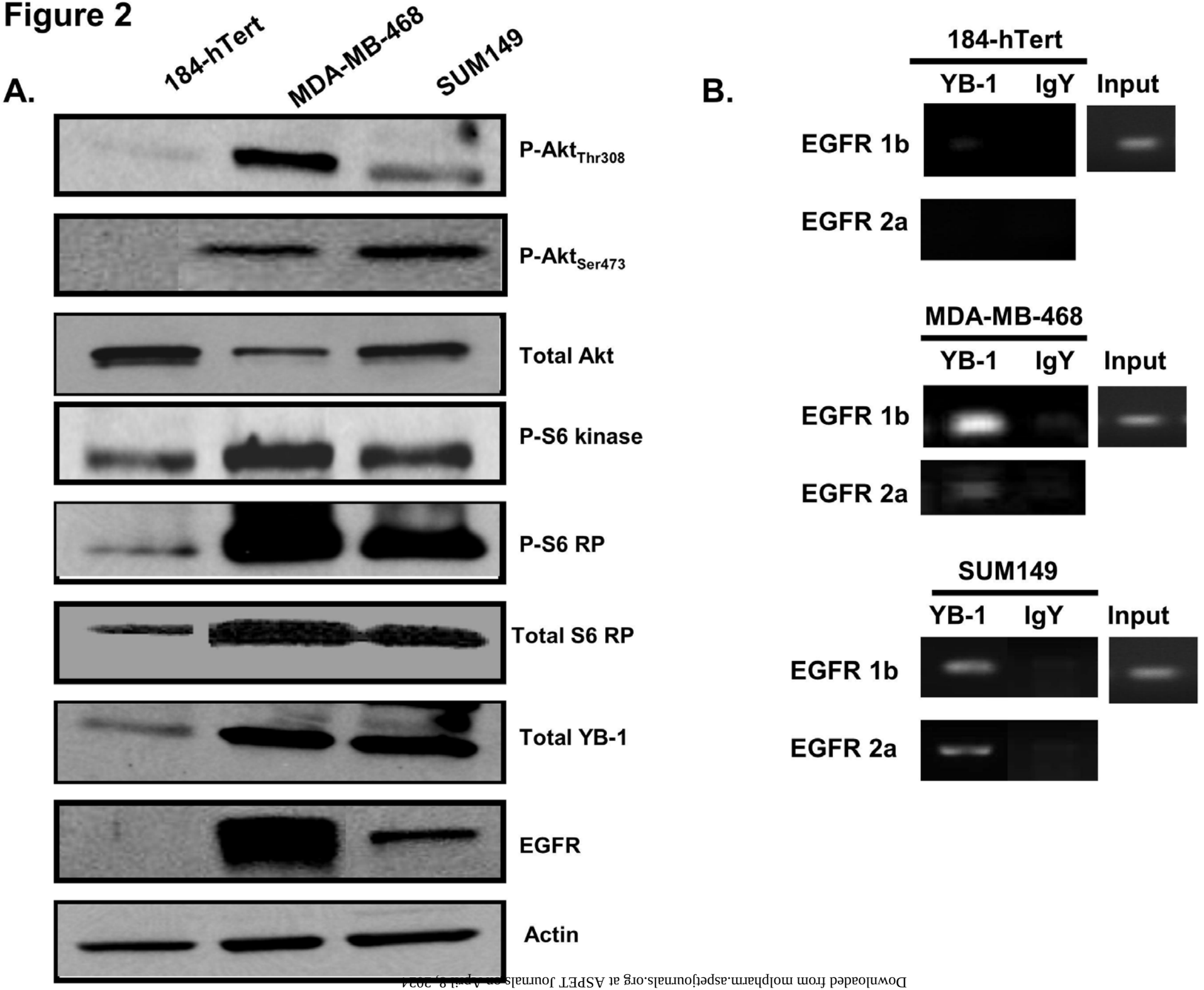


Figure 2



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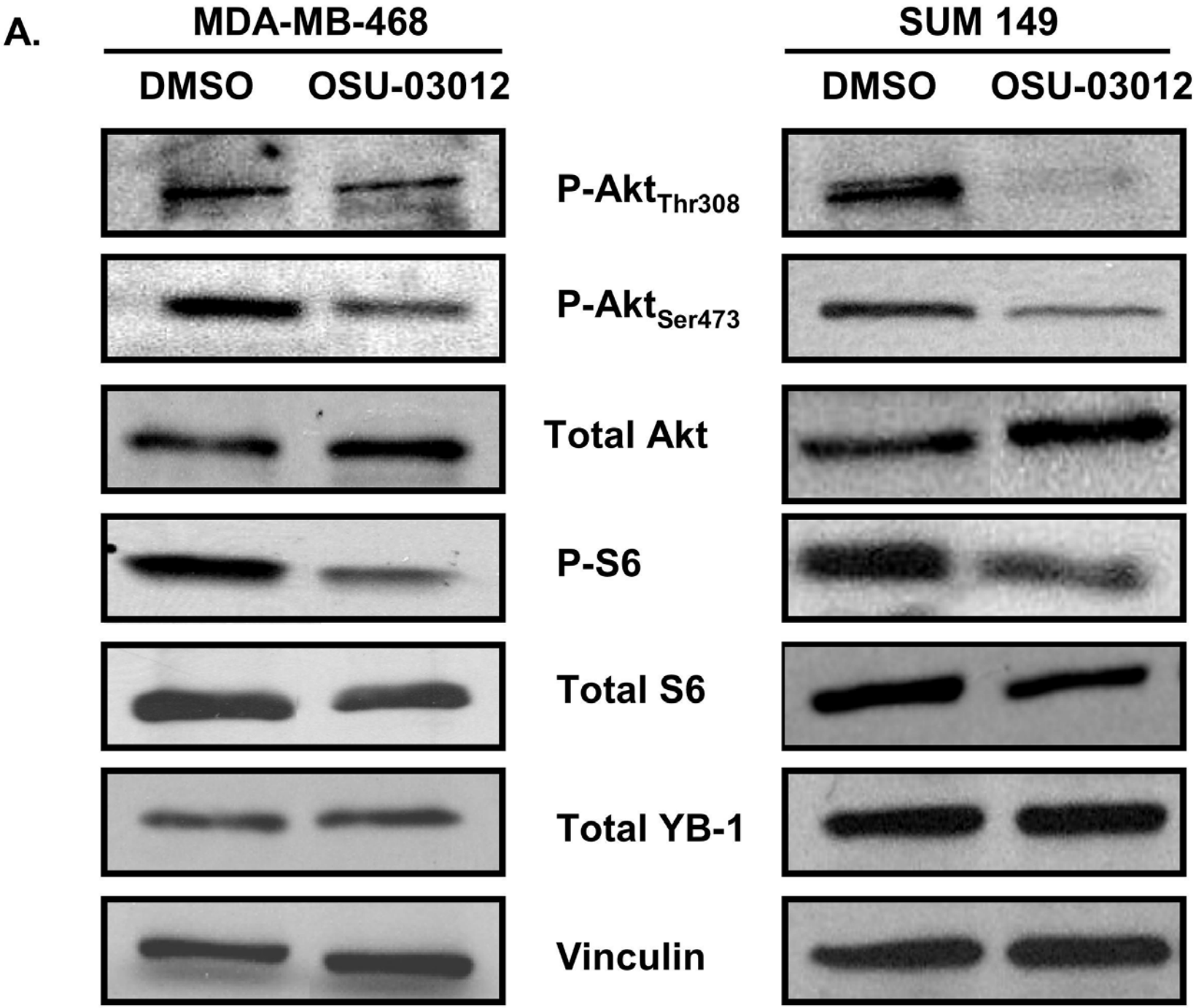

Figure 3

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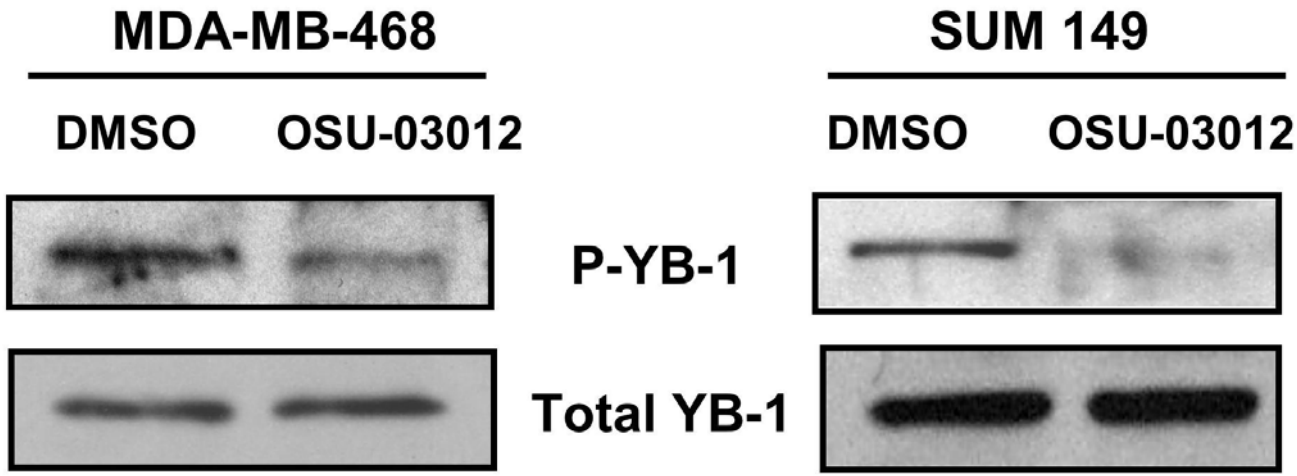

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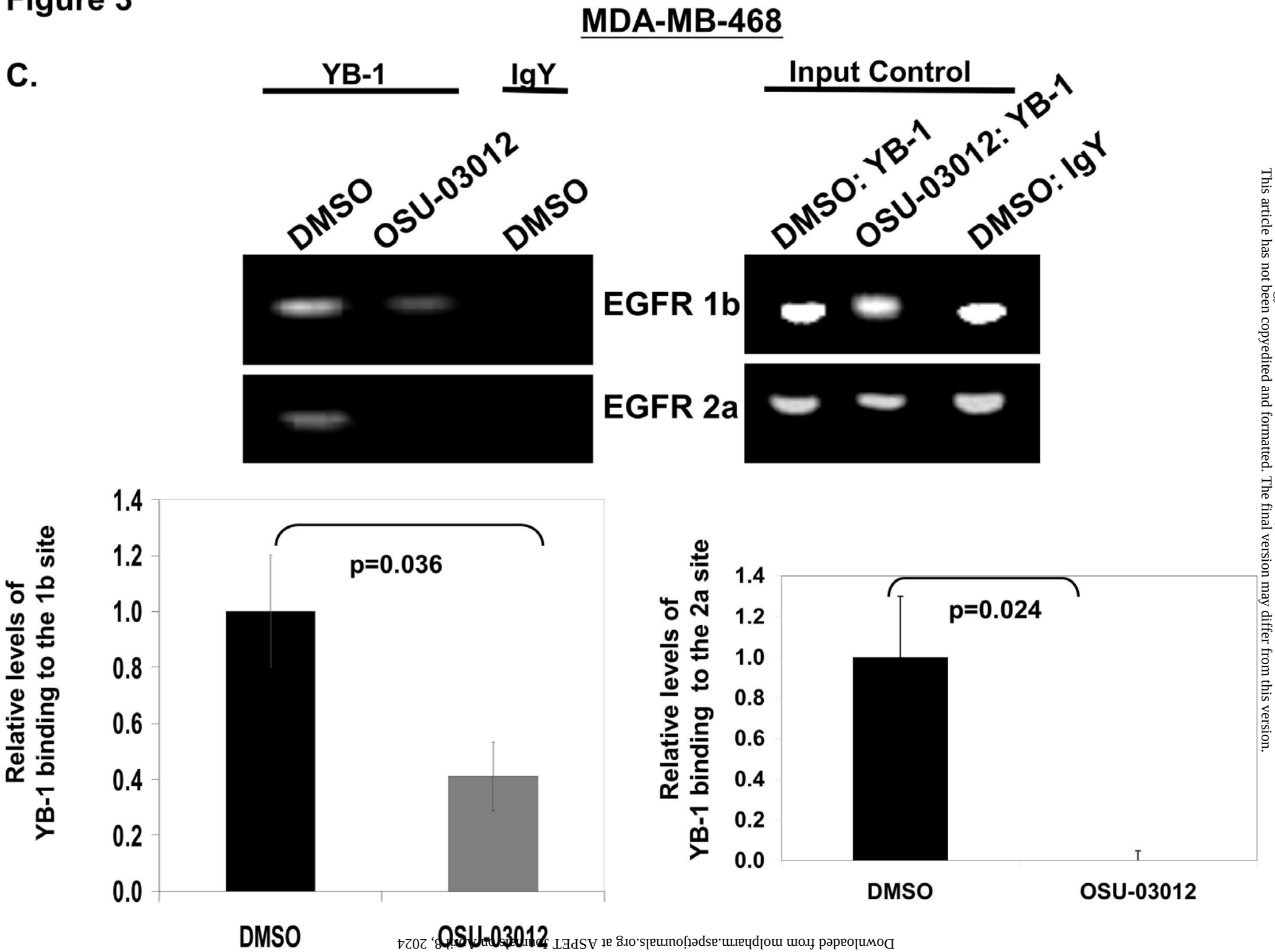
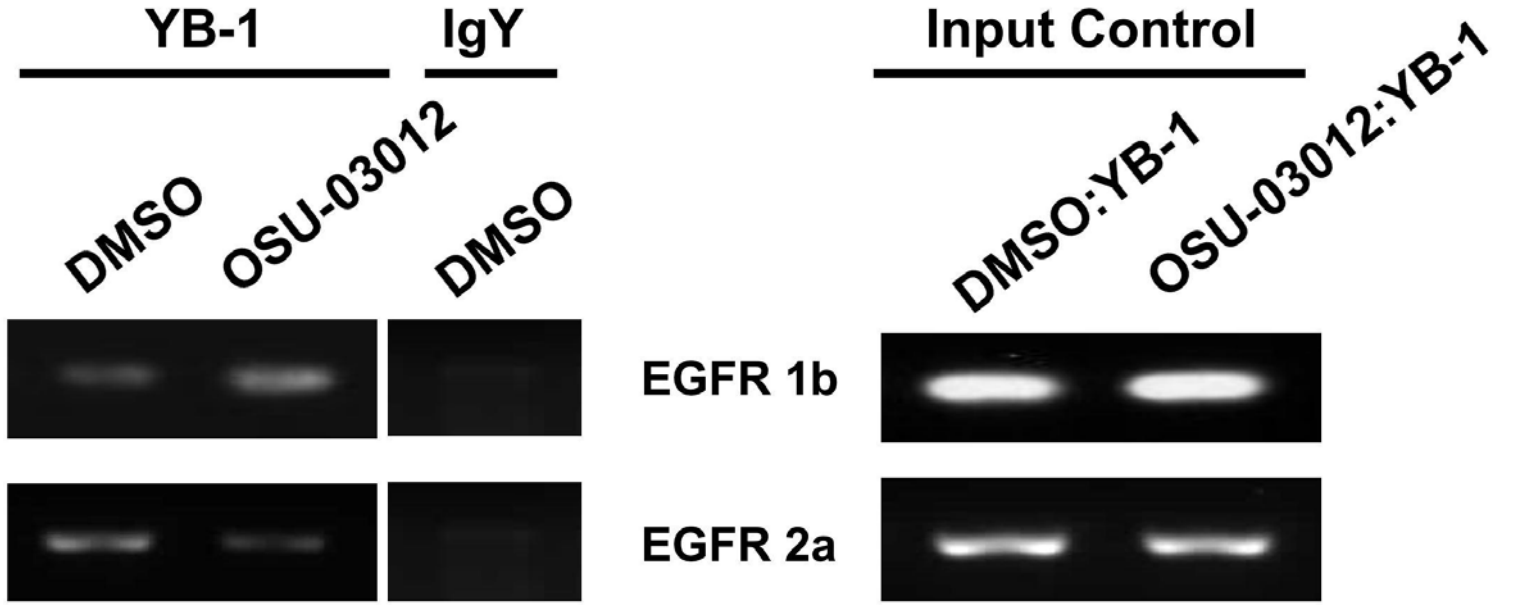
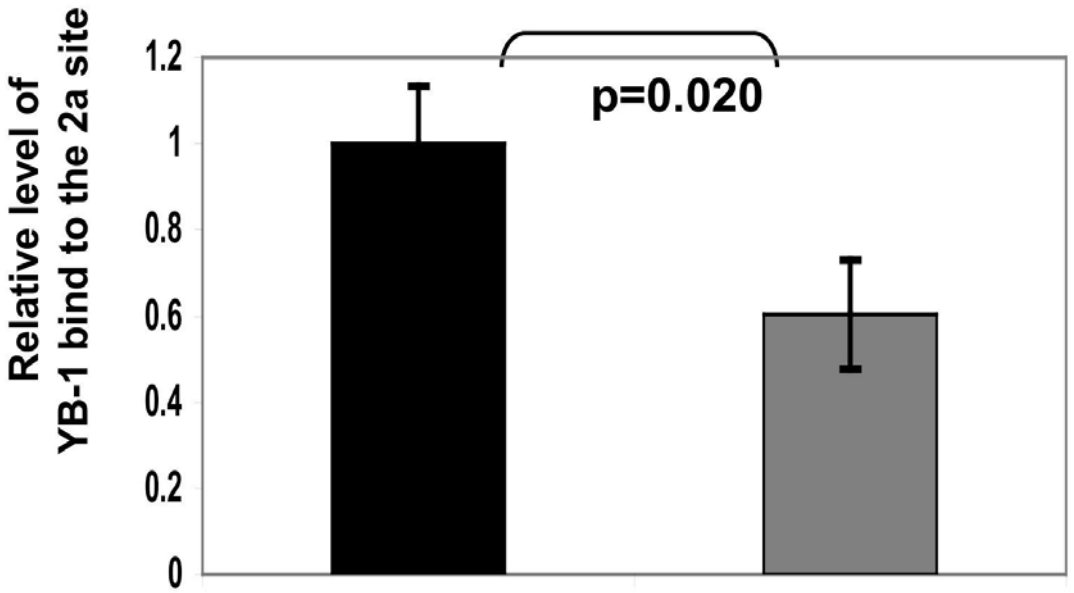


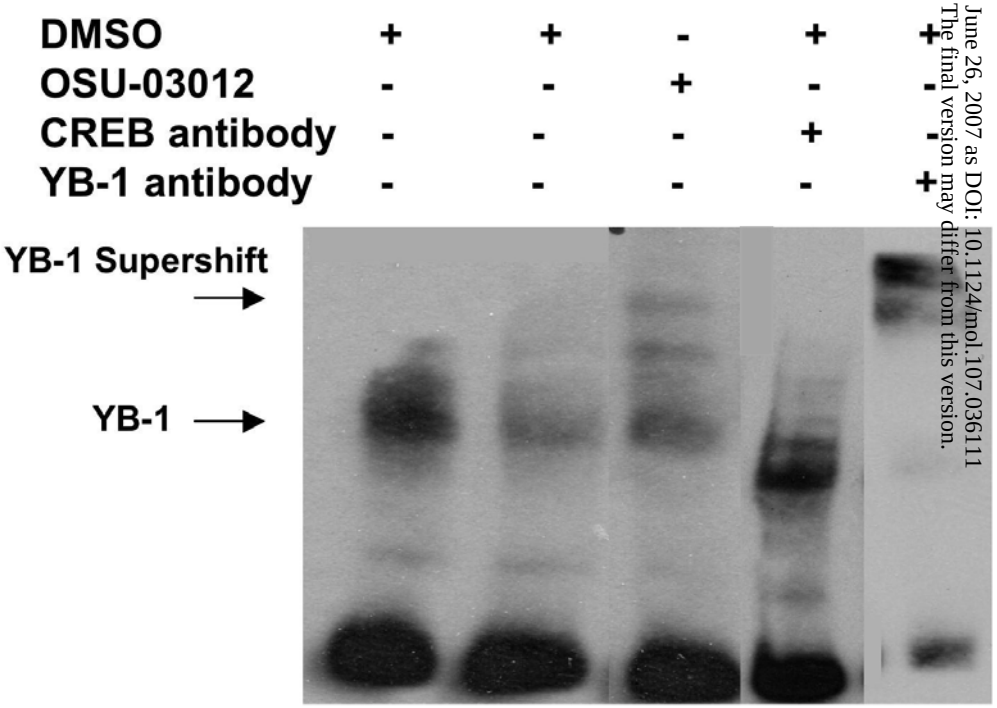
Figure 3

SUM 149

D.







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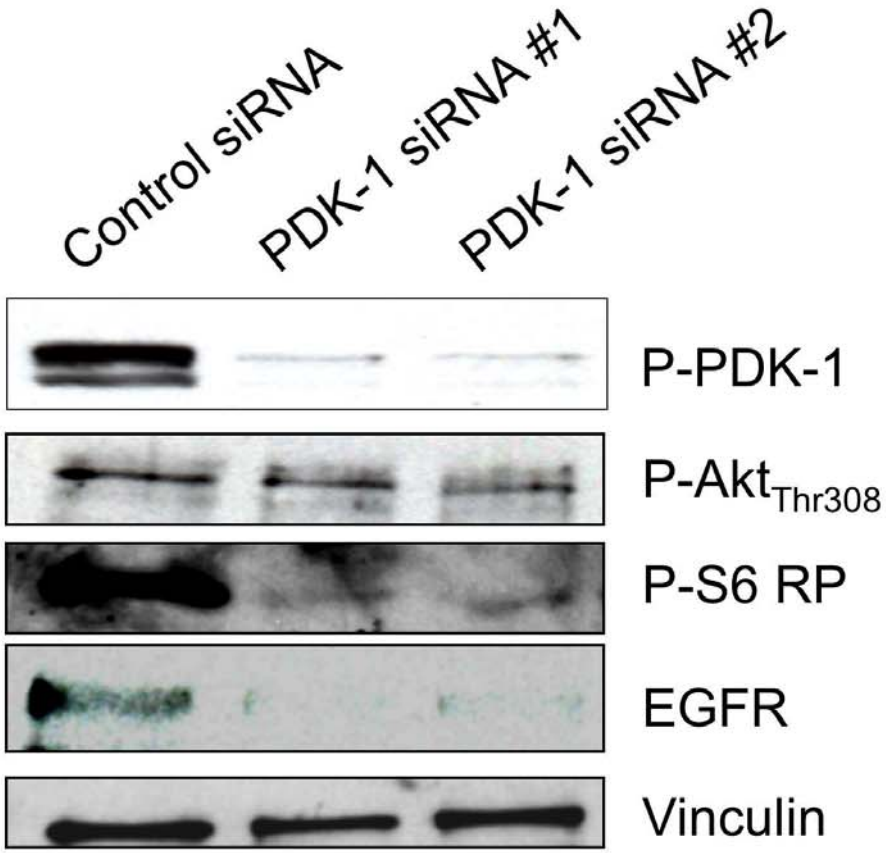

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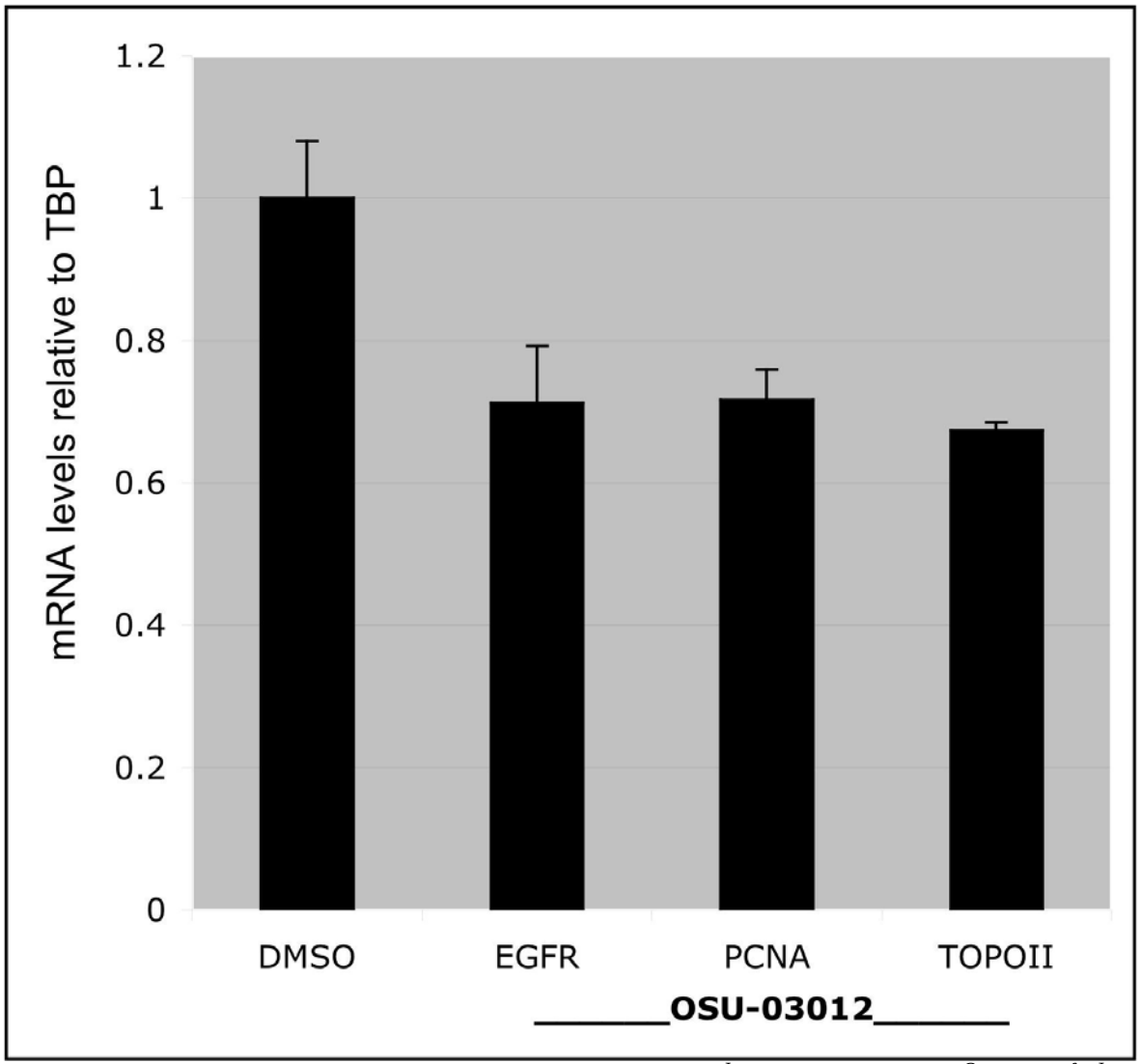


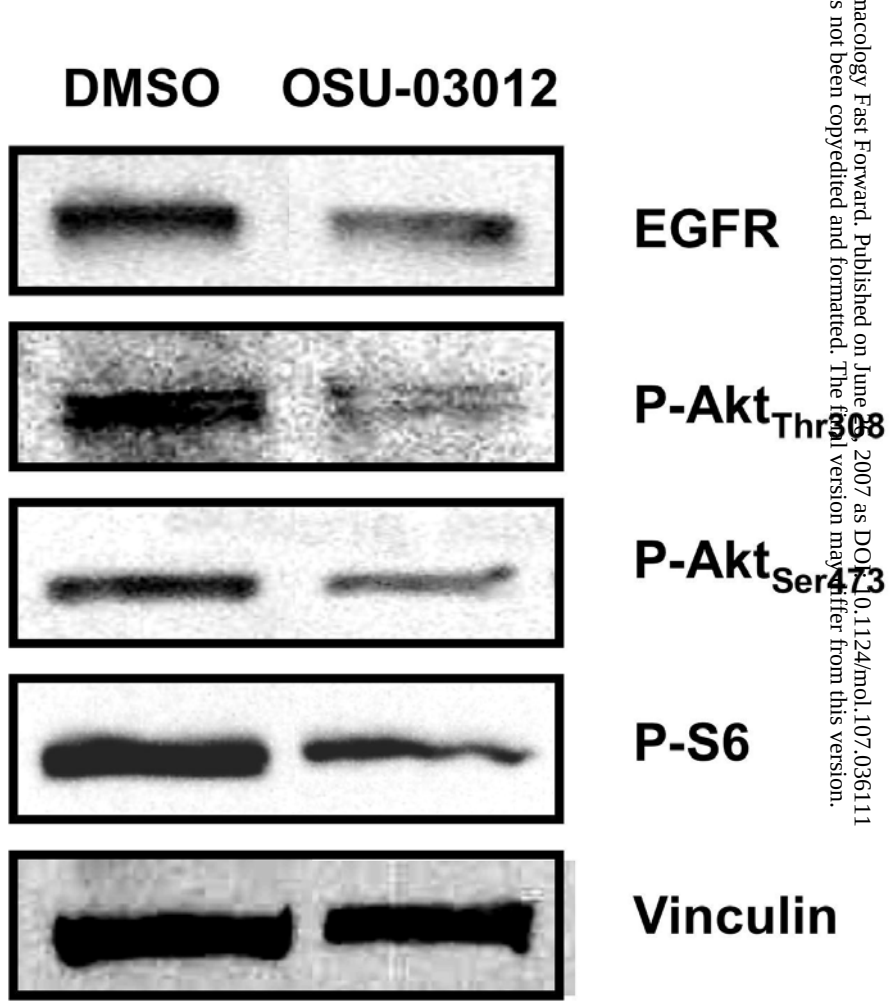


Figure 6

A.

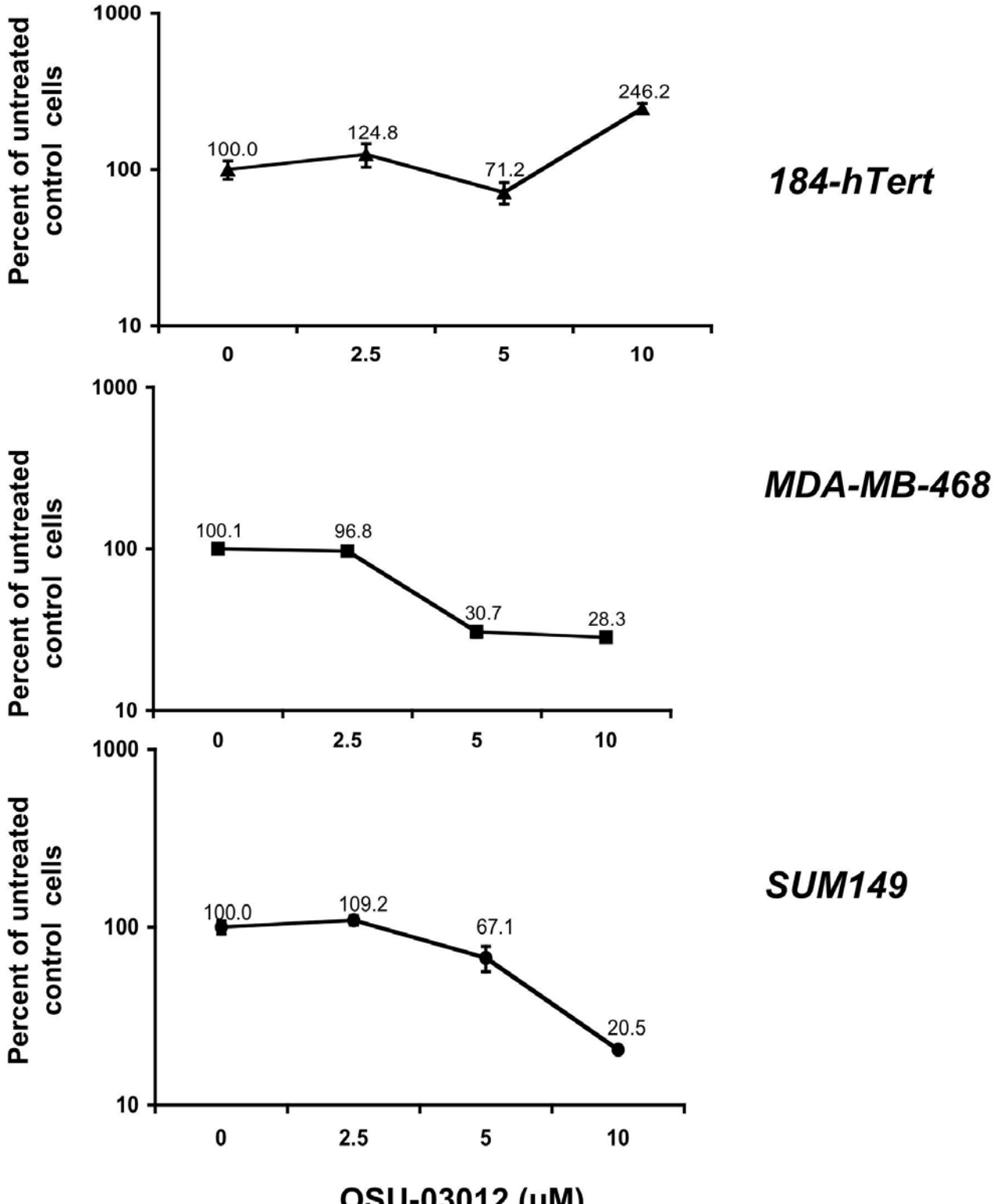
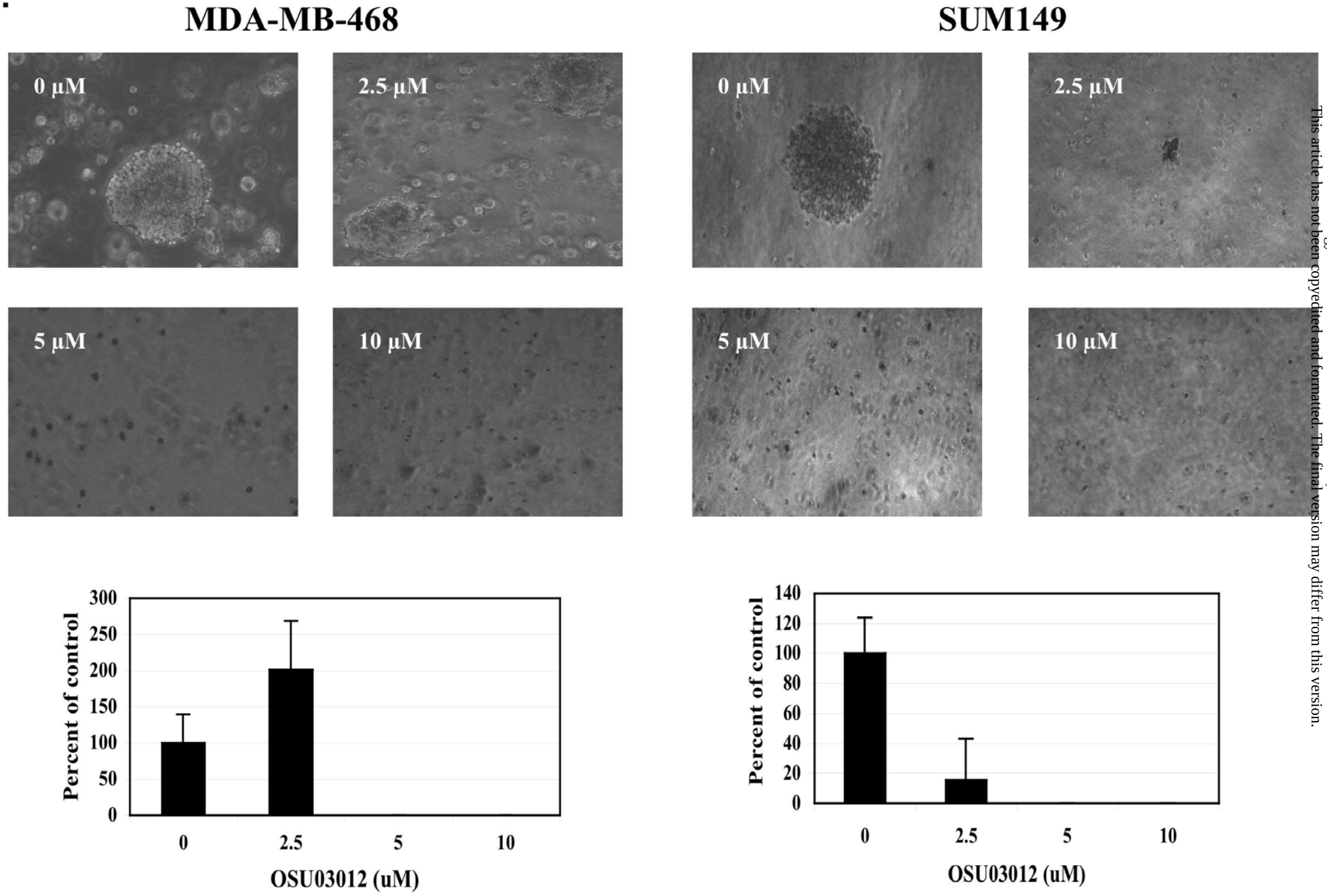


Figure 6

B.



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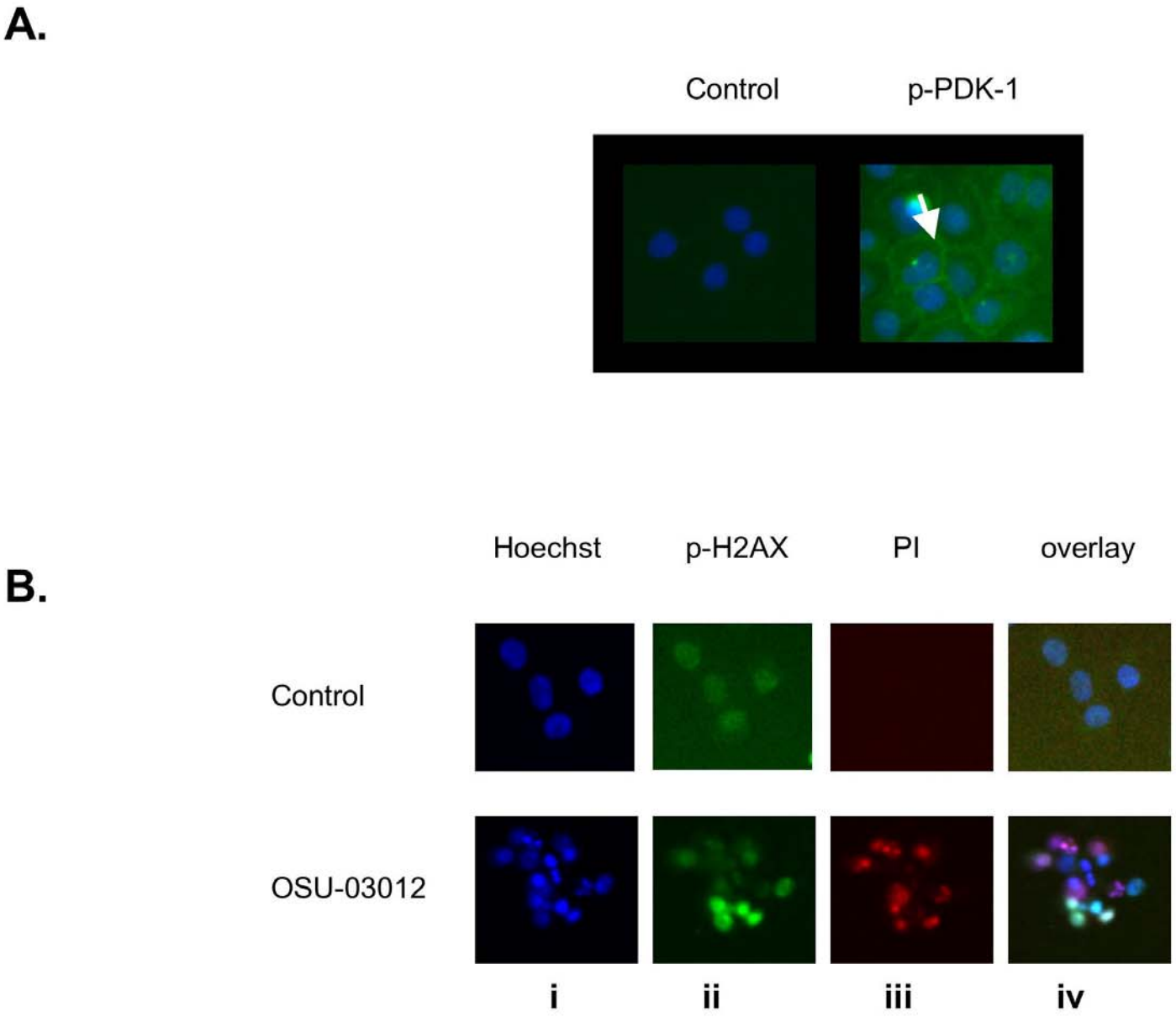

Figure 7

C.

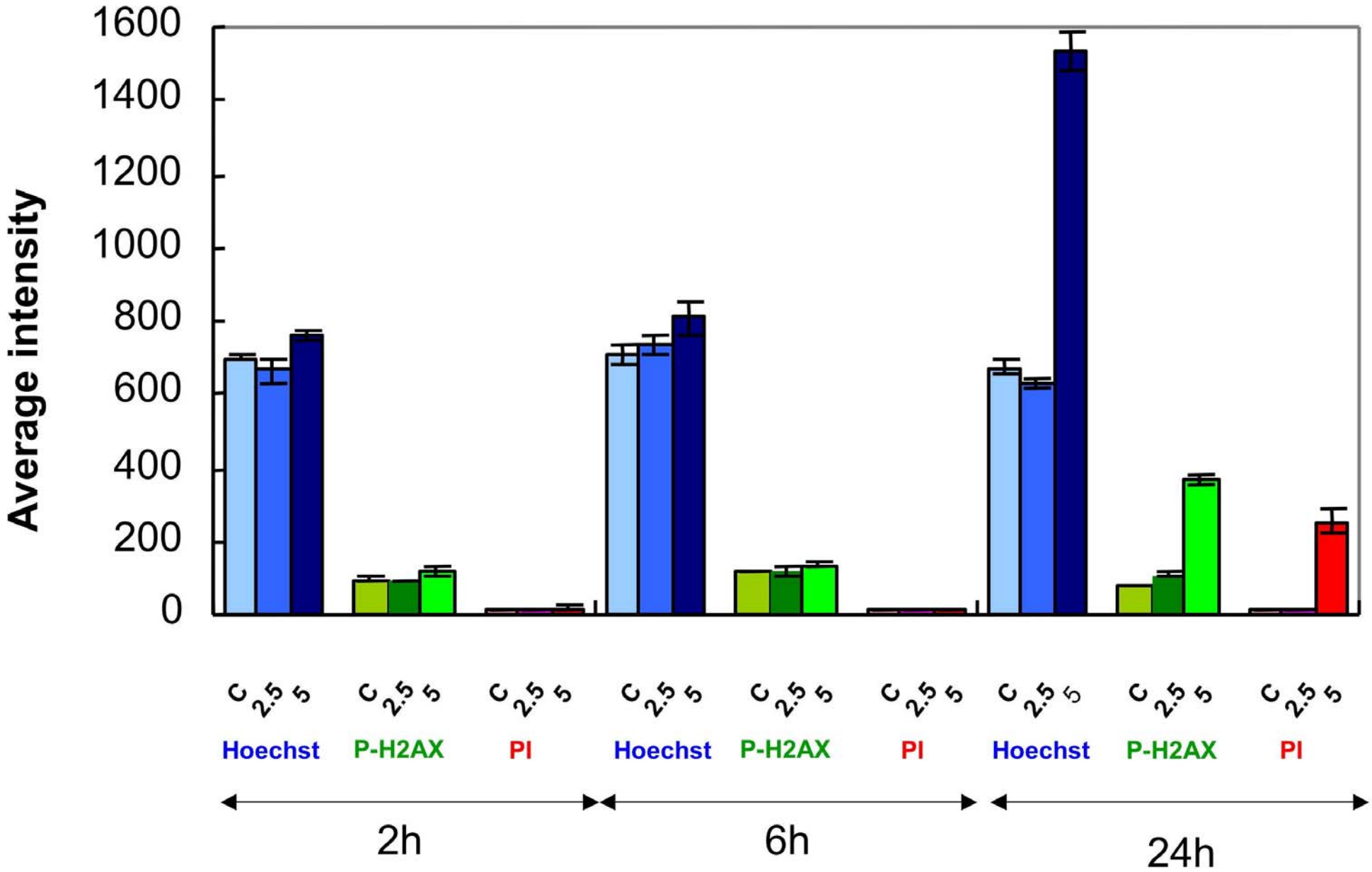

Figure 8