PROTEIN KINASE INHIBITORS TARGETING BREAST CANCER STEM CELLS

THE IDENTIFICATION AND CHARACTERIZATION OF PROTEIN KINASE INHIBITORS TARGETING BREAST CANCER STEM CELLS

By

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ABSTRACT

Breast cancer is the most common cancer among Canadian women with one in nine women expected to develop breast cancer in their lifetime. Until recently these breast tumors were thought to be a homogeneous cell population. Recent studies have shown that breast tumors contain a rare cell type termed breast tumor initiating cells (TICs) or cancer stem cells (CSCs) with the ability to elicit new tumor growth and metastases. These TICs exist apex of a tumor cell hierarchy and give rise to more TICs and non-tumorigenic cells. Traditionally, drugs were developed to target the highly proliferative cells population resulting in a decrease in tumor volume. However, these therapies spare the TICs, which results in tumor relapse demonstrating the need for new drugs that target the TICs. Because in cancer, mutated protein kinases are the controllers of cell proliferation, invasion and metastasis, they have become a target for drug development. Inhibition of these kinases could lead to the identification of compounds that selectively target breast TICs. Using mammary tumors from cancer prone mice propagated as non-adherent tumorspheres (TMS), which contain a high fraction of breast TICs and the same conditions to propagate the non-transformed mouse mammary epithelial stem and progenitor cells (MESC), as non-adherent mammospheres (MMS) a 240-kinase inhibitor library was screened using an AlamarBlue proliferation assay. Twenty percent of the compounds resulted in 75% decrease in proliferation of TMS derived cells and some of which were TMS-selective. Sunitinib, a multi-targeted kinase inhibitor, was one of the selective compounds identified and when administered to mice with subcutaneous mammary tumors resulted in tumor shrinkage. This was accompanied by an increase in apoptotic cells, decrease in proliferating cells and tumor vasculature, and a change in tumor morphology and composition. These findings show the efficacy of Sunitinib in shrinking mouse mammary tumors and suggest a potential use of Sunitinib for treatment of breast cancer.

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Abl	Abelson tyrosine kinase
α-SMA	Alpha smooth muscle actin
Alk	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
ATC	Adherent tumor cell
CMC	Carboxymethyl cellulose
CML	Chronic myeloid leukemia
CSC	Cancer stem cell
Csf-1r	Colony stimulating factor 1 receptor
DAPI	4',6–diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
Egfr	Epidermal growth factor receptor
ER	Estrogen receptor
ErbB2/Her2	Epidermal growth factor receptor 2
ERK	Extra-cellular signal regulated kinase
FACS	Fluorescent activated cell sorting
Fak	Focal adhesion kinase
FBS	Fetal bovine serum
Fgfr	Fibroblast growth factor receptor
Flt-3	FMS-like tyrosine kinase-3
GIST	Gastrointestinal stromal tumors
H&E	Hematoxylin and eosin
IC_{50}	Half-minimal inhibitory concentration
IGF-1R	Insulin-like growth factor 1 receptor
InsR	Insulin receptor kinase
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MESC	Mammary epithelial stem cell
MMS	Mammospheres
MMTV	Mouse mammary tumor virus
NOD-SCID	Non-obese diabetic-sever combined immune deficient
OICR	Ontario Institute for Cancer Research
Pdgfr	Platelet derived growth factor
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PTK	Protein tyrosine kinase

RCC	Renal cell carcinoma
RPTK	Receptor protein tyrosine kinase
SH2	Src homology-2
TIC	Tumor-initiating cell
TMS	Tumorspheres
Trk	Tropomyosin-receptor-kinase
Vegfr	Vascular endothelial growth factor

1.0 INTRODUCTION

1.1 Breast Cancer

Cancer is a disease that arises from cellular level genomic changes that ultimately result in malignant transformation. Cancer cell genome defects enable limitless replicative potential, growth factor self-sufficiency, evasion of apoptosis, insensitivity to growth-inhibitory signals, sustained angiongenesis, and tissue metastasis (1). Genetic aberrations occur as a result of a combination of accumulated contact with mutagens from the surrounding environment and/or innate inherited mutations in the genome (2). Breast cancer arises from the epithelial cells of the mammary gland. The human mammary gland, also known as the breast, is made up of two structures: the lobules and the ducts. Lobules are the milk-producing units of the glands, whereas the ducts act to transport milk from the lobules to the nipple during periods of lactation. The two major types of breast cancers are lobule carcinoma and ductal carcinoma further classified into “in situ” or non-invasive and invasive carcinomas (3).

Classification of breast cancer involves numerous criteria, each influencing treatment response and prognosis (4). Tumor staging is a method used to determine if the disease is pre-malignant; early cancer with good prognosis, or advanced cancer with or without metastases. Tumor grade determines how well differentiated tumor cells are. Cancerous cells lose the ability to differentiate and function as part of the organ; a high-grade tumor generally comprises poorly differentiated cells whereas a low-grade tumor comprises well-differentiated cells (5). Breast cancer cells may also express important receptors such as the epidermal growth factor receptor (erbB2/Her2), as well as the estrogen receptors (ER) and progesterone receptors (PR); the latter two determine whether cells likely require estrogen and progesterone for growth (4). Importantly, receptor expression measurements have an important clinical role, as ER/PR+ tumors generally respond to endocrine therapies, such as tamoxifen or fulvestrant,

whereas HER2+ tumors are treated with a humanized anti-HER2 antibody (Trastuzumab) (6). Lastly genomic technologies such as global gene expression profiling using DNA microarrays, which can be used to measure transcript abundance for thousands of genes, have resulted in a molecular classification of breast cancer. These large-scale micro-array studies resulted in the identification of 5 major molecular subtypes of breast cancer. These analyses dissected tumors into two branches based on expression of ER. ER+ tumors were classified as either luminal A or and luminal B, whereas ER negative were subdivided into the Normal-like, Basal-like and “Her-2+” subtypes (7, 8).

1.2 Treatment Options

In Canada, one in nine women are expected to develop breast cancer in their lifetime. With the current screening and treatment options the five-year survival rate has increased to 87% since 1986. Even with advances in treatments an average of one hundred women are expected to die weekly from the disease, indicating the need for better treatment options (9). An overview of the current available treatment options will be discussed in addition to some of the most common drugs used in treatment.

For breast cancer patients, surgery to remove the cancer is the primary treatment option. The surgical procedure is either a lumpectomy, removing the cancerous tissue and some of the surrounding tissue, or mastectomy, removing the entire breast, nipple and some axillary lymph nodes. Following surgery patients are given chemotherapy, radiation therapy, hormone therapy, and/or targeted therapy. The clinic is currently moving towards personalized medicines were diseases such as cancers are classified into subtypes based on distinguishing features which are used to select appropriate therapy.

Traditionally cancers were viewed as a homogenous cell population actively dividing. Chemotherapeutic drugs were generated to prevent cell

division, where dosing allowed a differential window to kill the fastest dividing cells, believed to be the most aggressive cancer cells. Alkylating agents (platinum compounds) anti-metabolite (purine and pyrimidines antagonists), plant alkaloids (taxanes, vinca alkaloids), anti-tumor antibiotics (anthrocyline), and topoisomerase inhibitors are all cell cycle specific and are cytotoxic to dividing cells (10). In breast cancer, chemotherapy is used in an adjuvant setting except for very aggressive late stage breast cancers, in which chemotherapy is used to reduce tumor size before surgery. The various combinations of chemotherapy include cyclophosphamide (alkylating agent), methotrexate and 5-fluorouracil (anti-metabolite), doxorubicin and epirubicin (anthracyclines), paclitaxel and docetaxel (taxanes) (11).

Hormone receptor positive breast cancer patients are started on hormonal therapy medicines that can be used before, after, or in conjunction with other breast cancer treatments. The mainstay of breast cancer hormonal therapy is tamoxifen a selective estrogen receptor modulator. Tamoxifen is an antagonist of the ER through its metabolite 4-hydroxytamoxifen which competes with and inhibits estrogen-ER complexes from translocating to the nucleus and activating downstream growth genes (11). Since the discovery of tamoxifen other selective estrogen receptor modulators have been developed include raloxifene, which acts directly on ER. Additionally, aromatase inhibitors, block the aromatase enzyme from converting androgen into estrogen, are currently used in post-menopausal patients (12).

The success story of targeted therapies for breast cancer comes from trastuzumab in the 1990s. Trastuzumab is a recombinant DNA-derived monoclonal antibody, which binds the epidermal growth factor receptor 2 (HER2/EGFR2) a tyrosine kinase from the EGFR family. HER2 is overexpressed in 20-30% of all breast cancers and forms homodimers or heterodimers with other EGFR family members, HER1, and HER3 to signal downstream activation of cell proliferation pathways (13). Trastuzumab has been shown to successfully

decrease HER2 signaling making it an effective single-agent treatment and in combination with chemotherapy it significantly improves survival in patients with metastatic breast cancer overexpressing HER2 (14). This targeted therapy demonstrates how molecularly targeted therapies have the promise for greater efficacy, with possibly less side effects. Understanding the biology and genetics of the patient and their cancer will help to develop specific cancer treatment (15).

1.3 Kinases in Cancer

Protein kinases mediate most of the signal transduction in eukaryotic cells; by transferring a phosphate group to the target protein, the change in enzyme activity, cellular location, or association with other proteins relays the signal within a cell. Protein kinases control many cellular processes including, transcription, differentiation, metabolism, cell cycle progression, apoptosis, and cell movement (16). About 20% of the 32,000 human coding genes encode protein involved in signal transduction; 8.5% of these genes encode the 518 protein kinases classified into groups based on sequence comparison (16). These proteins are normally tightly regulated and controlled because the perturbation of their signaling by mutation or any genetic alterations can lead to malignant transformation (17). Interestingly the comparison of kinase chromosomal mapping and known disease loci indicates that 164 kinases map to amplicons seen frequently in tumors (18). An overview of the protein kinases in cancer will be discussed and the role of some kinases in breast cancer will be highlighted.

Cancer does not just arise from increased cell proliferation rate but rather the loss of balance between cell proliferation and cell death. This integral concept of balance is observed early during normal embryonic development were signaling is precisely regulated to provide differentiation signals to prevent oncogenesis (19). Throughout life, DNA is exposed to mutagens and accumulation of replication errors that can lead to growth advantages to the cell.

These oncogenic mutations occur more frequently in certain classes of kinases, for example receptor protein-tyrosine kinases (RPTKs), especially ones governing pathways controlling development and differentiation. To date there are over 100 dominant oncogenes and protein kinases, specifically protein tyrosine kinases (PTKs), comprise the largest fraction (20).

PTKs are made up of 58 transmembrane RPTKs classified into 20 subfamilies and 32 cytoplasmic non-receptor PTKs in 10 subfamilies (21). RPTKs are cell surface receptors with a ligand binding domain and intracellular kinase activity. Upon ligand binding PRTKs dimerize or oligomerize leading to trans-phosphorylation on tyrosine residue, which functions to activate the catalytic activity and generates specific binding site for signaling molecules containing Src homology-2 (SH2) and protein tyrosine binding domain (17). Activation of kinase activity requires the relief of the inhibitory constraints exerted by the activation loop, the juxtamembrane and C-terminal regions (22). Phosphorylation of one, two or all of these inhibitory constraints allows the kinase to relax and disrupt the autoinhibitory interactions resulting in an active kinase. The autoinhibitory mechanism in an unstimulated cell illustrates the regulatory constraints provided as a safeguard to wrongful activation of kinase signaling.

Aberrant PTKs activation has been linked to human malignancies with over half of the RPTKs shown to be mutated or overexpressed in different tumors. Interestingly many of the genetic alterations that lead to deregulated kinase signaling are as a result of the relief of inhibition of control mechanisms found in normal cells (23). The mechanism of oncogenic activation occurs by four principle mechanisms; firstly, transduction by a retrovirus of a PTK proto-oncogene. An example can be seen in breast cancer-prone transgenic mice; these mice have a mouse mammary tumor virus (MMTV) promoter driving overexpression of the Her2/Neu oncogene whose overexpression results in tumor formation (24). Second, gene amplification and overexpression, commonly seen in breast and lung carcinomas, results in a constitutively active kinase. In

breast and lung cancer HER2 and EGFR are commonly overexpressed (23). The increase in receptor concentration promotes HER2 and EGFR random interaction with each other and other EGFR family allowing for activation in the absence of a ligand. The third form of oncogenic activation can occur from a chromosomal translocation (15). The product, an oncogenic fusion protein, contains the kinase catalytic domain and an unrelated protein resulting in a constitutively active kinase. In nearly all cases of chronic myeologenous leukaemia (CML) the Abelson tyrosine kinase (ABL) is fused to breakpoint cluster region (BCR) because of chromosomal rearrangement. The Bcr-Abl protein is believed to cause CML by eliciting uncontrolled activation of the MAPK cascade and inhibition of DNA repair causing genomic instability (25). Finally, gain-of-function mutations are observed in variety of human cancer. Stem cell factor receptor (KIT) kinase point mutations are observed in gastrointestinal-stromal tumors (GISTs), acute myeloid leukemias (AML), mast cell leukemias and melanomas (26). The activating mutations can occur in the intracellular juxtamembrane segment relieving the autoinhibitory constraints and the extracellular ligand-binding regions relieving the need for ligand binding for kinase activation. Overall, oncogenic activations observed in human malignancies obstruct regulatory inhibitions provided in normal cell.

In breast cancer, HER2 overexpression is a common oncogenic event, and targeting of HER2 has proven to be an effective therapy (13). This successful approach of molecular targeting suggests that understanding the biology and genetics of breast cancer can aid in the discovery of other potential therapies. Studies have shown other kinases including c-src, phosphatidylinositol 3-kinases (PI3K), and mitogen-activated protein kinase (MAPK) to be overexpressed, mutated, or constitutively activated in breast cancer (27, 28, 29). c-Src is a nonreceptor tyrosine kinase localized in the intracellular membrane. In breast cancer and other carcinomas c-Src is overexpressed or highly activated (27). In fact a study looking at breast tumor samples identified Src kinase activity to be 2

to 50-fold greater than in normal breast tissue due to an increase in protein levels (30). Unlike EGFR, overexpression of c-Src alone cannot transform cell cultures *in vitro* or maintain tumor growth *in vivo*. Although, in polyomavirus middle T transgenic mice the c-src is required for tumor formation suggesting that src promotes tumor growth by enhancing downstream mitogenic pathways activated by external growth factors or intracellular oncogenes (31). Interestingly, c-Src is found to be co-overexpressed with EGFR suggesting a cooperative interaction between the two. In breast cancer cell lines and tumor tissue, the co-overexpression of c-src and EGFR results in complex of these two kinases that leads to increased phosphorylation of EGFR effectors phospholipase C, and MAPK (32). Tumor size in nude mice from these cells lines were also larger in comparison to other cell lines that do not express both kinases. Acting synergistically their interaction drives unregulated growth and tumorigenesis.

The PI3K pathway has emerged in recent years as an important regulator of cell proliferation, growth and apoptosis. Deregulation of the pathway is characteristic in many human malignancies (33). PI3Ks are heterodimeric lipid kinases that are composed of two subunits; the catalytic (p110) and regulatory/adaptor (p85) subunit (28). PI3K can be activated by phosphorylated RTK through the SH2 domain found in the p85 subunit or through the interaction of the p110 subunit with activated Ras, a signaling molecule. Both forms of activation bring PI3K in close proximity to its substrate inositol phospholipids and relieve the inhibitory action of p85 on the p110. Once activated PI3K phosphorylate the 3'OH group of inositol phospholipids. The p110 subunit can be oncogenic when overexpressed or mutated as seen in ovarian cancer and in breast cancer and these activating mutations induce transformation (34). Furthermore, the PIP_3 the second messenger generated by PI3K helps activate another downstream kinase Akt. Akt is a small family of three related serine/threonine kinases (Akt1, Akt2, Akt3) that is recruited to the plasma membrane and binds to PIP_3 through its PH domain. Upon binding, a

conformational change occurs making it accessible to the PDK1 kinase, which phosphorylates the activation loop stabilizing Akt in an active confirmation. At this point Akt is not fully activated; full activation of Akt requires phosphorylation by a second kinase, PDK2 (35). The multiple steps required to activate Akt illustrates how tightly regulated this kinase is. Akt targets a wide range of substrate involved in cell cycle, cell growth and apoptosis. It comes as no surprise that Akt is dysregulated in a number of human cancers and in breast cancer Akt is overexpressed and its activity amplified (36). Additionally, the phosphatase and tensin homologue, a phosphatase that dephosphorylates the 3'OH group of phosphoinositides to prevent Akt activation, is mutated in some breast cancers (34). Interestingly, patients who overexpress Her2 with a PI3K mutation and/or mutated phosphatase and tensin homologue were associated with poor prognosis after Herceptin therapy (37). Furthermore, patients on Herceptin develop a resistance to the drug, which studies have linked an increase in PI3K pathway signaling to drug resistance (38).

The MAP kinase pathway is another integral pathway involved in cell proliferation and cell death. First identified as serine-specific kinases, these proteins are key for signal transduction activated by growth factors and mitogens. In cancer, deregulated MAP kinase signaling impinges on normal cell processes and play critical roles during development and progression of cancers (39). In fact, approximately half of breast cancers express more activated MAP kinase than the surrounding normal tissue (40). Activation of the MAP kinase occurs in a cascade of three kinases. Similar to the previously mentioned kinase, MAP kinase requires that the activation loop be phosphorylated on threonine and tyrosine residue by a dual specificity kinase, MAP kinase kinase (MAPKK). The MAPKK are themselves activated by phosphorylation by another class of kinases referred to as MAP kinase kinase kinase (MAPKKK). Although there are three different groups of MAP kinases, extra-cellular signal regulated kinase (ERK), Jun kinases (JNK), and p38 MAPKs, the ERK pathway is most relevant in breast

cancer (28). The components of the ERK pathway are Raf kinase (MAPKKK), MEK-1 and 2 (MAPKK), and ERK-1 and 2 (MAPK). Constitutive activation of ERK signaling observed in most cancers occurs upstream of the pathway, namely, overexpression of receptor tyrosine kinases, activating mutations in receptor tyrosine kinases, or sustained autocrine or paracrine production of activating ligands (38). The high frequency of activating mutations centered around this pathway suggests the importance of this pathway for cancer growth and progression (41).

1.4 Protein Kinase Inhibitors

In May 2001 a landmark event occurred when Imatinib (Gleeve, Novartis) a small molecule kinase inhibitor targeting Abl kinase was approved for clinical use in patients with CML carrying the Bcr-Abl mutation (42). The success of this drug led the way to more small-molecule kinase inhibitor drugs. Currently there are 10 small molecule kinase inhibitors approved for clinical use and many more in clinical trials (43). Initially there were concerns about developing selective kinase inhibitors because of the well-conserved ATP binding site, but novel compounds have been developed, which target structurally different points in kinases. Notably, some of the approved drugs have off-target activity proving to be advantageous. For example, Suntinib is a potent inhibitor for multiple kinases at clinically used concentrations (44).

Current kinase inhibitor drug discovery efforts target three broad groups, the RTKs, MAPK, and PI3K pathway (45). These three groups are linked because the MAPK and PI3K pathways are activated by RTKs as their primary signaling function. Over 50% of the kinases currently targeted in clinical trails are RTKs including Vegfr, Egfr, Her2, Kit, Met and FMS-like tyrosine kinase-3 (Flt-3) (46, 47). This is only a small fraction of the kinome illustrating a need for novel inhibitors that target diverse kinases.

The success of some kinase inhibitors can be attributed to oncogene addiction, a term first coined by Bernard Weinstein to explain the unexpected dependency of tumors cells on a single oncogene or oncogenic pathway for sustained activity despite the accumulation of a number of different mutations (48, 49). This Achilles heel of tumor cells, in the form of a mutationally activated kinase, results in drug-sensitivity. Identifying and targeting these kinases presents potential therapeutic opportunities. In fact, evidence of oncogene addiction can be seen in EGFR-mutant NSCLC cell lines in which inhibition of EGFR leads to inhibition of effectors of cell survival such as AKT and subsequently apoptosis (43). Furthermore, oncogene addiction suggests that these pathways are detrimental to tumor cells and small molecule kinase inhibitors may therefore target these pathways in tumor cells selectively sparing their normal cell counterparts.

1.5 Normal and Cancer Stem Cells

The intricate balance between self-renewal and differentiation is demonstrated early on in human development. Molecular signaling regulates these programs in order to produce specialized populations of terminally differentiated cells required for the parenchyma to function. Concurrently, a small population of cells is maintained which, is capable of self-renewing and differentiating termed somatic stem cells (50). Tissue growth is driven by these stem cells, which self-renew to maintain the stem cell pool or differentiate to produce the majority cell population of specialized tissue specific cells. Notably, stem cells can divide symmetrically to expand in number during development or injury or asymmetrically to maintain appropriate number of progeny (50).

In the mammary gland, mammary epithelial stem cells (MESC) self-renew to generate more stem cells or differentiate into mature myoepithelial and luminal cells required for mammary gland functions. A body of work has been published

on serial transplant assays showing the ability to generate a functional mammary gland from a single cell (51, 52) demonstrating the presence of MESC. Furthermore, these assays have also identified progenitor cells that can only give rise to outgrowths of one of the two structures that make up the mammary gland, lobules or ducts (52). This lead to the thinking that mutations in limited progenitors or differentiated cells must lead to a net increase in proliferative capacity or, lead to self-renewal capacity in order to have an impact on the entire mammary epithelia. However, any mutations in MESC will be passed on to the entire epithelial population and be inherited by stem cell progeny potentially leading to tumorigenesis.

The cancer stem cell hypothesis proposes that tumors are heterogeneous cell populations comprising a cellular hierarchy with cancer stem cells (CSCs) or tumor-initiating cells (TICs) at the apex of the tumor cell hierarchy (53). Parallel to normal tissue stem cells, these CSCs differentiate, albeit aberrantly, to give rise to all the various tumor cell types in the tumor. Only CSCs possess long term self-renewal and tumor reconstitution potential. The remaining cancer cells are capable of short-term proliferation for a finite number of cell division followed by senescence and are therefore unable to maintain tumor growth (54). This hypothesis takes in consideration the clonal nature in which tumors arise and explains why these tumors comprise heterogeneous cell population with diversity of morphologies, immunophenotypes and proliferative potentials (50). The term CSC does not preclude or imply that the cell originates from a stem cell. Like normal stem cells, CSCs are defined using functional assays where cells must be able to generate tumors in immunocompromised mice with small starting number of cells and the generated tumors must be serial passaged.

John Dick and colleagues were the first to demonstrate the existence of CSCs in AML (55). By using fluorescent activated cell sorting (FACS) and fluorescently tagged antibodies against cell surface proteins, they sorted AML into different populations of cells. Using the non-obese diabetic-severe combined

immunodeficient (NOD-SCID) mouse model they measured the growth of the transplanted human AML cells and identified tumorigenic cells and non-tumorigenic cell populations. Only those cells expressing CD34 and not CD38 were able to give rise to leukemia in the NOD-SCID mice, indicating that the CSCs were contained in the $CD34^{+}CD38^{-}$ cell fraction. This seminal study laid the basis of the strategy used to prospectively isolate and identify functional CSCs. This candidate CSC population must exhibit the ability to engraft and generate a tumor that recapitulates the morphological and functional heterogeneity of the original tumor. In addition, to measure self-renewal of the CSCs, the engrafted tumors must be serially transplanted (56). To date, CSCs have been identified in a number of solid tumors such as those of breast (57), brain (58), ovary (59), pancreas (60), colon (61), gastrointestinal (62), and skin (63).

In 2003 Michael Clarke's group discovered CSCs in human breast cancer (57). In adherence with the protocols taken from John Dick, mammary tumor cells were sorted based on cell surface markers (after sorting out the non-epithelial cells such as the hematopoietic, endothelial, and mesenchymal cells). The different cell populations were then transplanted into cleared fat pads of NOD-SCID mice. The subpopulation within $CD44^{+}CD24^{-/low}lineage^{-}$ phenotype gave rise to tumors, which gave rise to additional tumorigenic and non-tumorigenic cells as observed in the original human tumor (57). Furthermore, when 20,000 cells without this phenotype were used, they were unable to form a tumor. As few as 100 of the $CD44^{+}CD24^{-/low}lineage^{-}$ were required for engraftment and serial transplants of the tumor demonstrated the self-renewal capacity of these cells. Further fractionation of $CD44^{+}CD24^{-/low}lineage^{-}$ using ALDH expression has shown that as little as 20 $ALDH^{+}CD44^{+}CD24^{-/low}lineage^{-}$ could give rise to tumors (64). The ALDH cell phenotype in human breast tumors was also found to correlate with poor clinical outcome. More importantly, these experiments demonstrate that breast cancer does follow the stem cell model and that breast cancer stem cells (BCSCs) do drive tumor growth (65).

CSCs share many characteristics of normal stem cells; this includes a long quiescent lifespan, drug-transporting property through ABC transporter, active DNA-repair machinery, and resistance to apoptosis (53). All these properties result in the resistance of CSCs to conventional therapies such as chemotherapy and radiation (66). Analysis using clinical breast tumor biopsies before and after neoadjuvant chemotherapy revealed that docetaxel and doxorubicin/cyclophosphamide regimes increased the $CD44^{+}CD24^{-/low}$ subpopulation and enhanced their propensity for tumor formation in NOD-SCID mice in comparison to the pretreatment samples (67, 68). Additional studies have shown similar results in breast cancer cell lines; *in vitro* treatment with paclitaxel or 5-fluorouracil enriched for $CD44^{+}CD24^{-}$ cells. When subjected to radiation therapy, studies have shown that tumorigenic breast cancer cells contain lower levels of reactive oxygen than their non-tumorigenic counterparts along with increased activation of DNA damage checkpoint (69)

The discovery of CSCs has an important implication for cancer therapy. Novel therapies are needed that target and eliminate the CSCs in order to be effective and prevent tumor relapse. Unlike the traditional drug discovery approach, which targeted cell proliferation, novel drugs need to target stem cells. Initially these therapies would not necessarily result in tumor shrinkage, but would target the root of the problem and eventually result in tumor remission

1.6 *In vitro* model of MESCs and breast CSCs

Studying breast CSCs has proved inordinately difficult because these cells exist only as a rare cell fraction of human breast tumors. Even with FACS sorting techniques, the fractionate only contains about 5% CSC. In order to overcome this obstacle researchers have turned to other models of cancer stem cells in hopes of drug discovery. Dr. Hassell's lab has employed the use of

mouse mammary tumor cells from mouse mammary tumor virus (MMTV)-Her2/Neu transgenic mice.

In addition to recapitulating hallmarks of human disease these tumors are enriched in TICs. Limiting dilution cell transplant studies have shown that these tumors contain a high frequency (~15 to 50%) of tumorigenic cells that could form new tumors in syngeneic recipient mice. These transplanted subcutaneous tumors recapitulate the phenotype of the original tumor; composed of TICs defined by functional transplant assays and non-tumorigenic cells. In order to preserve these TICs, primary tumor cells isolated from the MMTV-neu tumors are cultured *in vitro* in suspension as spheres or tumorspheres (TMS) in a serum-free medium. This method was originally developed for culturing neuronal stem/progenitor cells (70). TMS represent a high frequency of tumor initiating cells (~15%) as determined by limiting dilution transplant assays with a sphere forming frequency of 1% (1 sphere in 100 cells). Alternately, primary tumor cells cultured as adherent tumor cells (ATCs) in serum containing media are devoid of TICs. As many as 100,000 cells were required to be transplanted in order to get a tumor, whereas single TMS derived cells were capable of engraftment Since ATCs comprise about 0.01% TICs, they provide a good model of the non-tumorigenic cell population in tumors. Using these cells provide a good model and renewable source of TICs and non-TICs that can be used to identify therapeutic agents.

In order to facilitate the study of mammary epithelial stem cells, researchers have tried various methods to culture these cells *in vitro*. Dontu et al. cultured human mammary epithelial cells as non-adherent mammospheres (MMS) (71). Human MMS comprise primarily progenitor cells, but these spheres could not be cultured for more than 3-5 passages in vitro. Dr. Hassell's lab and other labs have turned to mouse mammary cells culturing them *in vitro* in the serum-free media as MMS. The mouse model offers a number of advantages

over the human system, including extended propagation of stem and progenitor cells as MMS.

MMS-derived cells are capable of differentiating into luminal and myoepithelial cells *in vitro* and can seed the growth of complete mammary glands when transplanted othortopically. The newly formed mammary glands from these transplants can be serially transplanted resulting in fully functional mammary glands for up to 5 generations. This illustrates that MMS indeed comprise functional epithelial stem and progenitor cells as demonstrated by their self-renewal and differentiation capacity.

In primary mammary epithelial cells the frequency of MMS was determined to be approximately 0.08% (1 sphere in 1,250 cells). This approximates the frequency of functional stem cells in the mouse mammary gland (0.04% 1 sphere in 2,500 cells) (71). Furthermore, MMS were shown to originate from single cells using clonal assays. In established cultures, mammosphere-forming cells occurred at frequency of 1% (1 sphere in 100 cells) independent of passage number. This indicates that MMS can act as a renewable source of functional mammary epithelial stem and progenitors that can be used for drug discovery.

1.6 Thesis Objective

The success of Imatinib, a small molecule kinase inhibitor used to treat patients with chronic myeloid leukemia, demonstrated the validity of targeting kinases for effective cancer therapy. Several other drugs, including Gefitinib, Sunitinib, Sorafinib, and Lapatinib have also since been approved for clinical use and many more kinase inhibitors are in clinical trials. With over 500 kinases as potential targets and because many kinases function in common signaling pathways, a screen using small molecule kinase inhibitor libraries is likely to identify lead compounds. The aim of this research project was to identify kinase inhibitors that specifically target breast cancer TICs. To this end, medium-

throughput screens using the Ontario Institute for Cancer Research (OICR) kinase inhibitor library was carried out in TMS-derived cells to identify compounds that inhibit TMS-derived cell proliferation. Additional screens were carried out to identify which compounds selectively target TMS but not MMS as well as compounds that target the TICs in TMS and the non-tumorigenic cells in ATC. Identified TMS selective compounds were further characterized in follow-up assays.

2.0 MATERIAL AND METHODS

2.1 Isolation and propagation of mouse mammospheres, tumorspheres, and adherent tumor cells.

The #3 and #4 mammary glands were isolated from 6 to 8 week old female virgin FVB/N mice as describe previously (72). Immediately following isolation the tissue was minced in 2ml versene/gram and 4ml of RPMI 1640 [Gibco-BRL] supplemented with 1 mg/ml fungizone, 1% penicillin/streptomycin, 2% fetal bovine serum (FBS), 0.1% trypsin and 3-mg/ml collagenase [Invitrogen]. Following a 30-minute incubation at 37ºC an equal volume of RMPI lacking trypsin and collagenase A was added and the cells were filtered using a 40μm nylon strainer [Falcon]. The filtrate was then centrifuged for 15 minutes at 1500 rpm at room temperature and the pellet was washed twice with Ham's F-12 [Gibco-BRL] supplemented with 1 mg/ml fungizone and 1% P/S. The pellet was then resuspended in 10 ml of stem cell media (SCM) comprising of a 3:1 ratio of DMEM (low glucose) and Ham's F-12 supplemented with 1 mg/ml fungizone, 1% P/S, 4 mg/ml B-27, 20 ng/ ml EGF, 40 ng/ml bFGF and 4 ng/ml Heparin [all from Gibco-BRL]. A viable cell count was performed using trypan blue exclusion [Gibco-BRL] and the cells were incubated at 37ºC and 5% CO_2. Spheres were passaged every four days by centrifuging for 5 minutes at 1000 rpm to pellet the cells followed by mechanical dissociated in fresh SCM. Mammosphere-derived cells were plated at 30,000 cells/ml in 25 cm^2 culture flasks [Corning].

Mammary gland tumors arising from MMTV-Her2/neu transgenic mice were excised and processed in similar manner to the mouse mammary glands with a few exceptions. The tumor tissue was minced with 3 ml of versene/gram and placed in 7 ml of RPMI 1640 [Gibco-BRL] supplemented with 1 mg/ml fungizone, 1% penicillin/streptomycin, 2% FBS, 0.1% trypsin and 3 mg/ml collagenase [Invitrogen]. Primary tumor cells were counted, plated in stem cell medium and incubated at 37ºC and 5% CO_2. In addition to growing cells in stem

cell media, a fraction of the tumor cells were also grown in DMEM [Gibco-BRL] supplemented with 1 mg/ml fungizone, 1% penicillin/streptomycin, and 10% FBS at a density of 10,000 cells/ml as adherent tumor cells. These cells were passaged every 4 days by adding 5mL of 0.25% trypsin EDTA [Gibco-BRL] to 75 cm^2 culture flasks [Corning] and incubated at 37ºC for a couple of minutes to remove the cells from the plate. Once cells lifted off the plate 8 mL of DMEM media was added and cells were centrifuged for 5 minutes at 1000 rpm at room temperature to pellet the cells. Cells were then resuspended in fresh media.

2.2 High-throughput screen

Mammospheres, tumorspheres, and adherent tumor cell cultures at passage of 3 to 5 were used for carrying out proliferation assays. MMS and TMS were pelleted by centrifuging for 5 minutes at 1000 rpm and spheres were dissociated by mechanical trituration. Viable cells were counted using trypan blue exclusion [Gibco-BRL] and the sphere-derived cells were seeded at 20,000-cells/ml in fresh SCM. The adherent tumor cells were detached from plates using 0.25% trypsin-EDTA [Gibco-BRL] and suspended in media at density 20,000 cells/ml in high glucose DMEM with 10% FBS.

Kinase inhibitors were reconstituted in 100% dimethyl sulfoxide (DMSO) for 1 mM stock solutions, which were aliquoted into 96-well dishes. Columns 1 and 12 of the plates were empty. Assay plates were made up form these stock plates with each inhibitor at 250 μM concentration in 25% DMSO. Column 1 and 12 containing just 25% DMSO used for the controls. Prior to carrying out the assay, the compound plates were fully thawed to room temperature.

Using the Biomek 3000 [Beckman Coulter] for automated liquid handling 384-well black assay plates [Corning] were set up with 50 μL cells at 20,000 cells/ml density and 1 μL of inhibitor for a final concentration of 5 μM (final DMSO

concentration of 0.5%). Each inhibitor was tested in quadruplicate and on each assay plates there were 64 controls. Positive controls refer to 100% signal with wells containing cells (TMS or MMS derived) with 0.5% DMSO and negative controls comprised wells containing only media and 0.5% DMSO (no cells). Assay plates were incubated at 37ºC in a 5% CO_2 atmosphere for 24 hours after which 5 μL of almarBlue™ [BioSource] was added to each well. Plates were incubated for another 24 hours and then removed from the incubator and the fluorescence was measured (l_{ex} = 535 nm, l_{em} = 600 nm, mirror = general dual) using the Multimode Detector [Beckman Coulter](Figure 1).

The measured fluorescence (RFU) from tested compounds was converted to percent residual activity (%RA) using the following equation:

$$\%RA = \left(\frac{RFU - \mu_-}{\mu_+ - \mu_-} \right) \times 100 \qquad (73)$$

where μ_+ and μ_- are the average fluorescence of the positive and negative controls, respectively.

The quality of the screen was determined using statistical parameter Z`, which measures the size of the window used to identify active compounds.

$$Z` = 1 - \left(\frac{3\sigma_+ + 3\sigma_-}{|\mu_+ - \mu_-|} \right) \qquad (74)$$

σ_+ and σ_- are the standard deviation (σ) *%RA* positive (+) and negative (-) controls. A Z' value was calculated for each compound plate. A value greater than 0.5 indicates a high quality assay**.** Commonly, the ratio of the signal (reduced alamarBlue) between control wells bearing cells and those lacking cells was 10-20 fold.

For the follow-up assay a similar cell proliferation assay was also carried out. Compound plates for dose-response determinations were prepared from either the stock solution or from fresh aliquots of kinase inhibitors. Each plate contained 8 compounds with 11 dilutions in 25% or 5% DMSO depending on whether the compounds were from the stock solution or from fresh aliquots. The compound dilutions were 10.00, 6.324, 2.000, 0.6324, 0.2000, 0.0632, 0.020, 0.0063, 0.002, and 0.000632 μM. Assays were set up as previously mentioned except the incubation time was changed to 96-hour period. Following compound addition the cells were incubated at 37ºC in a 5% CO_2 atmosphere for 72 hours followed by almarBlue™ [BioSource] addition and an additional 24-hour incubation. Fluorescence reading was taken at the end of 96-hours.

2.3 Sphere forming assay with small molecule kinase inhibitors

Established MMS and TMS between passages 3 to 5 were centrifuged for 5 minutes at 1000 rpm to pellet the cells. The spheres were then mechanically triturated in fresh SCM and plated into 96-well plates at a density of 6,000 cells per well with 200 μL of media containing different inhibitor concentration (0.312, 0.625. 1.250, 2.500, 5.000 10.000, 20.000 μM). Each inhibitor concentration was done in triplicate and the control MMS and TMS contained media and 0.04% DMSO. Cells were incubated at 37ºC and 5% CO_2 for four days and on the fourth day spheres were counted.

2.4 *In vivo* Sunitinib treatment of mice with subcutaneous MMTV-*neu* tumors

Fifty thousand freshly isolated primary tumor cells were injected subcutaneously into 10 mice. Mice were monitored for the appearance of tumors.

Once the tumors reached ~ 1cm^3 treatment was commenced. Five mice were orally administered Sunitinib and the remaining five were orally treated with vehicle, carboxymethyl cellulose (CMC) solution (0.5% CMC, 1.8% NaCl, 0.4% Tween80, distilled water) (75). Mice were treated for five days and allowed to recover for two days for a period of two weeks. Tumor size was monitored throughout the treatment and at the end of the treatment mice were sacrificed. The remaining tumors and mammary glands were isolated and small portion was fixed in 2% w/v paraformaldehyde (PFA) [Merck] for 48-hours. Whatever was remaining of the tumor was frozen in 90% FBS [Sigma] and 10% DMSO [Sigma].

2.5 Immunohistochemistry

Fixed tumor and mammary glands were embedded in paraffin and 4 μM sections were placed onto slides. Prior to immunostaining, slides were dewaxed with xylene followed by rehydrating the cells. This was done by increasing the concentration of deionized water in absolute ethanol starting from 0% w/v to 30% w/v deionized water. After rehydration, slides were incubated at 85–95°C, citrate-based antigen retrieval solution [Vector] for 10 minutes and then allowed to cool. Slides were washed with PBS for 5 minutes and then blocked using a 3% v/v goat serum [Dako] in PBS containing 0.03% Tween20 [Sigma] for 45 minutes at room temperature. Primary antibodies to α-smooth muscle actin (α-SMA) (1:750) [Sigma] CK8 (1:20) [RDI], CK14 (1:500) [Sigma], and Vimentin (1:40) [Sigma] were incubated with slides for 1 hour at room temperature and then washed 3 times with PBS containing 0.03% Tween20 for 5 minutes. For secondary antibody, a fluorophore-linked secondary antibody that binds to the primary antibody was added to the slide for 45 minutes at room temperature. Slides were washed again with PBS containing 0.03% Tween20 and mounting media containing 4',6-diamidino-2-phenylindole (DAPI) [Vector] was added to the slides and covered with a coverslip [VWR]. Using OpenLab Improvisation software

[VWR] images were captured on a Leica inverted research microscope. As a control, slides were probed with secondary antibody only in order to determine background. PFA fixed tumor sections were also stained with hematoxylin and eosin stain (H&E) in addition to immunostaining for Ki-67 (counterstained with hematoxylin to identify the nucleus).

2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was carried out using TUNEL Apoptosis Detection Kit from Millipore to identify apoptotic cells. Protocols provided in the kit were followed. Briefly, sections were dewaxed and rehydrated as previously mentioned and incubated in antigen retrieval solution. Slides were washed with PBS for 30 minutes at 37ºC and then incubated with proteinase K for 20 minutes at 37ºC. Slides were washed again with PBS 4 times for 2 minutes to stop the proteinase K digestions. Slides were then blocked with a provided blocking buffer for 10 minutes at room temperature followed by incubation with terminal deoxynucleotidyl transferase and biotin-dUTP to label the free 3'-OH of cleaved DNA. After labeling the DNA, a fluorescein-conjugated avidin (avidin-FITC) was added to the slides for 30 minutes at 37ºC. A final wash with PBS at room temperature was done for 15 minutes and mounting media DAPI [Vector] was added to the slides and covered with a coverslip [VWR]. OpenLab Improvisation software [VWR] was used to capture images on a Leica inverted research microscope.

3.0 RESULTS

3.1 Small molecule kinase inhibitor screen identifies compounds that target tumor-initiating cells

Our goal was to identify kinase inhibitors, which selectively targeted tumor-initiating cells. To identify such compounds, we carried out a high-throughput screen of the OICR kinases inhibitor library in MMS, TMS and ATC. The OICR kinase inhibitor library comprises three 96-well plates with 80 compounds per plate for total of 240 distinct kinase inhibitors, some of which are approved drugs, compounds in clinical trials, and compounds used in lab settings to target kinases. Initially we completed a primary screen in all three-cell types, where each compound was tested in quadruplicate at a single compound dose of 5 μM (Figure 1). Controls were set up in each assay plate such that a total of 32 positive and negative controls were included. The positive controls comprised of cells and vehicle (DMSO) while the negative controls were media only with DMSO. 1,000cells were seeded into each well with 1 μL of kinase inhibitor at 5 μM and incubated for 24-hours. Following the 24-hour incubation, 5 μL of alamarBlue was added and incubated for another 24-hours. AlamarBlue is a non-fluorescent resazurin based dye that is converted to red- fluorescent resorufin by metabolically active cells. The amount of fluorescence produced is proportional to the number of live cells making it a useful indicator of cell proliferation.

For each assay plate positive and negative controls were analyzed to measure the size of the window used to identify active compounds. Z' value, a number between 0 and 1 which is calculated based on the mean and standard deviation of both positive and negative control, is calculated to determine the quality of the assay (74). A value of 0.5 or greater indicates a good assay in which there is a large signal to noise window. For all three compound plates the Z'-value in each cell culture was above 0.5 (Table 1) and there was a minimum of 20-fold difference in fluorescence between the positive and negative controls.

Cell viability was analyzed and the raw fluorescence readings were converted to percent residual activity (%RA). 100% RA indicated a compound that had no effect on the cells while 0%RA indicated a compound, which killed all the cells. Hits in this assay were defined as anything that reduced %RA by 75% or more. Based on this threshold 50 out of the 240 kinase inhibitors were identified as hits in TMS-derived cells resulting in a 20.83% hit rate (Figure 2A, Table 2). Similarly in the MMS-derived cells there was 14.17% hit rate and 9.58% in the ATC (Figure 2B-C). Interestingly, there were 16 that were hits in all 3-cell cultures, 17 in TMS and MMS, and 3 in TMS and ATC. Only 2 compounds came up as a hit just in the ATC and no compounds were identified as MMS only hit or MMS and ATC hits (Figure 2D). The compounds that were identified as hits targeted a diverse set of kinases. The OICR library is made up of multiple classes of kinase inhibitors with RTKs being the largest (25%) (Figure 3). Classifying the hit kinase targets indicates that 32% of the inhibitors targeted are also RTKs including fibroblast growth factor receptor (Fgfr), EGFR, and Kit (Figure 4). The PI3K pathway, cell cycle kinases, and MAPK pathway are also targeted by the hit kinase inhibitors. Abl and Src kinase make up most of the non-receptor-tyrosine kinases targeted. Furthermore, some of the compounds identified in the screens, such as Sunitinib, Imatinib, and Sorafenib are cancer drugs approved to treat various other human malignancies.

3.2 Secondary screen validates hit compounds and identifies selective compounds

To further refine the list of hits and measure compound selectivity; a secondary screen was carried out in MMS, TMS, and ATC cultures at multiple concentrations. Similar to the primary screen the assay was a proliferation assay using alamarBlue reduction as a measure of live cells. TMS hit compounds were assayed in an 11-point serial dilution starting as high as 10 μM to 0.100 μM

(some of the compounds could only be tested at 5 μM for the highest dose because of a relatively dilute stock solution). Cells were plated at a density of 1000 cells per well and 1μL of inhibitor was added at an appropriate concentration. Each compound concentration was tested in quadruplicate. In the secondary screen the assay was carried out over a 4-day period unlike the primary assay. The change in incubation time was done to allow more time for the inhibitors to have an effect on the cells. Following cell plating and compound addition cells were incubated for 72-hours and then alamarBlue was added. After 24-hour incubation fluorescence was measured.

For each assay the Z' was measured to ensure a value 0.5 or more and fluorescence was converted to % RA. The results of this assay were first used to validate the primary screen; 86% of the primary hits were confirmed. The half-maximal inhibitory concentration (IC_{50}) for each compound was also calculated. Futhermore, compounds that target TICs but not their normal counterparts were termed selective compounds and were defined by a ratio of MMS to TMS IC_{50} of 2 or more. Six compounds were identified: TAE-684, FAK Inhibitor 14, Sunitinib, Masitinib, Sorafenib, and CI-1033, which inhibits anaplastic lymphoma kinase (Alk), focal adhesion kinase (Fak), Kit (both Sunitinib and Masitinib), and Raf respectively (Figure 5). The most selective inhibitor identified was the TAE-684 compound with a MMS to TMS IC_{50} ratio of 6.72 (Table 2). Interestingly, the TAE-684 target Alk is not expressed at the gene level in TMS, MMS, or ATC as determined by global gene expression. The FAK Inhibitor 14, Sunitinib, Masitinib, and Sorafenib all had MMS to TMS IC_{50} ratios between 2 and 3. Sunitinib, Masatinib and Sorafenib all target Kit kinase with Kit being a secondary target of Sorafenib (Figure 5). Imatinib, Gefitinib, and Ryuvidine were also moderately selective inhibitors. At the highest concentration of the compound, 10 μM, the inhibitor did not inhibit MMS proliferation by 50% or more. Imatinib and Gefitinib target RTKs, but Imatinib also targets Abl kinase. Ryuvidine on the other hand targets CDK4.

The second group of compounds identified in the screen were compounds that do not have any effect on ATC at the highest concentrations; 16 inhibitors were identified (Figure 6). Interestingly, these compounds inhibited TMS and MMS proliferation with some compounds having IC_{50} values in the nanomolar range ((5Z)-7-Oxozeaenol and Dovitinib). Notably, these inhibitors that did not inhibit ATC proliferation did not target a specific class of kinases. The remainder of the compounds targeted all three cells types. This group can subdivided into compounds that target all three cells types with equal potency (AZD0530 and API-1 were not tested in ATC) (Figure 7) and those that are slightly selective for TMS and MMS (Figure 8). Again, the inhibitors in this group did not target any specific class of kinases; no trend was observed among the targets of the inhibitors that targeted all three cell types or TMS and MMS.

3.3 Kinase inhibitors inhibit sphere-formation in TMS and MMS-derived cells

Having discovered kinase inhibitors that were moderately selective in TMS compared to MMS, we carried out sphere forming assays to further characterize these compounds. A sphere-forming assay is an *in vitro* surrogate assay for self-renewal. TMS and MMS-derived cells between passage 2 and 5 were mechanically dissociated and plated in 96-well plates at a density of 30,000 cells/mL (6,000 cells/well). Appropriate inhibitor concentration was added to each well and DMSO was added to the control wells. Following a 4-day incubation period spheres were counted. In this fashion, TAE-684, Masatinib, A-443654 and Sunitinib were tested.

Although TAE-684, an ALK inhibitor, was selective in the alamarBlue proliferation assay, it was not selective in multiple sphere-forming assays (Figure 9A). The compound IC_{50} in a sphere-forming assay was 0.350 μM in MMS-derived cells and 0.264 μM in TMS-derived cells. The TMS IC_{50} in this assay was

similar to what was observed in the proliferation assay but the MMS IC_{50} was 3.630 μM in the proliferation assay about 10-fold higher IC_{50} value than that in a sphere-forming assay.

A-443654 compound was not selective in the sphere-forming assay but was one of the very potent PI3K inhibitors (TMS IC_{50} 0.47 μM) and therefore it was tested in sphere-forming assay. Interestingly, this compound was moderately selective in a sphere-forming assay (Figure 9B). The IC_{50} in TMS was 0.408 and 1.01 in MMS; a 2 fold selectivity ratio.

Masatinib and Sunitinib, Kit inhibitors were also assayed in sphere forming assays (Figure 9C). Similar to TAE-684, Masatinib was not selective in this assay with the IC_{50} in MMS and TMS at 3.39 μM and 2.51 μM respectively. On the other hand, Sunitinib was selective in multiple sphere forming assays with an IC_{50} in MMS of 3.21 μM and 0.805 μM in TMS displaying a 4-fold selectivity (Figure 9D). Interestingly this compound not only inhibited sphere-formation in TMS-derived cells it also changed cell morphology (Figure 10). TMS cells normally exist as spheroids in suspension. Treatment with a compound can either inhibit sphere formation and kill cells or have no effect on the spheres. Sunitinib inhibited sphere formation and caused the cells to adhere to the plate displaying morphology reminiscent of adherent cells and at higher compound concentrations cells were killed. Similarly, the MMS displayed a change in morphology in the presence of Sunitinb but this did not occur until much higher concentrations.

3.4 *In vivo* treatment with Sunitinib shrinks tumors in mice with subcutaneous MMTV-Her2/neu Tumors.

Sunitinib is a small molecule RTK inhibitor that targets Kit, Pdgfr, and vascular endothelial growth factor receptor (Vegfr) and is currently used to treat gastrointestinal tumors (GISTs) and renal cell carcinoma (RCC). As previously

mentioned this compound was discovered to be selective in kinase inhibitor screen and in the follow-up sphere-forming assay. Because Sunitinib is an approved drug and the compound's *in vivo* toxicity is known, the compound was tested in mice with subcutaneous MMTV-Her2/*neu* tumors.

Primary MMTV-Her2/*neu* tumors were isolated from mice and mechanically and enzymatically dissociated. The cells were counted and 50,000 cells were injected subcutaneously into 6 to 8 week old syngenic FVB mice. The mice were monitored for tumors and once tumors arose and reached an approximate size of 1 cm^3 treatment with Sunitinib was initiated. Sunitinib treatment entailed 5 days of treatment with 2 days of no treatment for duration of 2 weeks. The compound was orally administered at 80mg/kg dissolved in CMC solution. Mice in the control group were treated with the vehicle, CMC solution.

Treatment with Sunitinib resulted in tumor shrinkage regardless of parental tumor (811 and 007) (Figure 11). In fact, following the first week of 5-days of treatment and 2-days of no treatment, a drastic change in tumor volume was observed (Figure 12). Notably, some of the tumors shrunk to 0% of the initial volume. On the other hand, vehicle treated tumors increased in size with final volumes ranging from 200% to 500% of the initial tumor volume (Figure 11). Lastly, treatment of mice with Sunitinib showed no toxicity to the mice. Both mice cohorts appeared healthy with no visible signs of weight loss.

3.5 Treatment with Sunitinib results in change in tumor morphology and cellular composition

To study the effect of Sunitinib on subcutaneous tumors, any residual tumors following the treatment period were isolated along with the tumors from vehicle treated mice. Tumors were fixed in paraformaldehyde, embedded in paraffin, and sectioned at 5 microns. Sections were stained with Harris' H&E to

analyze their histology and stained with antibodies to markers of proliferation, differentiation, and apoptosis (TUNEL).

H&E of treated tumor revealed cell free areas (Figure 14 arrows) and cluster of cells surrounded by cell free-areas or tumor islands (Figure 14 asterisks). Additionally, mice that appeared to have experience complete tumor regression were euthanized and mammary glands were examined. In some cases, small tumor nodules were found which were also stained with H&E. This revealed that these nodules were mostly cellular debris of dying cells. Analysis of the H&E stained sections of primary and subcutaneous tumor treated with vehicle showed similar tumor histology with both showing a high nucleus to cytoplasmic ratio and abundant vasculature (Figure 13). Comparison of the H&E sections of treated tumors and controls showed a decrease in vasculature in the treated sections (Figure 14) keeping with Sunitinib's antiangiogenic properties. Furthermore, to determine the affect of Sunitinib on the mammary glands, the #4 mammary glands were isolated, fixed, and stained with H&E (Figure 15). The histological appearance and morphology of the treated and control glands were very similar.

In order to investigate whether there were proliferating or apoptotic cells in Sunitinib treated tumors, tumor sections were stained with Ki-67 and a TUNEL assay was carried out. Ki-67 is a nuclear protein required for cell proliferation and therefore used as marker for proliferating cells. Using Ki-67 stained sections the number of proliferating cells in Sunitinib treated and vehicle-treated mice was quantified (Figure 16). Sunitinib treated cells contained much lower frequency of Ki-67 positive cells (3.5%) compared to control tumors which contained 21.14% Ki-67 positive cells. Furthermore, Sunitinib treated tumor sections contained more apoptotic cells as determined by TUNEL assay (Figure 17). TUNEL assay labels the terminal end of nucleic acids of DNA fragments, found in programed cell death and is therefore used as a biomarker for apoptosis. An average of 54.01% of treated cells were TUNEL positive unlike the 1.05% of cells found in the control

treated mice suggesting that Sunitinib elicited an apoptotic program in the cells. Taken together, these data suggest that Sunitinib caused tumor regression by both inhibiting cell proliferation as well as inducing apoptosis.

We also measured whether Sunitinib affected the differentiation status of the MMTV-neu derived tumors. MMTV-neu tumors are mainly composed of luminal cell markers, which can be observed by staining tumor sections with luminal cell markers such as CK8. In addition, a small number of cells stain for the α-SMA marker, which stains myoepithelial cells and fibroblasts. Subcutaneous tumors treated with vehicle follow this staining pattern as seen in Figure 18A-B. However, upon treatment with Sunitinib this staining pattern changes. The number of α-SMA positive cells increases and are localized around the edge of the tumor island surrounding CK8 positive cells located in the center (Figure 18C-D). In fact some cells stain for both CK8 and α-SMA. Because α-SMA identifies both myoepithelial cells and fibroblast cells, CK14 a marker that also stains for myoepithelial cells was used to distinguish between these 2 cells types. Similar to α-SMA, CK14 does not stain MMTV-neu tumors or subcutaneous tumors treated with vehicle (Figure 19A-B). When treated with Sunitinib, very few cells stained for CK14. In fact, only very sporadic cells stained positive for CK14 and some double stained for CK14 and CK8 (Figure 19 C-D). The staining pattern of these rare CK14+ cells was somewhat sporadic but was generally present around the edge of cell clusters. To determine whether the α-SMA positive cells were fibroblast cells, tumor sections were stained with vimentin, a fibroblast marker. Both treated and control tumor sections did not stain for vimentin (Figure 20). Overall, this data shows that Sunitinib treatment of mice with subcutaneous tumors decreased tumor volume, increased TUNEL positive cells, decreased Ki-67 positive cells, and changed cellular composition of the tumors.

4.0 DISCUSSION

4.1 High-Throughput Screen

The goal for this project was to identify selective kinase inhibitors that target tumor-initiating cells. A high-throughput screen measuring cell proliferation was carried out in order to identify compounds that targeted breast TICs. TMS, MMS, and ATC were assayed in an initial screen at a single concentration and hits were defined as any compound that inhibited proliferation in TMS derived cells by 75% or more. The caveat with using a proliferation assay for this is screen is only cytotoxic compounds are identified. Compounds that have a cytostatic effect or inhibit cell proliferation but do not kill cells will not result in a decrease in residual activity and therefore using the criterion set for this screen these compounds may not be identified as hits. This mentioned, 50 compounds were identified as hits in the TMS-derived cells (20% hit rate). Of these 50 compounds, 34 of them overlapped with hits identified in MMS and 23 identified in ATC. Given that transformation can occur through an activating mutation in kinases that control cell proliferation signaling pathways, inhibition of these kinases would reduce proliferation. Additionally, if these pathways that control cell proliferation were normally active in the cells, it would make sense that inhibiting these pathways would kill normal MMS-derived cells. However, the compound concentration required may differ and therefore follow-up screens were carried out to determine whether this was the case. One thing that should be noted from this screen is that only 23 compounds were identified to target the ATC compared to the 50 identified in TMS-derived cells. As previously mentioned the tumor cells cultured in the presence of serum as ATCs are devoid of tumorigenic cells unlike the TMS-derived cells, which are made up of TICs. This suggests the tumorigenic cells are potentially more sensitive to these compounds than their non-tumorigenic counterparts. If this is in fact true, the compounds that target TICs could potentially be used in combination with current chemotherapeutic drugs in order to eliminated both tumorigenic and non-tumorigenic cell populations.

Notably, another explanation for what is observed could be due to the presence of serum in the ATC cell media. Work in the lab has shown that a compound that potently inhibits TMS derived cells proliferation but not ATC, can be made to target these ATC in the absence of serum in the media. Serum is known to contain multiple growth and survival factors, such as PDGF, which may protect cells from general compound toxicity (76).

Hits identified in TMS-derived cells were all classified into kinase groups; the biggest being RTKs. This would be expected because a large number of inhibitors are made to target these kinases. As previously mentioned, RTKs have provided the proof-of-concept for small molecule kinase inhibitor and therefore a large number of small molecules have been designed to target them. RTK signaling does control a number of cellular functions importantly cell proliferation and therefore inhibiting this signaling will prevent cell proliferation. Some of the other kinase groups identified are PI3K pathway and the MAPK pathway, which have linked to human breast cancer (28, 29). These results further confirm the validity of this assay and the tumor model used for the screen. Both pathways are activated downstream of Her2/neu signaling; the sensitivity of TMS-derived cells to the inhibitors of PI3K and MAPK pathway could suggest an oncogenic addition to the pathway or a kinase in the pathway (49).

Small molecule kinase inhibitors are not all specific for a single kinase. In fact most kinase inhibitors are known to target a number of related kinases. The lack of specificity comes from a well-conserved ATP-binding site of kinases and inhibitors are developed to target this site (44). This results in off-target activity of the inhibitor. In the screen a number of kinase inhibitors targeted ALK, Trk, and Flt-3. These are not expressed in TMS-derived cells as determined by global gene expression profiling suggesting that the inhibitor is functioning through a different kinase. Although selectivity is a characteristic that is strived for, a number of approved kinase inhibitor drugs, such as Dasatinib and Sunitinib, target multiple kinases at concentrations used in the clinic. This is thought to be

advantageous because the drug can target multiple activated processes such as cell survival and angiogenesis. Conversely, selective compounds might be less toxic than multi-kinase inhibitor (43).

Follow-up screens were carried out in TMS, MMS, and ATC to determine IC_{50} values for each hit compound and to verify the identified the hits. Of the 50 initial hits, 85% of these reproduced. The IC_{50} values were used to identify a concentration window in which the drugs selectively targeting TMS-derived cells but not the MMS-derived cells. Selective compounds were defined as compounds that have a MMS to TMS IC_{50} ratio of 2 or more. Based on this criterion, 9 compounds were identified. Interestingly, of these selective compounds, 4 compounds, Imatinib, Sunitinib, Sorafenib, and Masatinib, share Kit and PDGFR as a common target. TMS and primary MMTV-*neu* tumor cells highly expressed Kit at the mRNA level as determined by gene expression profiling, but express PDGFR at very low levels if at all. Because all these compounds have been identified in this screen to be selective and they share a common target that is expressed in TMS-derived cells this suggest that Kit is important for TMS survival and proliferation. Kit is a receptor tyrosine kinase encoded by a proto-oncogene composed of an extracellular ligand-binding domain, a single transmembrane-spanning domain and an intracellular domain composed of a hydrophilic juxtamembrane region, a conserved tyrosine kinase domain and a hydrophilic C-terminus (77). Kit belongs in the subclass III of RPTKs, along with PDGFRa/b, CSF-1, and Flt-3 kinases grouped based on structural similarity. Hematopoietic and gametogenic precursors cells express and depend on Kit signaling for development. Kit is also expressed in mammary epithelial cells. Recently, over 30 gain-of-function mutations have been observed in Kit in highly malignant tumors in the form of single amino-acid changes or deletion of few amino acids. These mutations occur in two regions, the juxtamembrane region as seen in GIST or the kinase domain as seen in mast-cell/myeloid leukemias. Both forms of mutations result in constitutive ligand-independent kinase activation (78). The consequence

of this is downstream activation of the PI3K pathway. Although no activating Kit mutations have been observed in breast cancer but Kit kinase is expressed in breast carcinomas (79). Results from this screen suggest Kit might be important for cell proliferation and survival.

A focal adhesion kinase (FAK) inhibitor was also identified to be selective in TMS-derived cells. FAK is implicated in development of a number of cancers including breast (80). The result from the screen suggesting that FAK is required for TMS survival/proliferation is consistent with work by Luo et al (81). In this work they showed that deletion of FAK in a mouse mammary epithelium significantly suppressed mammary tumorigenesis in a mouse breast cancer model. Furthermore, deletion of FAK in these mice reduced the cancer stem cell and progenitor pool along with impairing self-renewal and cell migration *in vitro* (81). Taken all together, FAK is target that should be furthered investigated.

One thing that should be noted from the screen is the number of PI3K pathway inhibitors (PI3K and AKT) that were found to be potent inhibitors (relative to other inhibitors) with IC_{50s} of less than 1 μM in all cell types. The PI3K pathway is activated by a number of RTKs, as well as being a commonly mutated in breast cancers (33). Because PI3K is downstream of a number of RTKs implicated in breast cancer, it makes for an advantageous target. Inhibiting PI3K would inactivate the upstream signals from multiple RTKs. Although selectivity is an issue, this is an aspect that may be overlooked. Currently breast cancer patients are subjected to mastectomies in hopes of saving their lives. If compounds that lack selectivity are characterized and shown to just target breast TICs and MESCs but not other stem cells in the body these compounds can be considered as potential drugs.

Sphere-forming assays were carried out as follow-up assay using the some of the kinase inhibitors identified in the screen. TAE-684 was the most selective compound identified in the screen and therefore was tested. Unlike the

cell proliferation assay, in multiple sphere-forming assays TAE-684 was not selective. The IC_{50} in TMS was similar to what was observed in the alamarBlue assay but the MMS IC_{50} was about 10-fold lower. The difference in IC_{50} could be explained by the difference in the readout of the 2 assays. The alamarBlue assay determines percent proliferation while the sphere-forming assay is readout of sphere-formation a surrogate assay for self-renewal. Given the difference in the IC_{50} this suggests that the TAE-684 compound might be inhibiting sphere-formation in MMS-derived cells but not killing the cells and therefore there is no reduction in alamarBlue. Furthermore, TAE-684 is only known to target ALK; this kinase is not expressed at the mRNA level based in gene expression profiling. This suggests that it must be functioning by an alternate method. ALK shares a high sequence homology with insulin receptor kinase (InsR) and the insulin-like growth factor receptor (IGF1R) (82), which could be the off targets of the TAE-684 inhibitor. In breast cancer, InsR is implicated in pathogenesis and IGF-IRs are overexpressed in all breast cancer lines and in some human breast tumor tissue (83).

In addition to TAE-684, Masatinib, a Kit inhibitor was also selective in the alamarBlue cell proliferation assay but no the sphere-forming assay. Kit kinase was inhibited by a number of compounds in the screen including Sunitinib, Sorafenib and Imatinib. In multiple sphere-forming assays, Sunitinib was selective in addition to causing a change in cell morphology. Sunitinib inhibited sphere formation and caused the cells to form a monolayer of cells reminiscent of adherent cells. The effect of Sunitinib on the TMS-derived cell was novel and had not been observed in any compounds previously tested. Therefore, Sunitinib was selected for further characterization.

4.2 Sunitinib

Treatment of subcutaneous MMTV-*neu* tumors with Sunitinib, a class III RTK inhibitor, caused tumors to regress and in some mice completely disappear. The consequence of Sunitinib treatment was the decrease in proliferating cells, an increase in the level of apoptotic cells, and a decrease in tumor vasculature. The increase in apoptosis and decrease in proliferation in response to Sunitinib treatment might explain the reduction in tumor size. The anti-angiogenic effect of Sunitinib has been observed in several cancers and is characteristic of the drug (44). Sunitinib inhibits angiogenesis by inhibiting signaling through VEGFR1, VEGFR2, and PDGFRb. Work by Mendel and other groups have shown that Sunitinib inhibits the VEGF-dependent mitogenic response of human umbilical vein endothelial cells and prevents migration of these cells (84). *In vivo,* Sunitinib decreased tumor microvessel density along with inhibiting neo-vascularization in tumor vascular-window model (a mouse model which allows for measurement of tumor vascularity by use of fluorescent and light microscopy) and significantly inhibited tumor growth in the bone of MDA-MB-435HAL-Luc mouse mammary carcinomas model (85, 86). The multi-targeted approach of this kinase inhibitor is evident in the MMTV-*neu* tumors, simultaneously targeted the tumor and surrounding cells. An advantage of this multi-targeted approach is resistance that results due to mutations or overexpression of components of signaling pathways are less like to occur.

Mice with subcutaneous MMTV-neu tumors treated with Sunitinib did not display any visible toxicity from the drug or any weight loss. In fact, comparison of control and treated mammary glands did not exhibit any histological change. The morphology appeared normal with ductal structures surrounded by adipocytes. Hence, the dosing developed for a different study was efficacious in inhibiting mammary gland tumors and sparing the mammary glands (75). This suggests that it may be possible to adjust the dose of Sunitinib to selectively target TICs in breast cancer patients.

Treated tumors displayed a change in tumor morphology and change in the tumor cell composition suggesting more than just an apoptotic program was elicited by the drug. MMTV-neu tumors are composed of primarily luminal cells as determined by CK8 antibody staining. Treatment with vehicle did not change this staining pattern. However, treatment with Sunitinib resulted in expression of α-SMA positive cells and small fraction of cells to express CK14. MMTV-neu tumors are suspected to arise from the transformation of a bipotent alveolar progenitor cells (87). In fact MMTV-neu tumor cells have the capacity to produce progeny that resemble luminal and myoepithelial cells indicative of cells with bipotent potential. An example of the bipotent potential of these cells can be seen in treated MMTV-neu subcutaneous tumors with MRK003, a Notch signaling inhibitor. Control tumors stain uniformly for CK8 and no cells express myoepithelial cells and upon treatment some cells stain for CK8 and some stain for α-SMA and CK14 myoepithelial makers (88). This suggests that Sunitinib may be causing the MMTV-neu cells to differentiate towards the myoepithelial lineage. In fact a recent study has shown that Sunitinib induces monocytic differentiation of acute myelogenous leukemia cells (89). It is important to mention that that α-SMA is also a marker for fibroblasts and a larger fraction of cells stain for α-SMA marker than CK14. However, when sections of Sunitinib treated tumors were stained with vimentin also a marker for fibroblasts no cells were positive. Although these 2 markers, α-SMA and CK14, do stain for myoepithelial cells, α-SMA stains a more mature myoepithelial cells as compared to CK14, which stains a primitive myoepithelial cell. Furthermore, many cells double stained for both for CK8 and α-SMA, suggesting that a-SMA cells were not host fibroblasts, but of tumor origin.

Differentiation is a process by which a less specialized cells becomes more specialized cell types required for tissue function. A number of studies have shown that differentiation is dependent on caspases; specifically, caspase 3 which regulates differentiation indirectly by inactivating proteins that maintain

stem cell self-renewal/pluripotency (90). Work by Larsen et al. have shown caspase signaling during the differentiation process leads to the activation of specific nuclease caspase-activated DNase (CAD) resulting in DNA strand breaks and in fact inhibiting this process inhibits differentiation. It is therefore plausible based on the data that Sunitinib may be causing differentiation through this process but the DNA strand breaks are detrimental to these cells resulting in cell death. In addition to the staining showing differentiation, TUNEL assay confirms the presence of double strand break.

One characteristic observed in Sunitinib treated tumors is appearance of cell clusters or tumor islands with some having an organized appearance. These tumor islands are visible in H&E stained sections and cell specific marker stained sections. Notably the α-SMA and CK14 staining are localized to the edge of these tumor islands. Evident in the immunofluorescence staining, all the cells of the tumor islands stain positive for CK8 except for the cells localized around the edges of these tumor islands. These edge cells stain positive for α-SMA and in some cases double stain for both markers. In the CK14 stained sections, a very small fraction of these edge cells stain positive. The encircling of myoepithelial cells of luminal cells is reminiscent of the normal mammary gland. In fact, work by several groups has shown that myoepithelial cells are natural tumor suppressors by inhibiting invasion and metastasis (91, 92). Interestingly, myoepithelial cells are relatively resistant to transformation and tumors originating from these cells are uncommon and usually benign (91). This phenotype observed in Sunitinib treated tumors is very interesting and warrants further investigation.

4.3 Proposed mechanism of action of Sunitinb

Sunitinib, a multiple targeted kinase inhibitor, effectively inhibited tumor growth of MMTV-neu tumors and caused tumor regression. Along with an

increase in apoptotic cells and decrease in proliferating cells, treatment resulted in change in tumor morphology and cellular composition. Sunitinib is characterized by its antitumor and antiangiogenic properties (84). A number of studies in GISTs and RCC have shown that Sunitinb inhibits Kit, VEGFR, and PDGFR signaling respectively. Inhibition of Kit kinase results in antitumor effects and inhibition of VEGFR and PDGFR inhibits angiogenesis (44). In MMTV-*neu* tumors Sunitinib function is two fold; it inhibits tumor proliferation/causes an apoptotic program and it inhibits angiogenesis. MMTV-neu tumors highly express Kit kinase as determined by global gene expression profiling. Therefore it is plausible that Sunitinb is inhibiting Kit signaling in these cells resulting in programed cell death. Furthermore, through the inhibition of VEGFR and PDGFR, Sunitinib is inhibiting angiogenesis in these tumors. Work by Lim *et al.* have reported KIT to be overexpressed in basal cancers and BRAC1-associated cancers (93). These BRAC1 mutated tumors contain an expanded luminal progenitor population. Similarly, the MMTV-neu mammary tumor model highly correlates with the luminal cell progenitor signature (94). The efficacy of Sunitinib in this model suggests that similar results may be observed in basal cancers and BRAC1-associated cancers. Additionally, Sunitinib may also be effective for treatment of metastatic breast cancer. In fact, clinical trails carried out in patients with pretreated metastatic breast cancer and in combination with capecitabine resulted in partial response in these patients (95). Taken together, this data suggests that Sunitinib can potentially be used to treat breast cancer patients.

4.4 Future Work and Conclusion

The goal of this project was to identify kinase inhibitors that target TICs using a cell proliferation screen. A number of compounds were identified in the screen and some of these compounds were followed up. Sunitinib a mutli-targeted kinase inhibitors was one of the consistently selective compounds *in*

vitro and *in vivo* the compound inhibited tumor growth of established subcutaneous mammary gland tumors. In addition to shrinking tumors, Sunitinib treatment resulted in a change in tumor cell morphology, decrease in tumor vasculature and change in tumor cell composition. Having shown that Sunitinib is effective in a mouse mammary tumor model the next step would be to learn whether Sunitinib targets the TICs fraction. This would require transplanting treated tumor cells and vehicle treated cells into syngenic mice and monitoring the mice for tumors. If in fact Sunitinib does target TICs then the treated cells should not give rise to tumors. Furthermore, Sunitinib is known to function through inhibition of Kit, PDGFR, and VEGFR using western blot analysis the target of Sunitinib in MMTV-neu tumor can be elucidated. To determine whether Sunitinib is efficacious in human breast tumors, mice with xenograft tumors seeded by multiple human breast cancer cell lines should be treated with the drug.

The Sunitinb data presented in this project, suggest that there is potential efficacy of this drug in breast cancer patients. The results seen in the MMTV-*neu* tumor model make a good case for this drug to further be tested. Having already been approved and used in the clinic makes Sunitinib an even more appealing breast cancer drug candidate.

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Table 1- Z' values for kinase inhibitor screens

	Plate 1	Plate 2	Plate 3
TMS	0.590	0.808	0.748
MMS	0.749	0.768	0.784
ATC	0.760	0.500	0.600

Table 1- Percent Residual Activity of Hit Kinase Inhibitor Compounds in MMS TMS ATC

Compound Names	Target	Percent Residual Activity		
		MMS	TMS	ATC
(5Z)-7-Oxozeaenol	MAPKKK	28.03	15.54	100.36
10-DEBC hydrochloride	Akt	26.31	4.22	15.96
5-Iodotubercidin	Adenosine Kinase	23.87	19.83	101.97
A-443654	Akt	8.30	1.27	0.60
API-2	Akt	6.88	6.85	8.12
AZD0530	Abl	57.53	22.96	6.22
BI 2536	Plk	0.70	0.35	9.58
BI 78D3	JNK	1.49	2.24	100.84
BIBU 1361 dihydrochloride	EGFR	0.69	0.68	80.85
BIBW-2992	EGFR	0.29	0.49	2.94
BIBX 1382 dihydrochloride	EGFR	8.47	0.93	70.82
Bosutinib	Abl	0.36	0.24	27.34
CGK 733	ATR, ATM	2.28	9.06	110.82
CGP-74514A hydrochloride	CDK	0.43	0.06	15.73
Chelerythrine chloride	PKC	63.87	17.48	45.65
CI-1033	EGFR	13.05	1.40	3.62
Dorsomorphin	TGFBR	69.72	3.58	89.71
Dovitinib	FGFR	1.13	1.82	77.57
ER 27319 maleate	Syk	10.25	12.41	3.87
FAK Inhibitor 14	FAK	0.27	1.09	1.53
Gefitinib	EGFR	51.53	18.04	95.89
IKK 16	IKK	0.38	0.66	98.29
Imatinib Mesylate	Abl	102.53	25.18	97.07
Imidazolo-oxindole	PKR	13.34	25.25	102.22
IPA 3	PAK1	8.32	6.52	3.97
K-252a	Trk	5.71	3.40	10.39
Lestaurtinib	Trk	8.12	2.94	11.05

Table 3- Summary of secondary screen used to identify IC_{50} for inhibits in tumorspheres, mammospheres, and adherent tumor cells

Compound Names	Target	Target Classification	Highest Assay Conc (uM)	IC50				Gene Expressed		
				MMS	TMS	ATC	MMS/TMS	MMS	TMS	ATC
API-2	Akt	PI3K	10.00	0.40	0.42	N/T	0.94	Y	Y	Y
A-443654	Akt	PI3K	10.00	0.36	0.47	1.73	0.77	Y	Y	Y
TX-1918	eEF2K	Protein synthesis	5.00	0.45	0.49	N/A	0.91	Y	Y	Y
TAE-684	ALK	RTK	10.00	3.63	0.54	N/A	6.72	N	N	N
ER 27319 maleate	Syk	NRTK	10.00	0.83	0.78	1.58	1.07	N	Y	Y
(5Z)-7-Oxozeaenol	MAPKKK	MAPK	5.00	0.47	0.85	N/A	0.56	Y	Y	Y
PIK-75	PI3K	PI3K	10.00	0.85	0.86	0.95	0.98	Y	Y	Y
FAK Inhibitor 14	FAK	RTK	10.00	1.92	0.95	N/T	2.03	Y	Y	Y
PD173074	FGFR-3	RTK	10.00	0.20	0.97	N/A	0.20	Y	Y	Y
Dovitinib	FGFR	RTK	5.00	0.75	0.98	N/A	0.77	Y	Y	Y
IKK 16	IKK	TGFR Pathway	5.00	0.85	1.01	N/A	0.84	Y	Y	Y
CGP-74514A	CDK	Cell cycle kinase	10.00	1.40	1.44	5.51	0.97	Y	Y	Y
NSC 663284	CDK	Cell cycle kinase	5.00	1.28	1.47	N/A	0.87	Y	Y	Y
Ro 31-8220 mesylate	PKC	PKC	10.00	1.42	1.70	N/A	0.84	Y	Y	Y
BIBU 1361	EGFR	RTK	10.00	2.07	1.78	9.76	1.16	Y	Y	Y
BI 78D3	JNK	MAPK	5.00	1.36	1.97	N/A	0.69	Y	Y	Y
Lestaurtinib	Trk	RTK	10.00	2.57	2.49	N/A	1.03	N	N	N
AZD0530	Abl	NRTK	10.00	2.51	2.51	N/T	1.00	Y	Y	Y
Ryuvidine	CDK4	Cell cycle kinase	5.00	N/A	2.69	N/A	Y	Y	Y	Y
Sunitinib Malate	c-Kit	RTK	10.00	9.12	2.76	N/A	3.30	Y	Y	Y
Vandetanib	VEGFR	RTK	10.00	2.89	2.81	7.19	1.03	Y	N	N
BI 2536	Plk	Cell cycle kinase	10.00	1.57	2.88	8.91	0.55	Y	Y	Y
OSU-03012	PDK1	Cell metabolism	5.00	2.24	2.95	N/A	0.76	Y	Y	Y
Imidazolo-oxindole	PKR	Other	5.00	3.09	3.00	5	1.03	Y	Y	Y
BIBW-2992	EGFR	RTK	10.00	3.72	3.27	3.98	1.14	Y	Y	Y
5-Iodotubercidin	Adenosine Kinase	Cell metabolism	5.00	3.97	3.36	N/A	1.18	Y	Y	Y
Masitinib mesylate	c-Kit	RTK	10.00	9.12	3.57	N/A	2.55	Y	Y	Y

Compound Names	Target	Target Classification	Highest Assay Conc (uM)	IC50				Gene Expressed		
				MMS	TMS	ATC	MMS/TMS	MMS	TMS	ATC
Sorafenib	Raf	MAPK	10.00	7.94	3.81	N/A	0.94	Y	Y	Y
Dorsomorphin	TGFBR	TGFR Pathway	5.00	2.56	3.89	N/A	0.77	Y	Y	Y
CI-1033	EGFR	RTK	10.00	6.02	4.06	N/A	0.91	Y	Y	Y
PHA 665752	Met	RTK	5.00	3.13	4.44	N/A	6.72	N	Y	Y
Bosutinib	Abl	NRTK	10.00	3.76	4.59	N/A	1.07	Y	Y	Y
CGK 733	ATR, ATM	Other	5.00	4.27	5.00	N/A	0.56	Y	Y	Y
Gefitinib	EGFR	RTK	10.00	N/A	5.03	N/A	Y	Y	Y	Y
VX-680	Aurora	Cell cycle kinase	10.00	5.28	5.11	N/A	2.03	Y	Y	Y
BIBX 1382	EGFR	RTK	10.00	8.03	5.35	N/A	0.20	Y	Y	Y
10-DEBC	Akt	PI3K	10.00	6.83	5.72	N/A	0.77	Y	Y	Y
Imatinib Mesylate	Abl	NRTK	10.00	N/A	6.37	N/A	Y	Y	Y	Y
TWS119	GSK3	Other	10.00	6.49	6.59	N/A	0.97			
Chelerythrine	PKC	PKC	10.00	9.60	6.98	N/A	0.87	Y	Y	Y
MLN-518	FLT3	RTK	10.00	N/A	7.71	N/T	Y	N	N	N
NH125	eEF-2	Protein synthesis	10.00	N/A	N/A	N/A		Y	Y	Y
K-252a	Trk	RTK	10.00	N/A	N/A	N/A		Y	N	N
SIS3	TGFBR	TGFR Pathway	10.00	N/A	N/A	N/A		Y	Y	Y
Sphingosine	PKC	PKC	10.00	N/A	N/A	N/A		Y	Y	Y
R 59-022	DGK	PI3K	5.00	N/A	N/A	N/A		Y	Y	Y
IPA 3	PAK1	MAPK	5.00	N/A	N/A	N/A		Y	Y	Y
PD 198306	MEK	MAPK	5.00	N/A	N/A	N/T		Y	Y	Y
Staurosporine	PKC	PKC	10.00	TBT	TBT	TBT		Y	Y	Y
SGI-1776	PIM	Other	10.00	TBT	TBT	TBT		Y	Y	Y

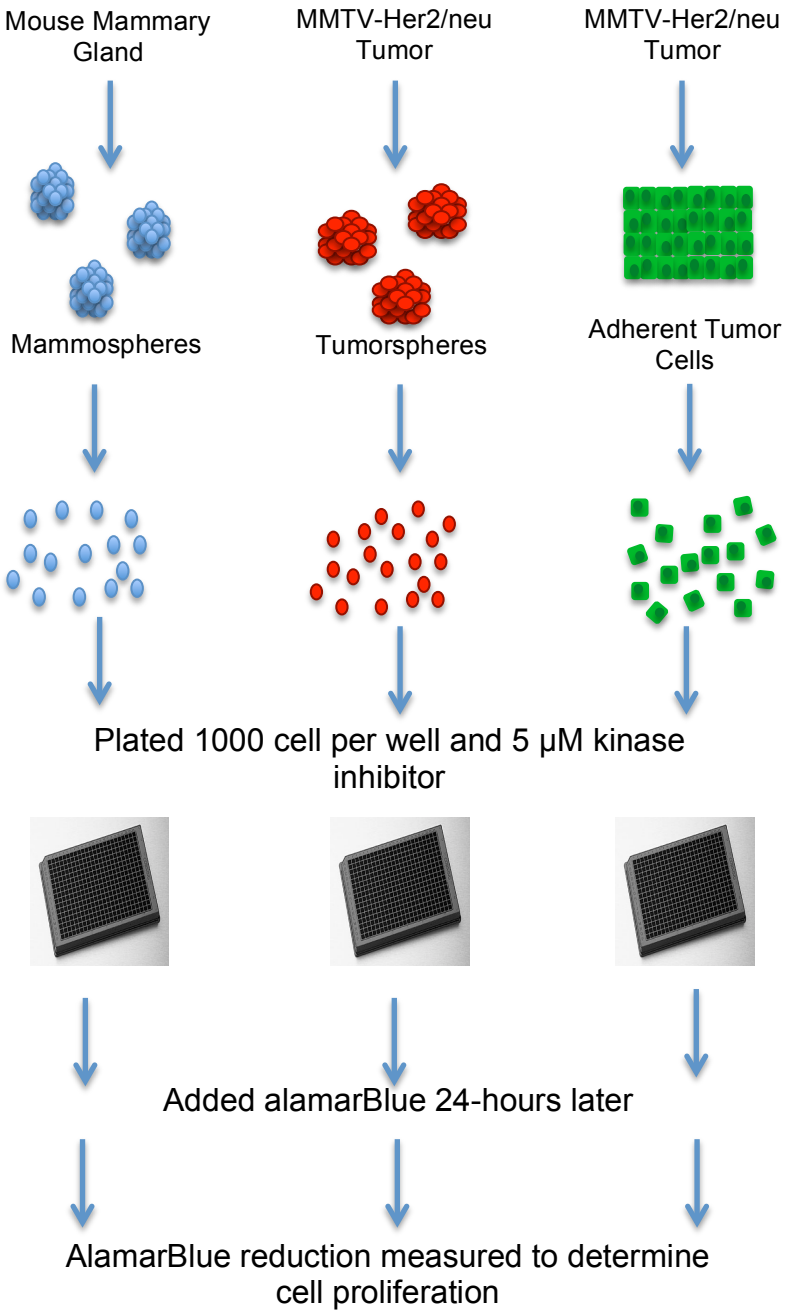


Figure 1- High-throughput screen procedure- MMS, TMS, ATC were dissociated to seed dispersed cells for the assay and plated at density of 1,000 cells per well with media. Appropriate concentration of kinase inhibitors was added to each well and cells were incubated for 24 hours. Following this incubation period alamarBlue was added to each well. Cells were incubated for another 24 hours and then fluorescence was measured. Hits from this assay were defined as those, which inhibited alamarBlue reduction by 75% or more.

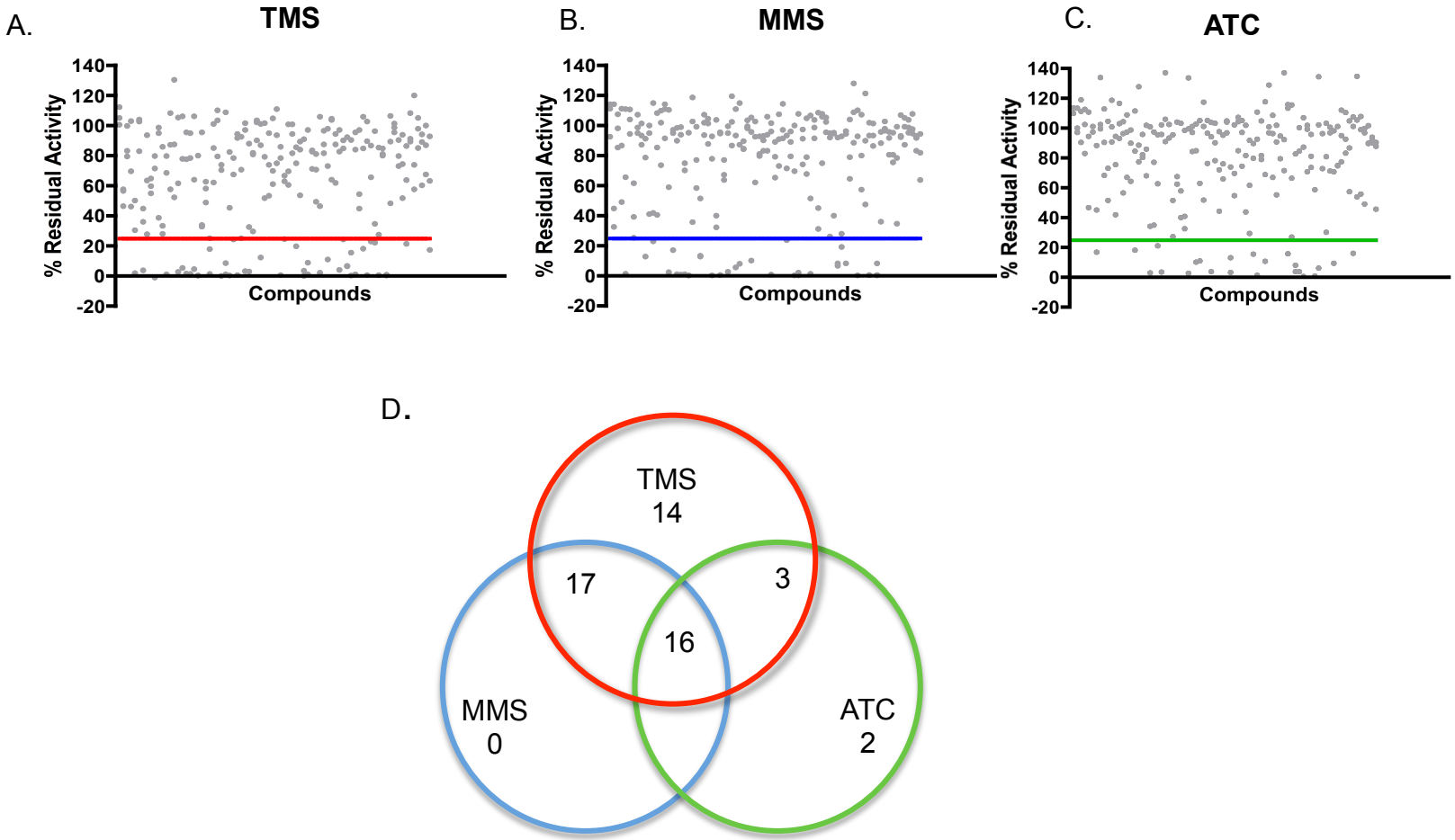


Figure 2- Primary screen identifies compounds that target tumor-initiating cells- Illustrated is the TMS, MMS, and ATC percent residual activity for each of the tested kinase inhibitors (A-B). The Venn diagram illustrates the overlap of hit compounds amongst the three cells cultures (D).

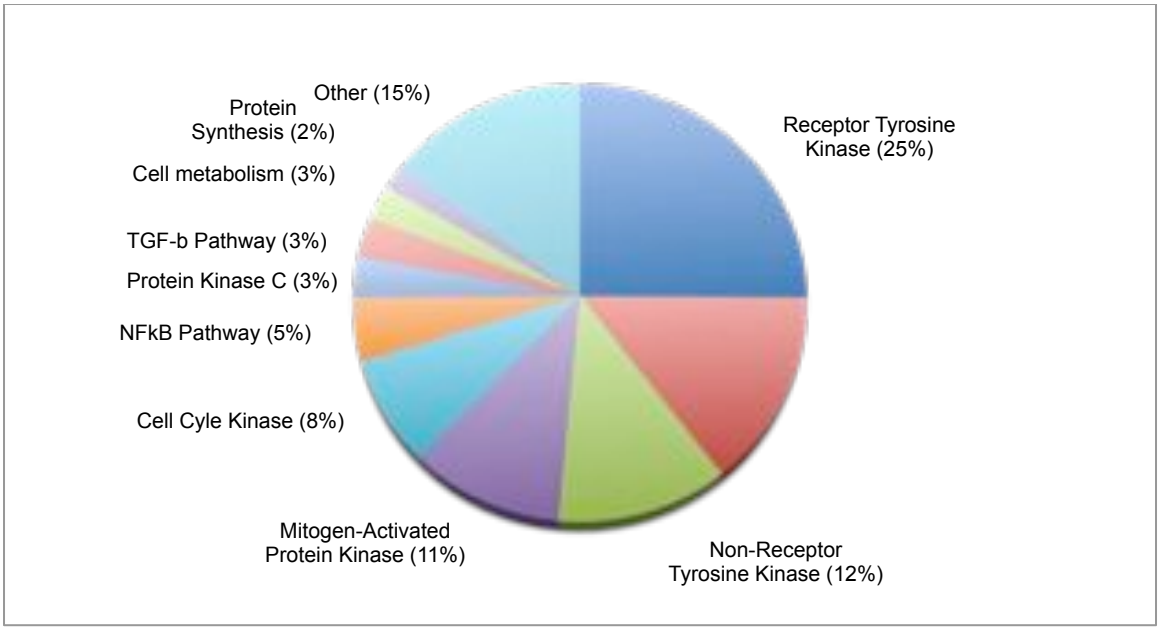


Figure 3- Target classification of OICR kinase inhibitor library- The targets of the kinase inhibitors were classified into groups of related kinases. The largest group of inhibitor targets was receptor tyrosine kinases.

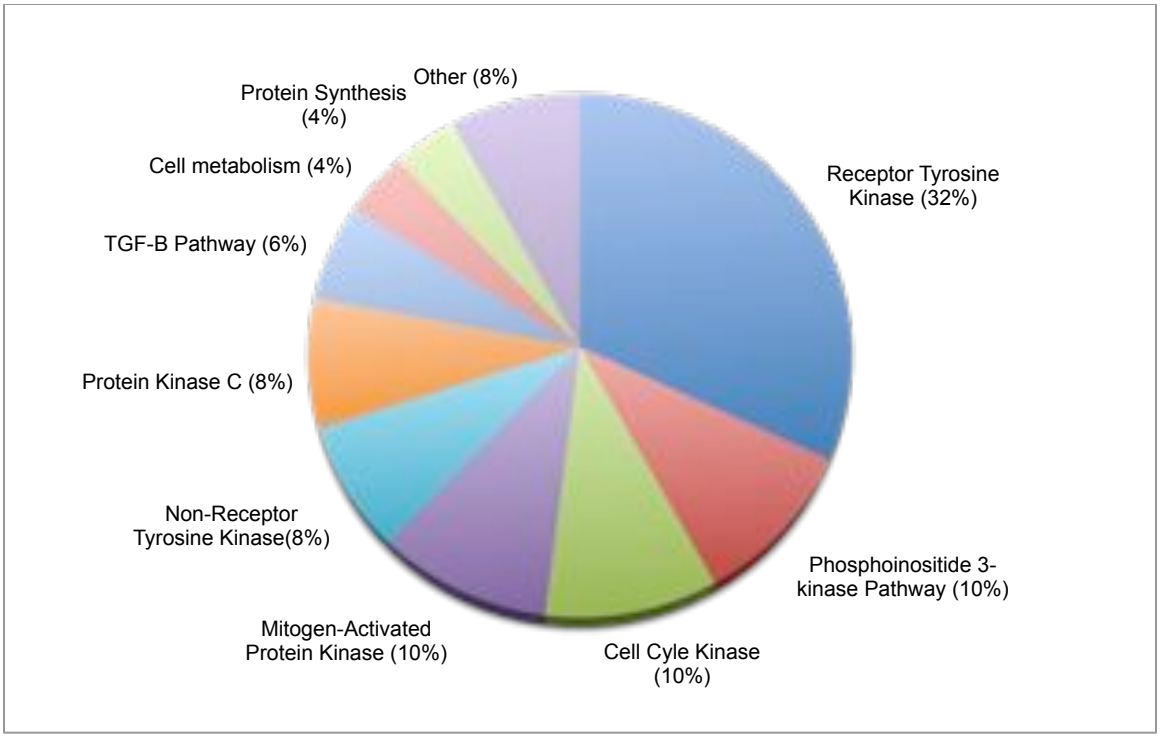


Figure 4- Target classification of identified kinase inhibitors - The targets of compounds identified in the kinase inhibitor screen were classified into groups of related kinases.

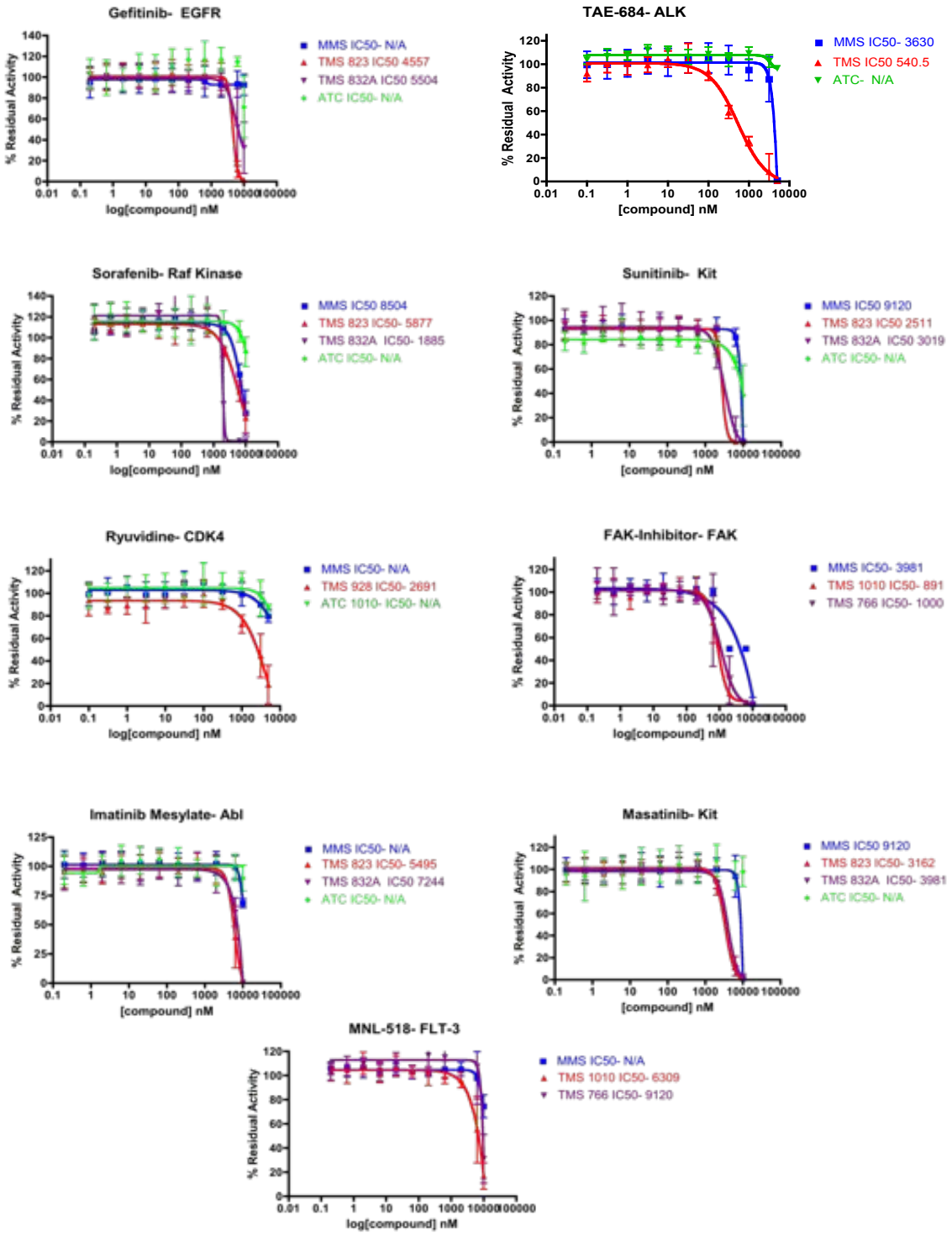
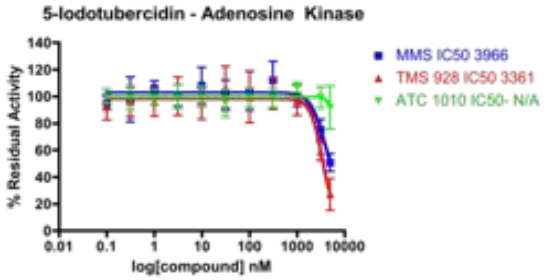
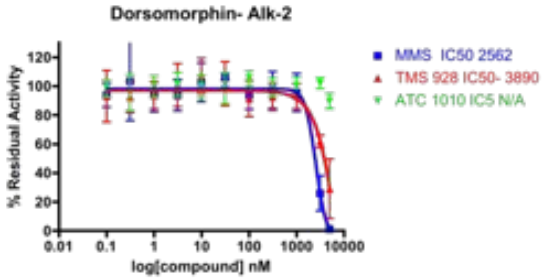
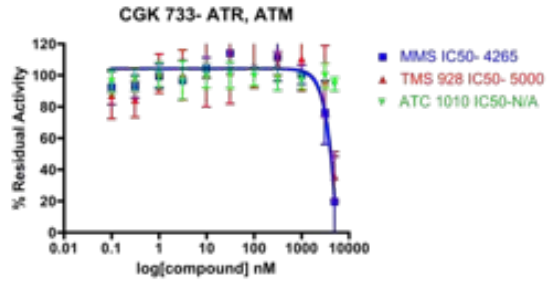
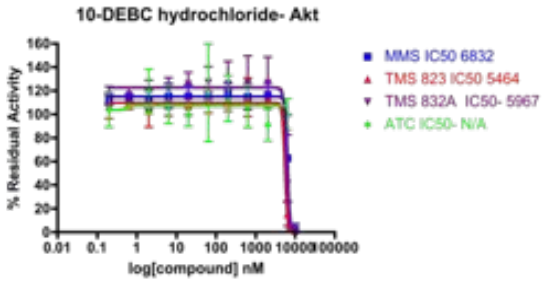


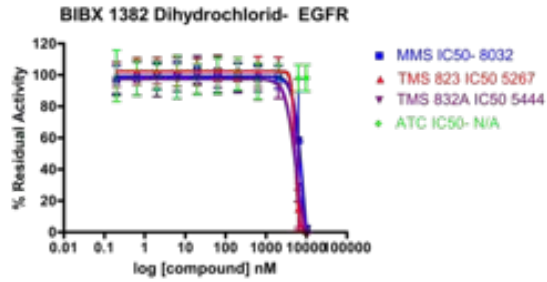
Figure 5- Tumorsphere selective kinase inhibitors- All the above compounds were identified to be TMS-selective based on a 2 or more MMS to TMS IC_{50} ratio.

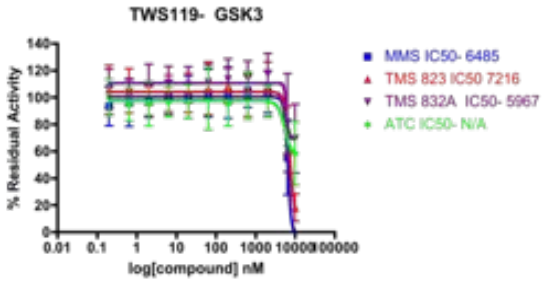

5-Iodotubercidin - Adenosine Kinase
% Residual Activity
log[compound] nM
MMS IC50 3966
TMS 928 IC50 3361
ATC 1010 IC50- N/A

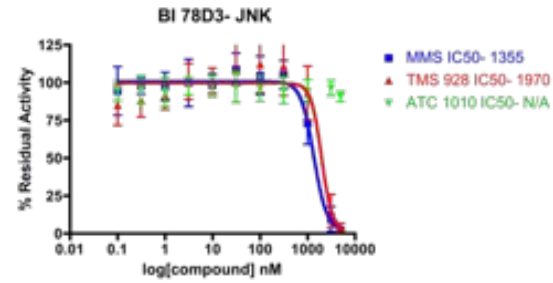

Dorsomorphin- Alk-2
% Residual Activity
log[compound] nM
MMS IC50 2562
TMS 928 IC50- 3890
ATC 1010 IC5 N/A

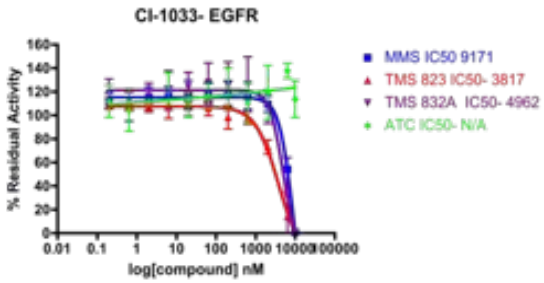

CGK 733- ATR, ATM
% Residual Activity
log[compound] nM
MMS IC50- 4265
TMS 928 IC50- 5000
ATC 1010 IC50-N/A

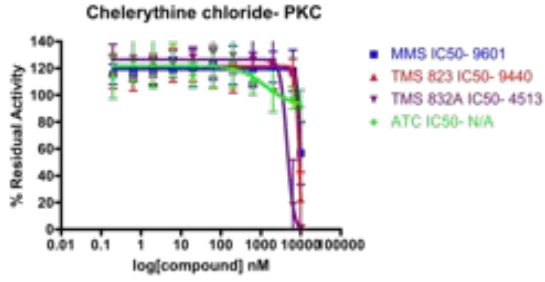

10-DEBC hydrochloride- Akt
% Residual Activity
log[compound] nM
MMS IC50 6832
TMS 823 IC50 5464
TMS 832A IC50- 5967
ATC IC50- N/A

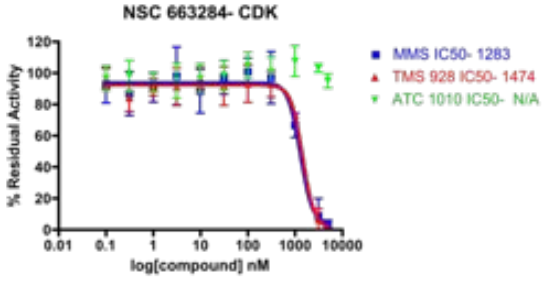

BIBX 1382 Dihydrochlorid- EGFR
% Residual Activity
log [compound] nM
MMS IC50- 8032
TMS 823 IC50 5267
TMS 832A IC50 5444
ATC IC50- N/A


TWS119- GSK3
% Residual Activity
log[compound] nM
MMS IC50- 6485
TMS 823 IC50 7216
TMS 832A IC50- 5967
ATC IC50- N/A


BI 78D3- JNK
% Residual Activity
log[compound] nM
MMS IC50- 1355
TMS 928 IC50- 1970
ATC 1010 IC50- N/A


CI-1033- EGFR
% Residual Activity
log[compound] nM
MMS IC50 9171
TMS 823 IC50- 3817
TMS 832A IC50- 4962
ATC IC50- N/A


Chelerythine chloride- PKC
% Residual Activity
log[compound] nM
MMS IC50- 9601
TMS 823 IC50- 9440
TMS 832A IC50- 4513
ATC IC50- N/A


NSC 663284- CDK
% Residual Activity
log[compound] nM
MMS IC50- 1283
TMS 928 IC50- 1474
ATC 1010 IC50- N/A

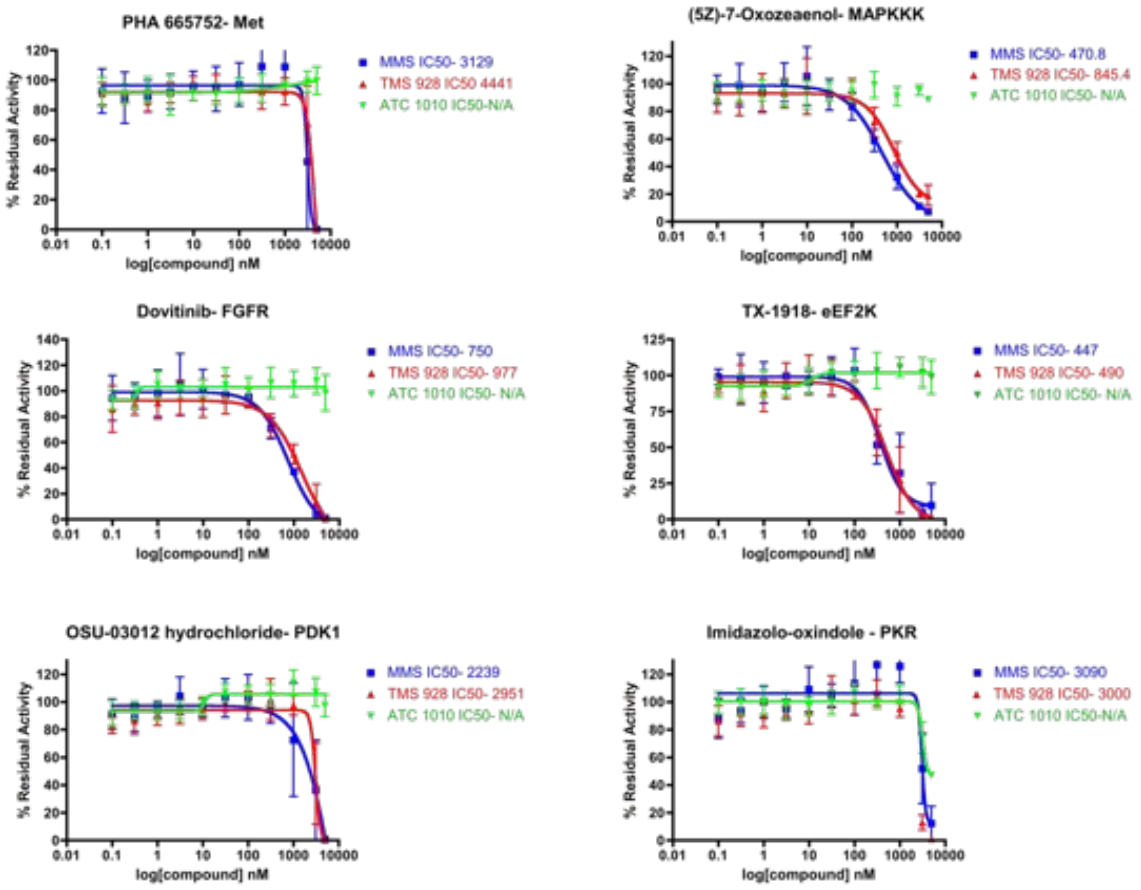


Figure 6- Kinase inhibitors that showed no effect on adherent tumor cells at the highest assay concentration - All the above compounds had no affect on ATC proliferation at the highest concentration (5 or 10 μM). On the other hand, these compounds completely inhibited TMS and MMS proliferation at the highest compound concentration.

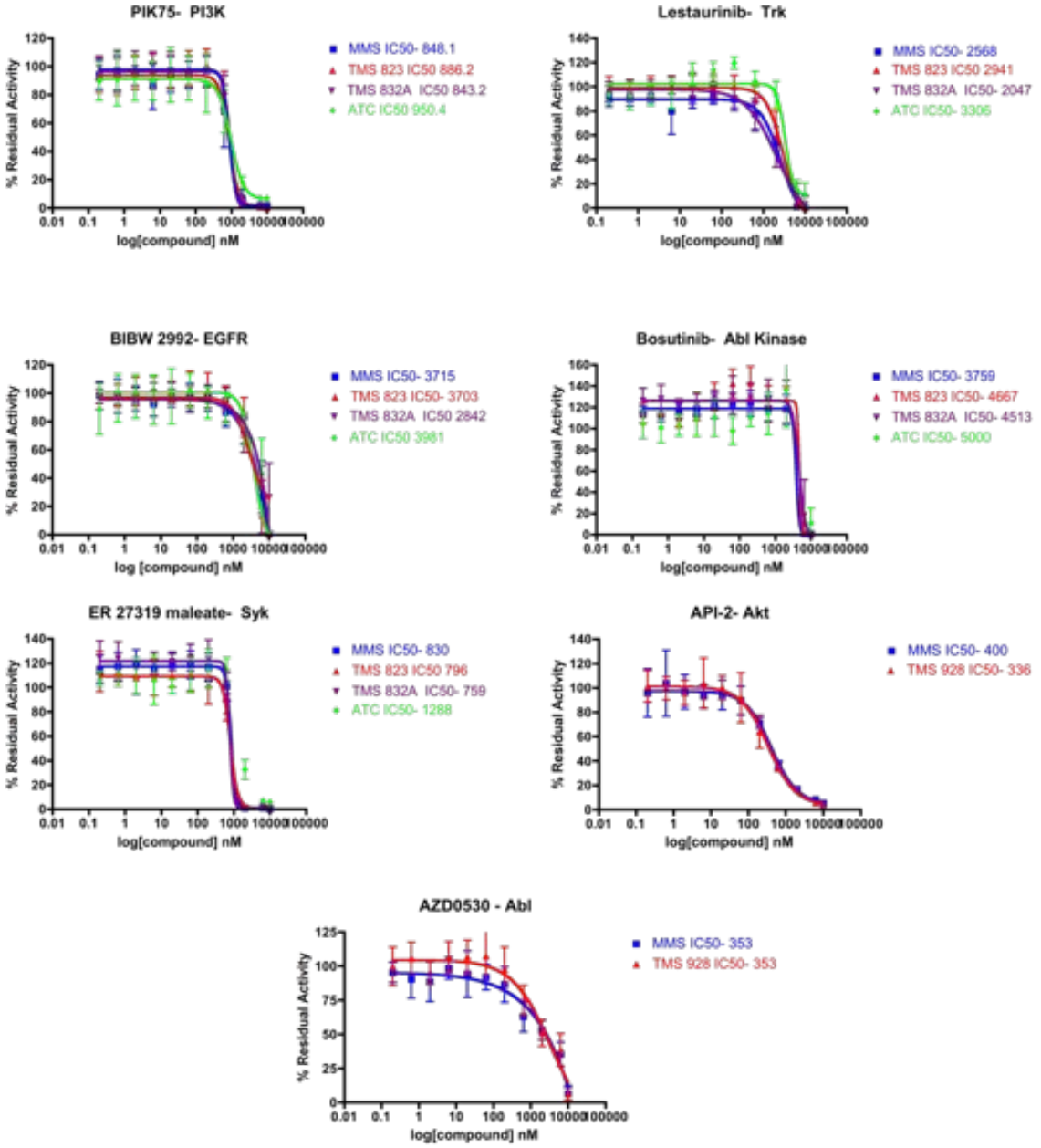


Figure 7- Kinase inhibitors with no selectivity for any of 3-cell cultures- All the above compounds showed no selectivity in any of the TMS, MMS or ATC cultures. In fact they inhibited proliferation in all 3-cell types at similar IC_{50} concentrations. AZD0530 and API-1 were not tested in ATC.

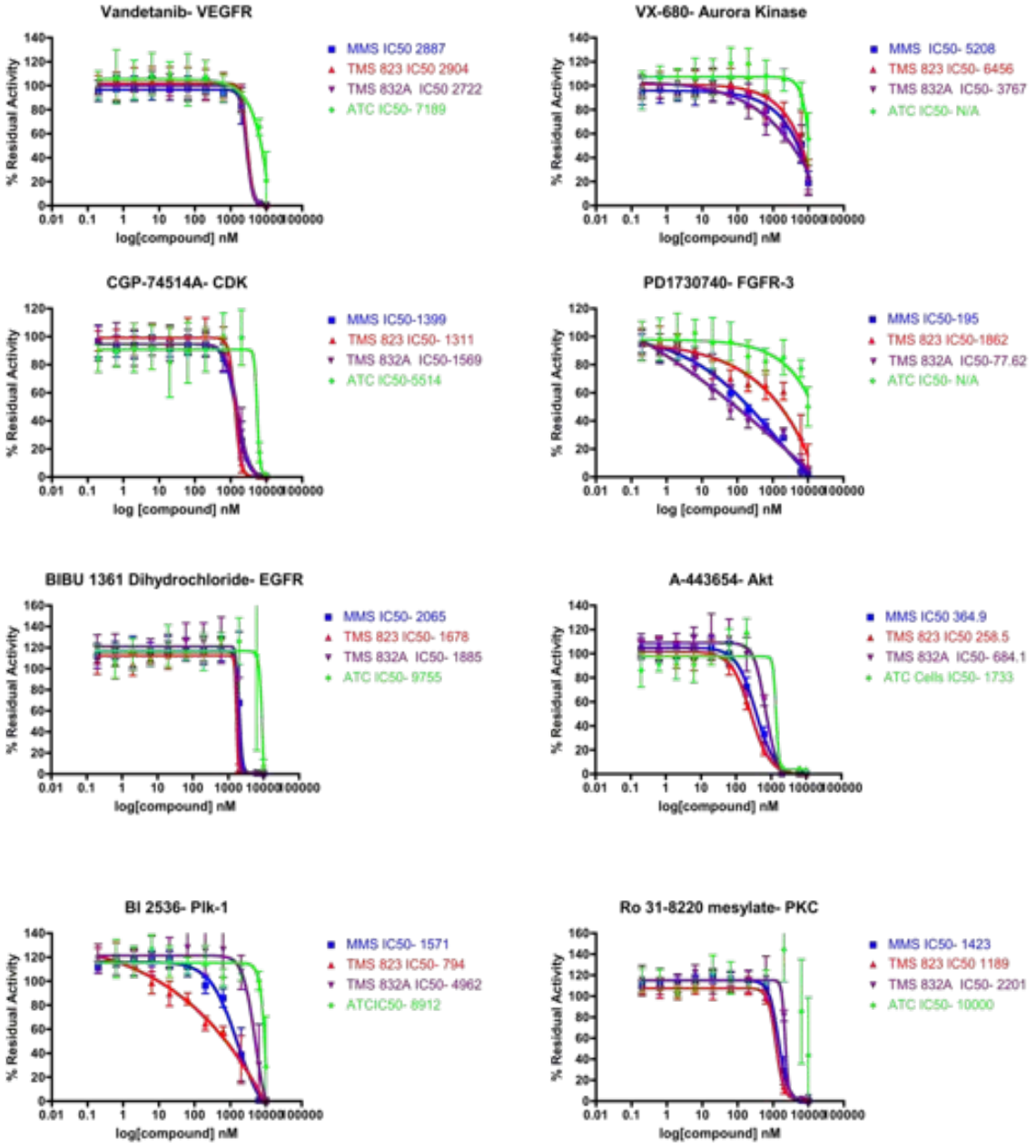


Figure 8- Kinase inhibitors with moderate selectivity for tumorspheres and mammospheres compared to adherent tumor cells- All the above compounds targeted all 3 cell cultures with selectivity for TMS and MMS. The TMS to ATC or MMC to ATC IC_{50} ratio was 2 or more.

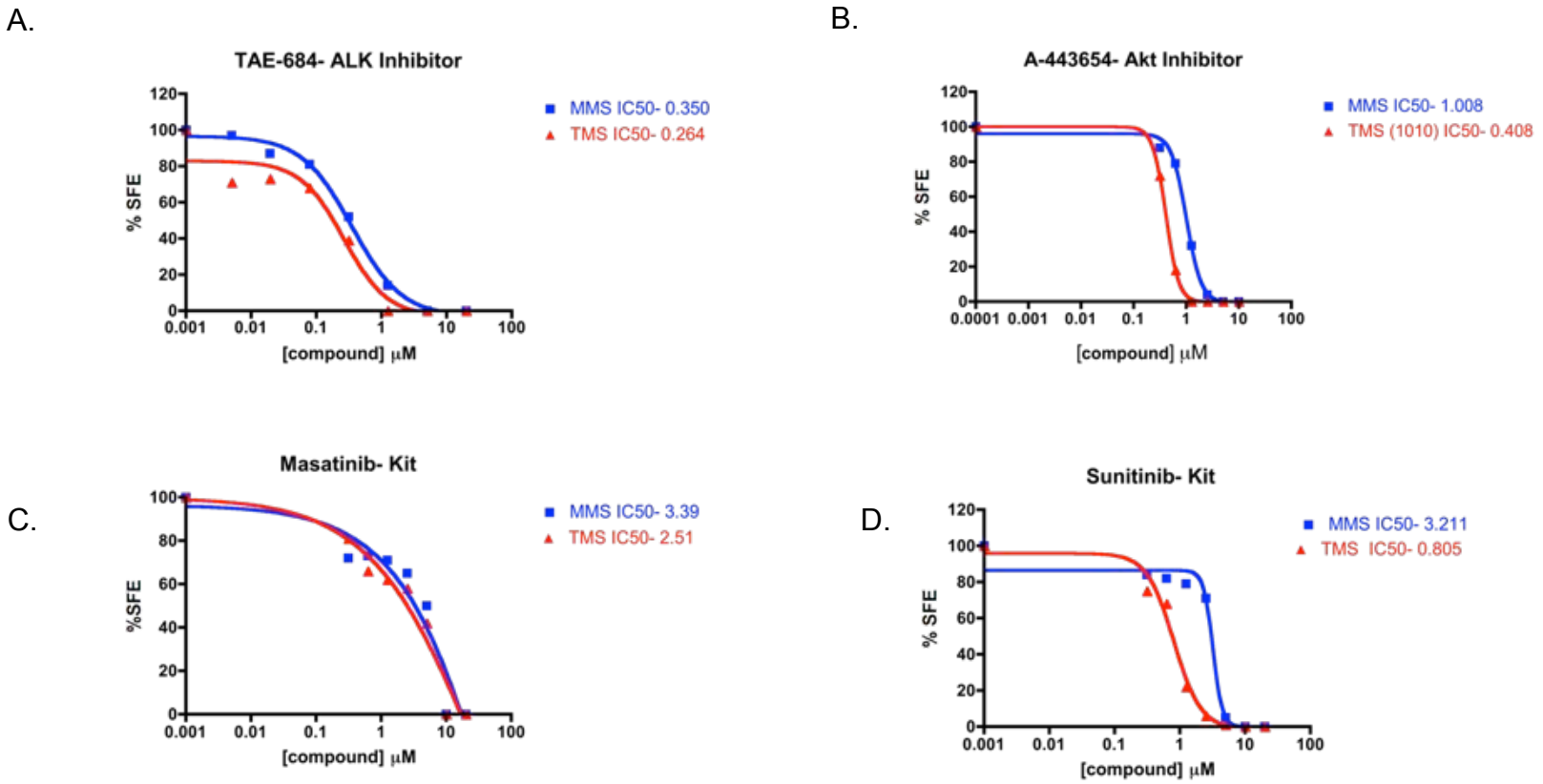


Figure 9- Follow-up sphere forming assays identifies Sunitinib as a tumorsphere selective kinase inhibitor- Sphere forming assays were carried out with inhibitors identified from the screen. The ALK and Kit inhibitor, TAE-684 and Masatinib were not selective in sphere-forming assays unlike in the cell proliferation assay (A, B). The Akt inhibitor A-443654 was not selective in previous assays but was moderately selective in sphere-forming assays. Sunitinib was 4-fold selective in the sphere-forming assays.

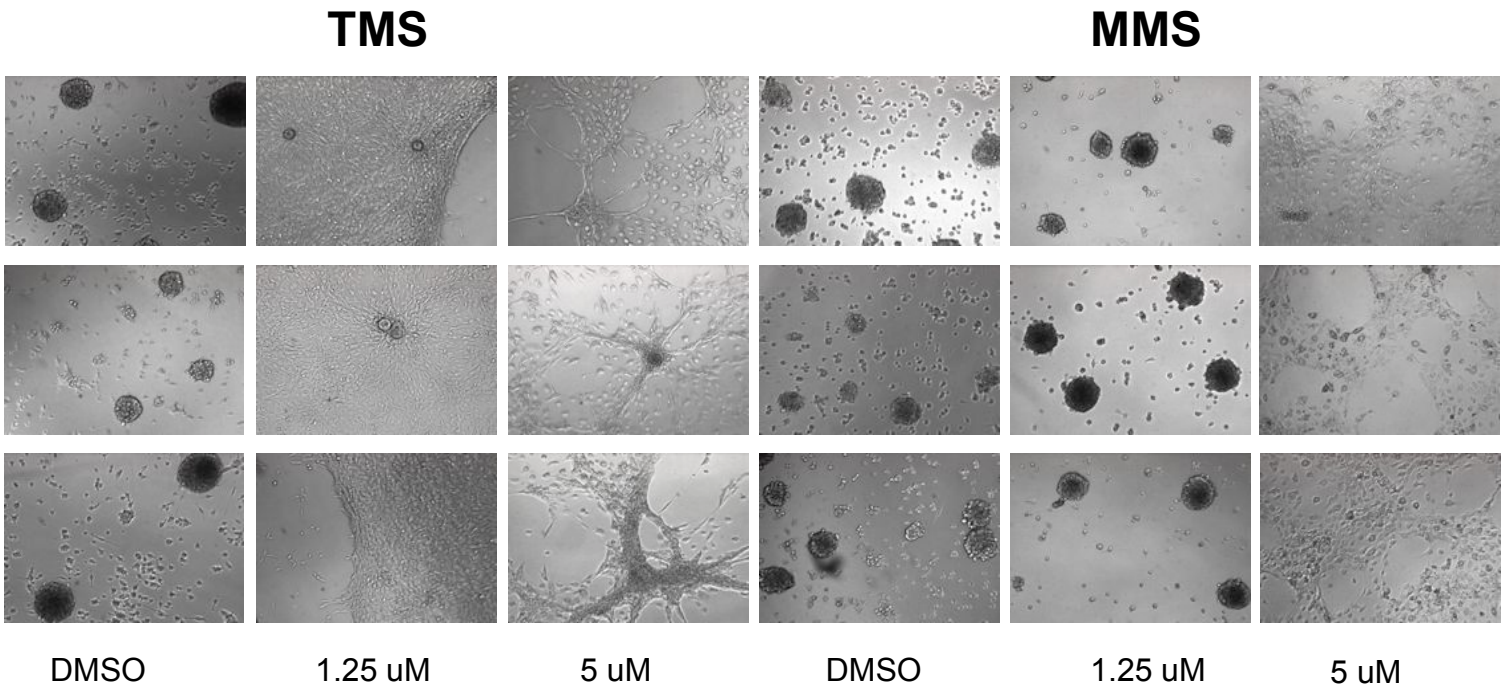


Figure 10- Sunitinib inhibits sphere formation in tumorsphere-derived cells. Treating TMS derived cells inhibits sphere formation and results the cell to adhere to the plate displaying an adherent cell type phenotype. This is also seen in MMS-derived cells but at higher concentrations.

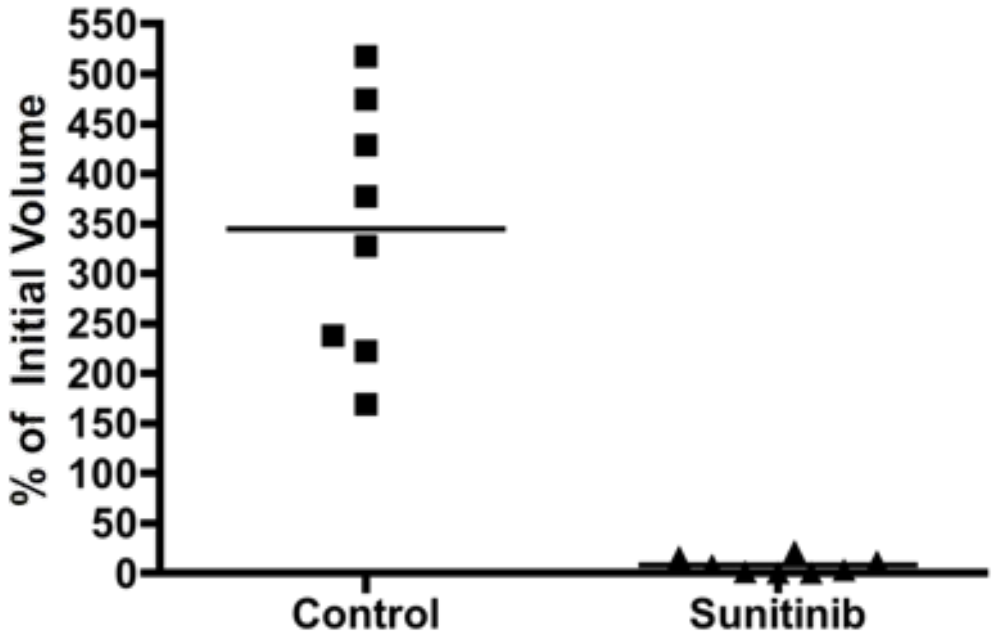


Figure 11 - Sunitinb shrinks MMTV-*neu* subcutaneous tumors- Subcutaneous tumors were seeded by 2 primary tumors 811 and 007 and allowed to grow to about 1 cm^3. Treatment was then started with 2mg of Sunitinib per mouse or carboxymethylcellulose solution, the control, for two-weeks with 5 days on 2 days off. Treatment with Sunitinb resulted in tumor shrinkage while tumors of mice treated with control grew. The difference in tumor volume was statistically signification with a p-value < 0.0001.

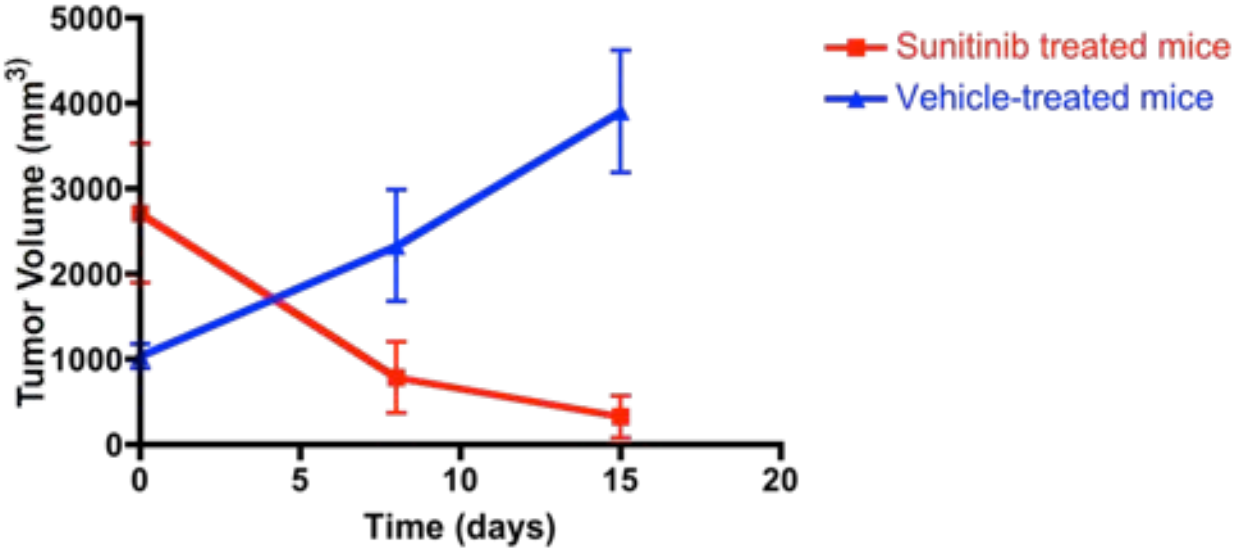


Figure 12- Change in tumor volumes of subcutaneous treated tumors. Tumor volumes of Sunitinib treated mice drastically shrank in size after only one week of treatment. Meanwhile control or vehicle treated tumors grew (Tumor 007, n=4).

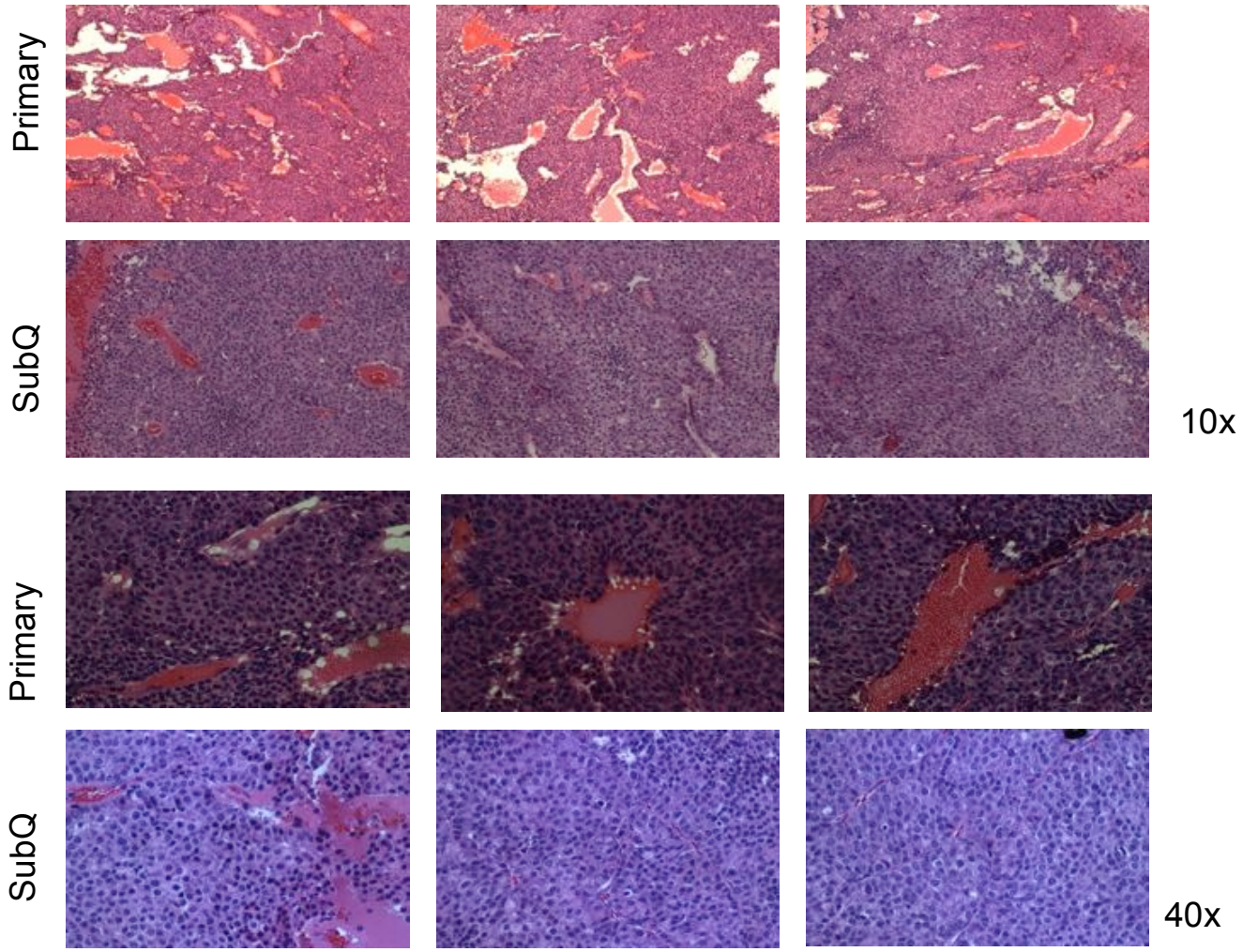


Figure 13 - H&E staining of primary and subcutaneous tumor sections. Primary tumor and subcutaneous (SubQ) tumors seeded by the primary tumors have similar histology with a high nucleus to cytoplasmic ratio and abundant vasculature.

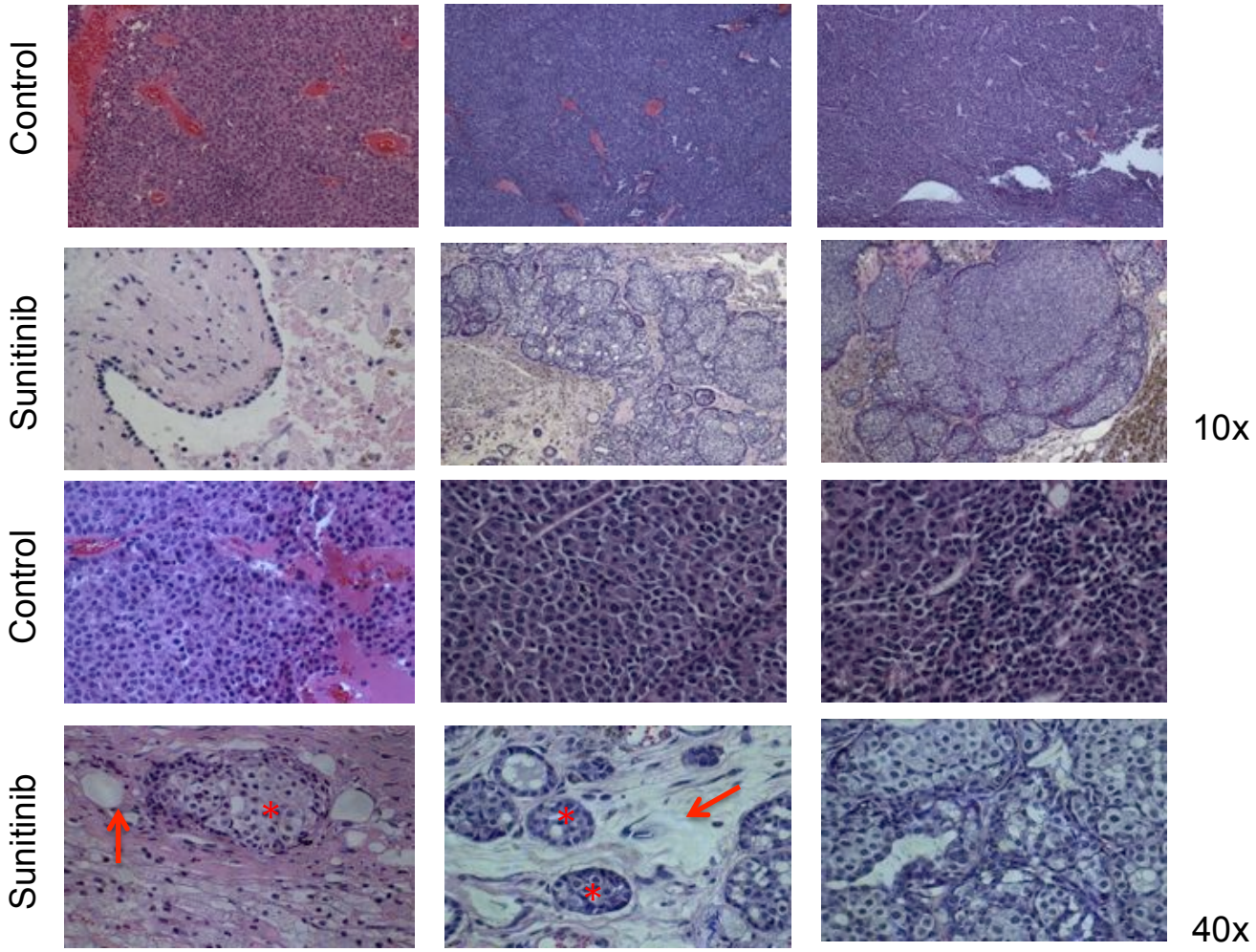


Figure 14 - H&E staining of tumor sections shows a change in tumor histology of Sunitinb treated tumors-Sunitinib treated tumors contained less vasculature, more cell-free areas, and what appears as tumor cell cluster or islands (asterisk) surrounded by cell-free areas (arrow).

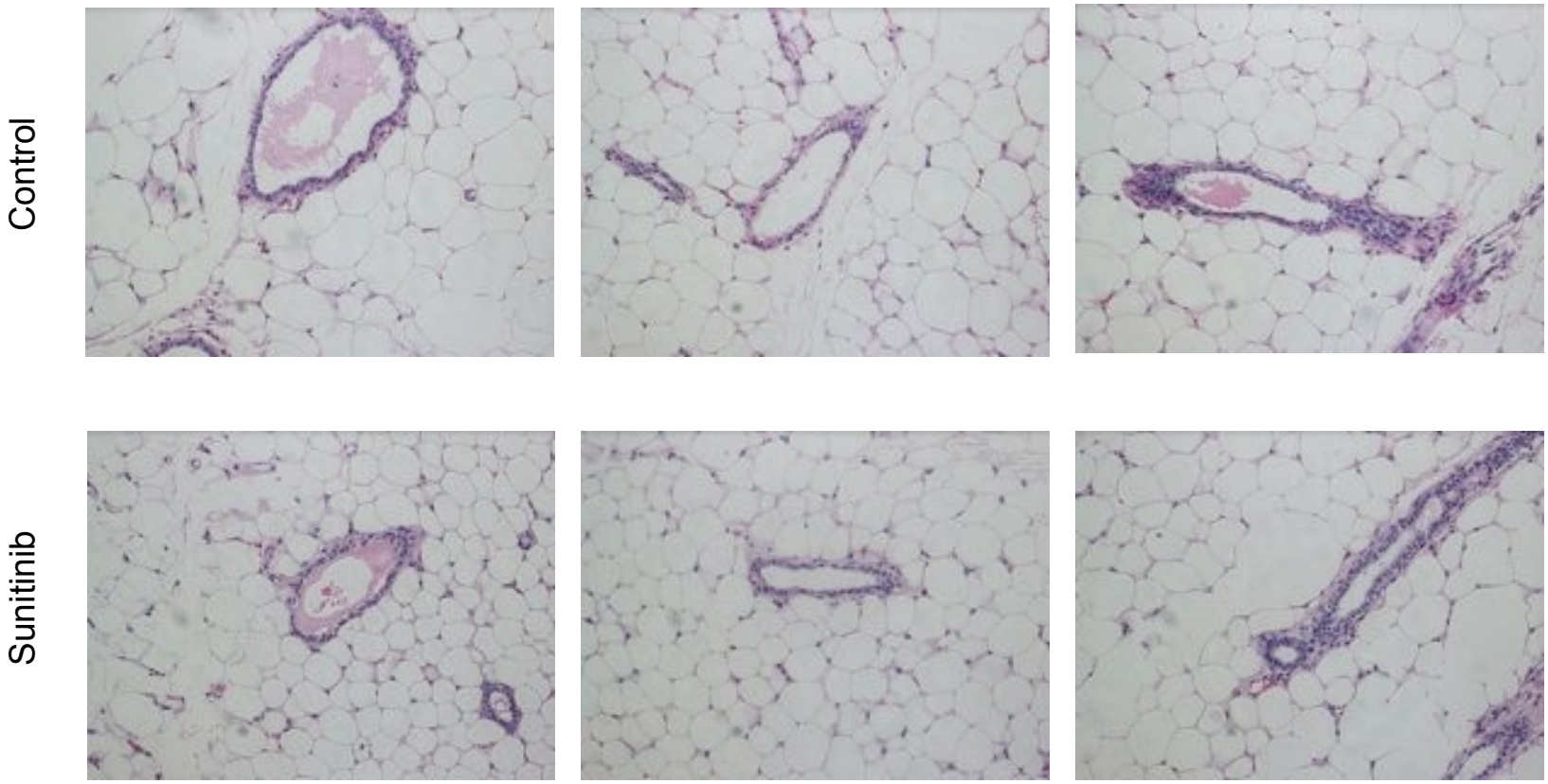


Figure 15 - H&E staining of mammary glands from Sunitinib treated and control mice show no change in gland morphology- Mammary glands from treated and control mice have similar histology suggesting no effect of the drug on the glands (20x).

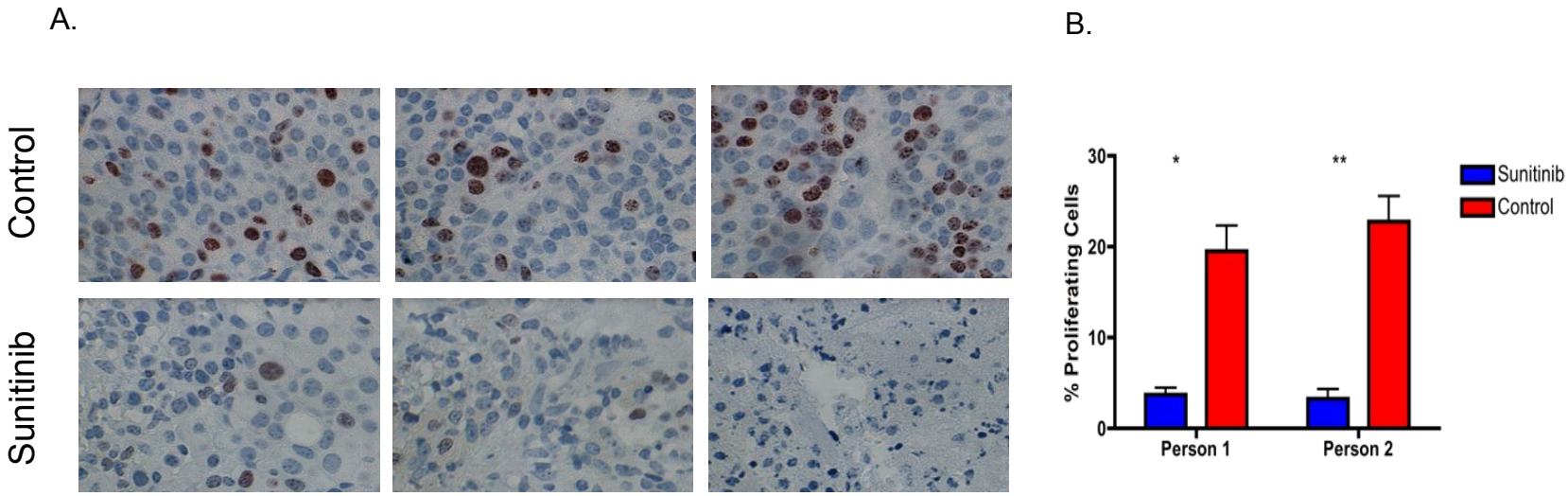


Figure 16- Ki-67 staining of Sunitinib treated tumor sections shows a decrease in proliferating cells- (A) Control and Sunitinib treated sections were stained for Ki-67 to determine the fraction of proliferating cells. (B) Quantification of Ki-67 cells in sections of vehicle treated and Sunitinib treated tumors (* p-value = 0.0387, ** p-value= 0.0061) (40x).

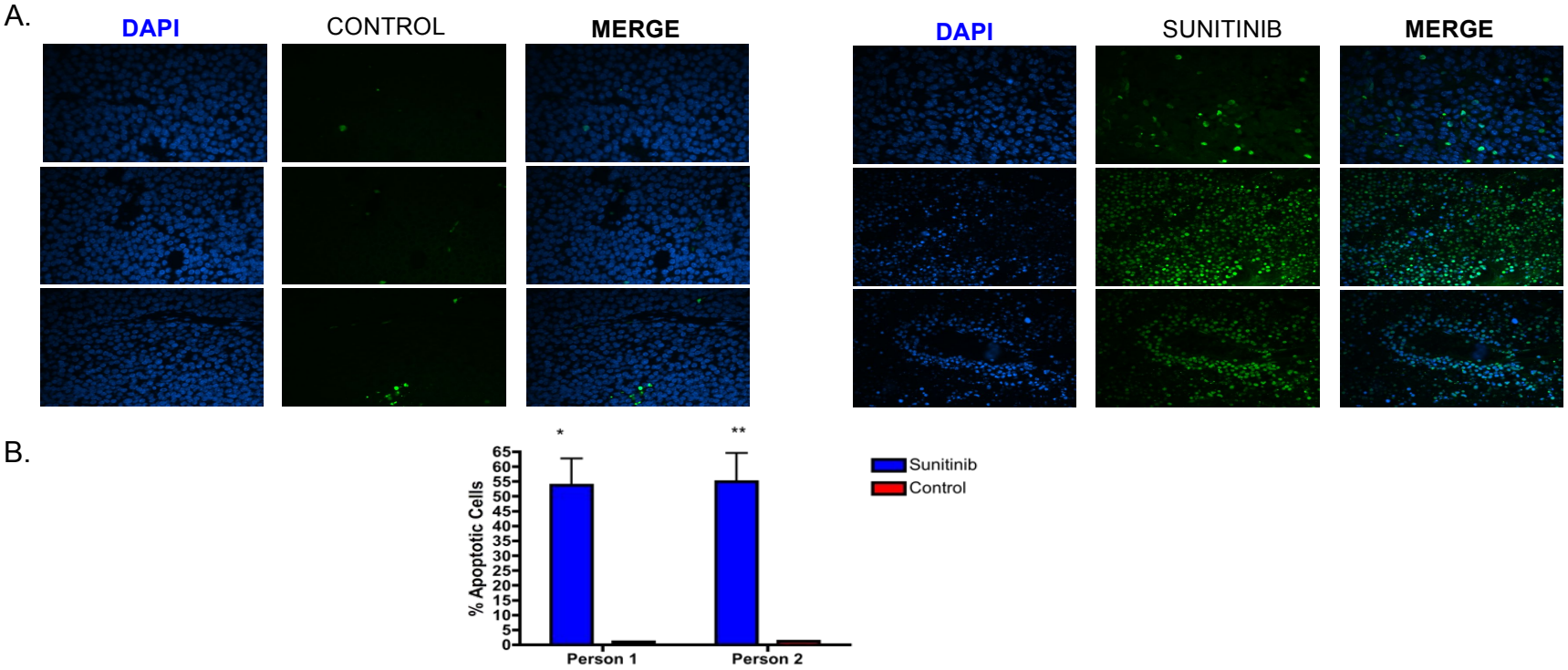


Figure 17- TUNEL staining of Sunitinib treated tumor sections shows an increase in apoptotic cells- (A) Sections of control and Sunitinib treated tumors were stained using a TUNEL assay to determine the fraction of apoptotic cells. (B) Quantification of apoptotic cells in sections of vehicle treated and Sunitinib treated tumors (*p-value =0.0454, **p-value=0.0391) (40x).

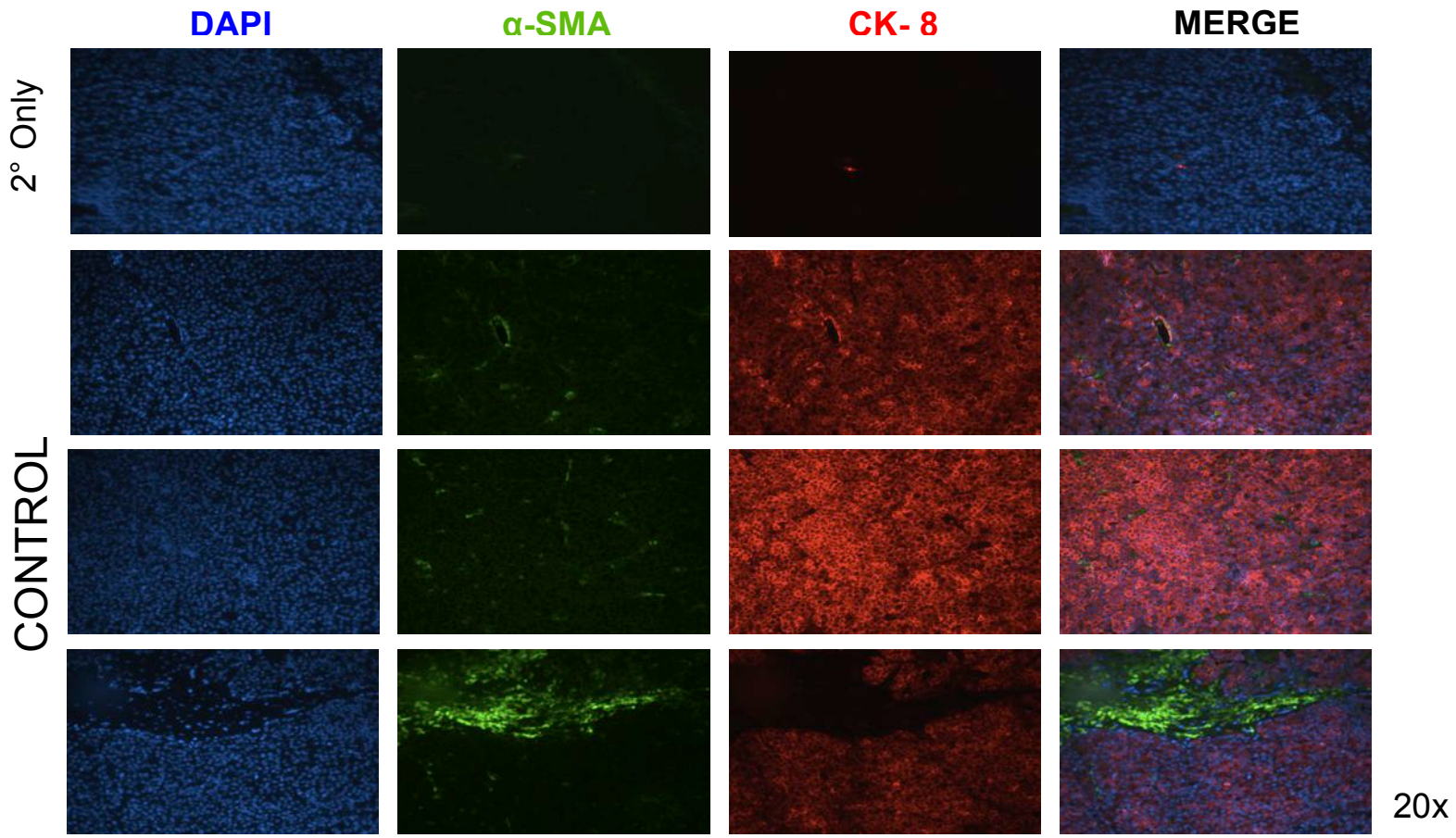


Figure 18 - α-SMA and CK8 staining of Sunitinib treated tumor sections shows a change in cellular composition- (A, B) Tumor section from control treated mice stained for CK8 and a few cells stained for α-SMA. (C, D) Tumor sections from Sunitinib treated tumors stained for CK8 and α-SMA. α-SMA staining was localized around tumor cell clusters or islands.

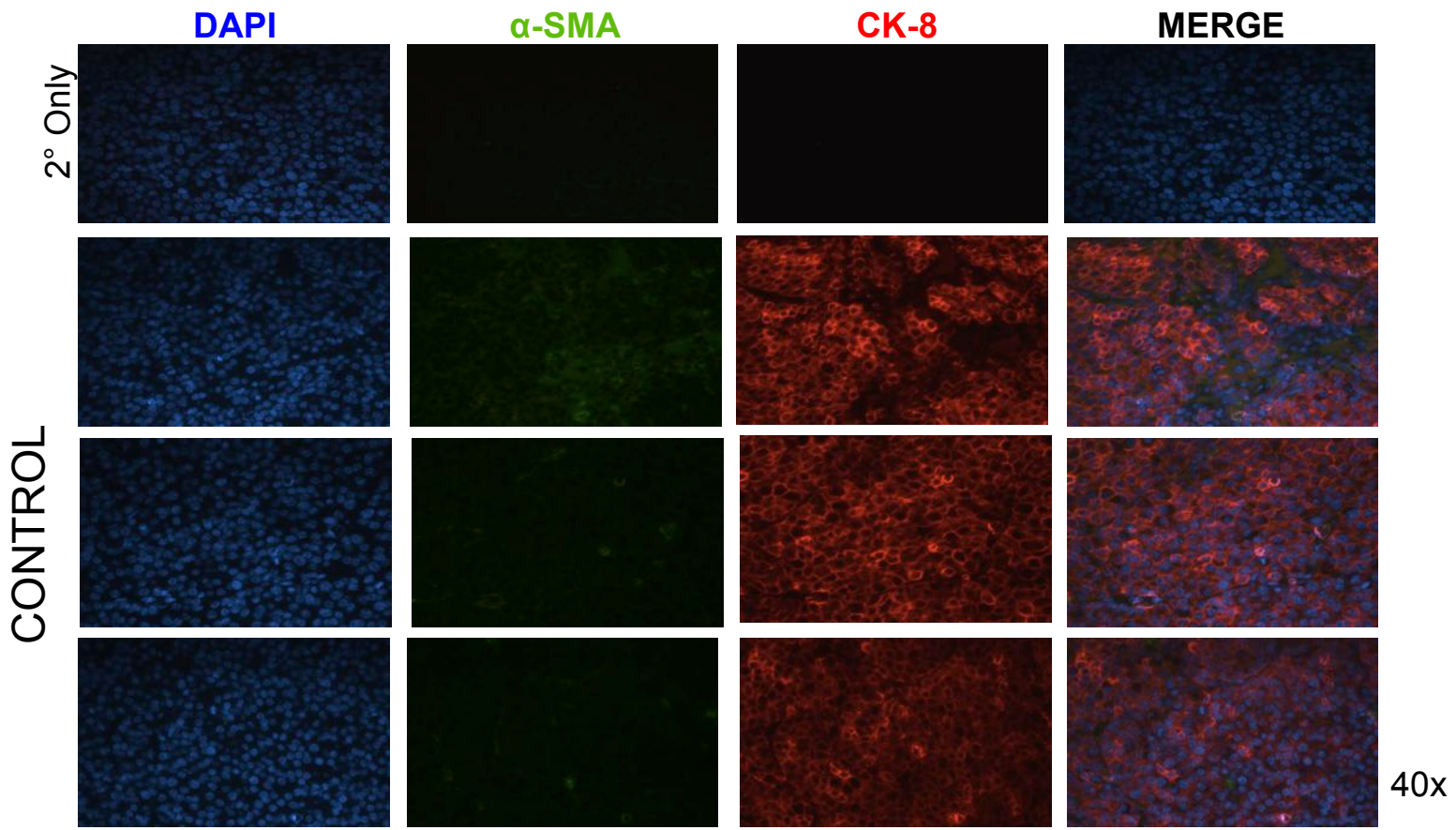


Figure 18 (B)

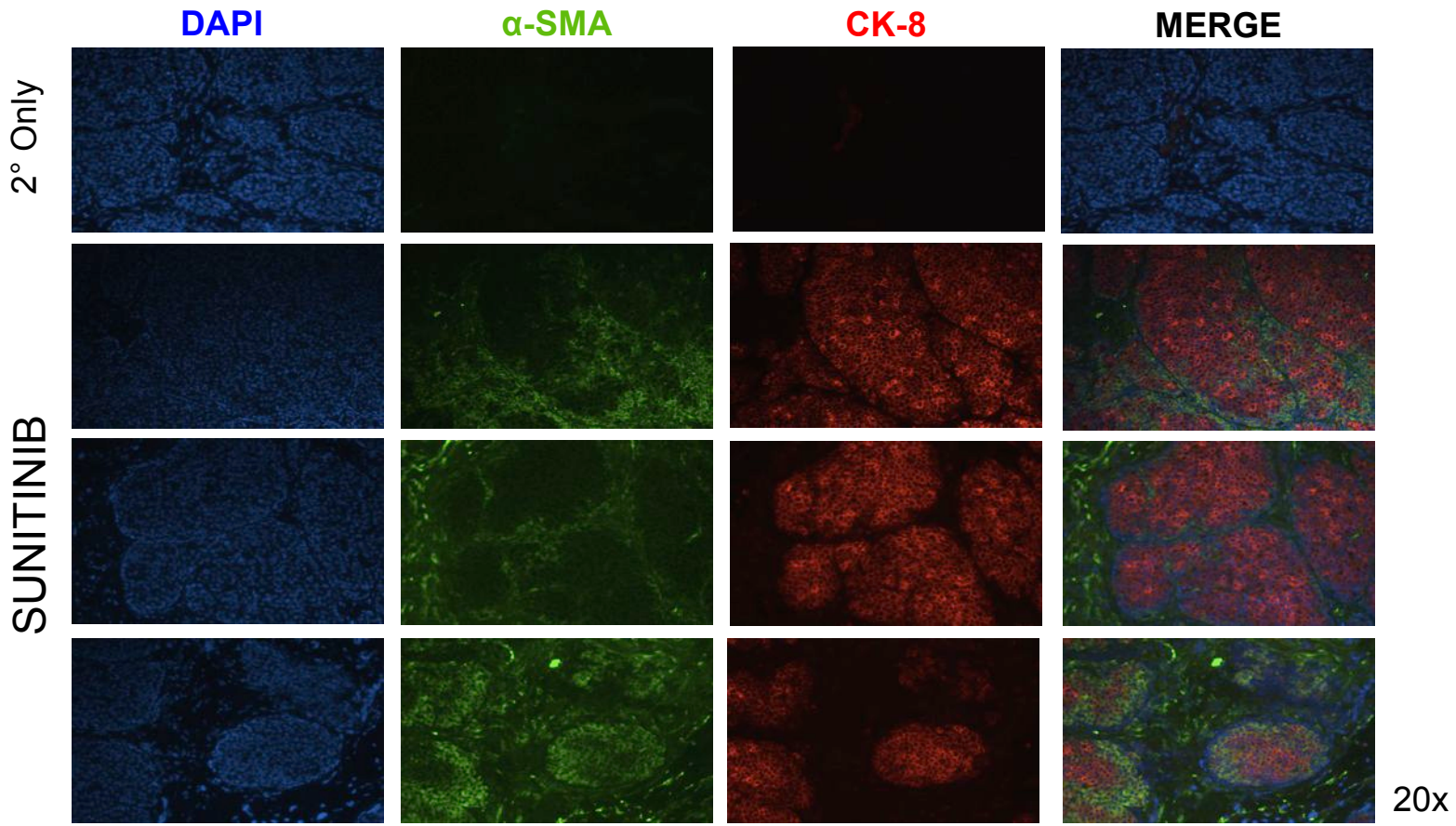


Figure 18 (C)

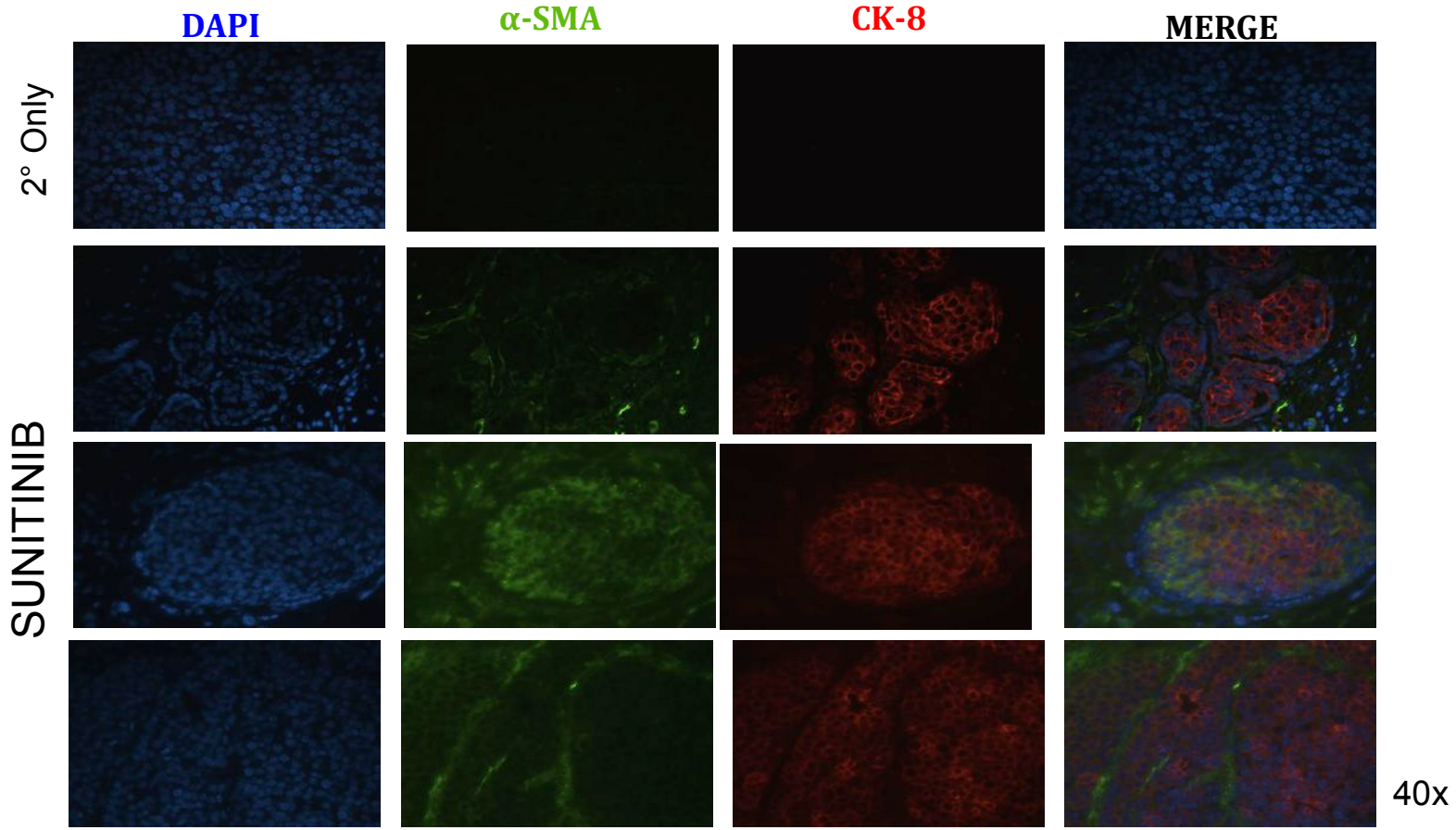


Figure 18 (D)

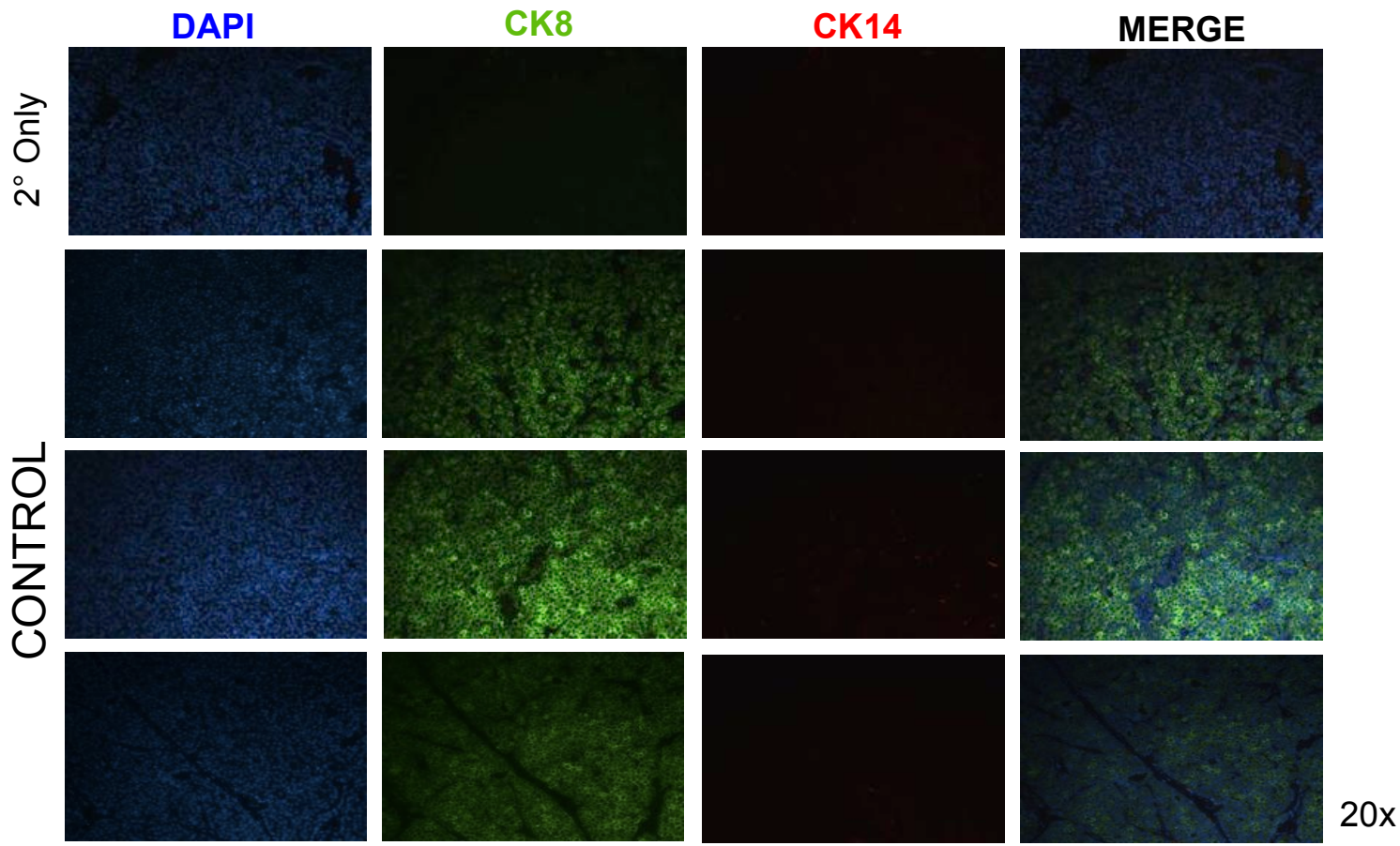


Figure 19- CK14 and CK8 staining of Sunitinib treated tumor sections shows a change in cellular composition- (A, B) Tumor sections from control treated mice stained uniformly for CK8 and not for CK14. (C, D) Treated tumor cell sections contained some rare cells that stained for CK14, a majority stained for CK8 and some cells that did not stain for either marker.

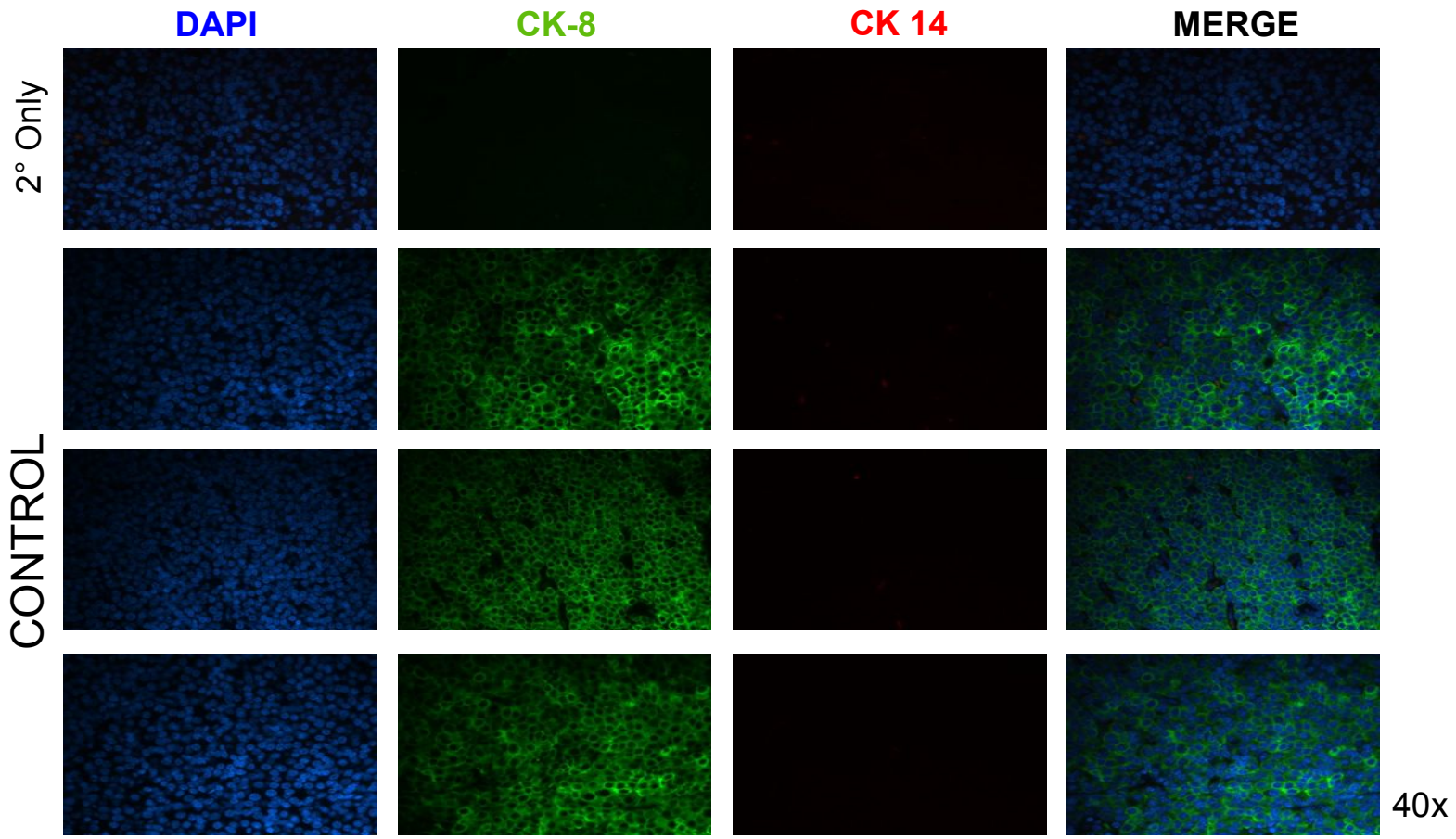


Figure 19 (B)

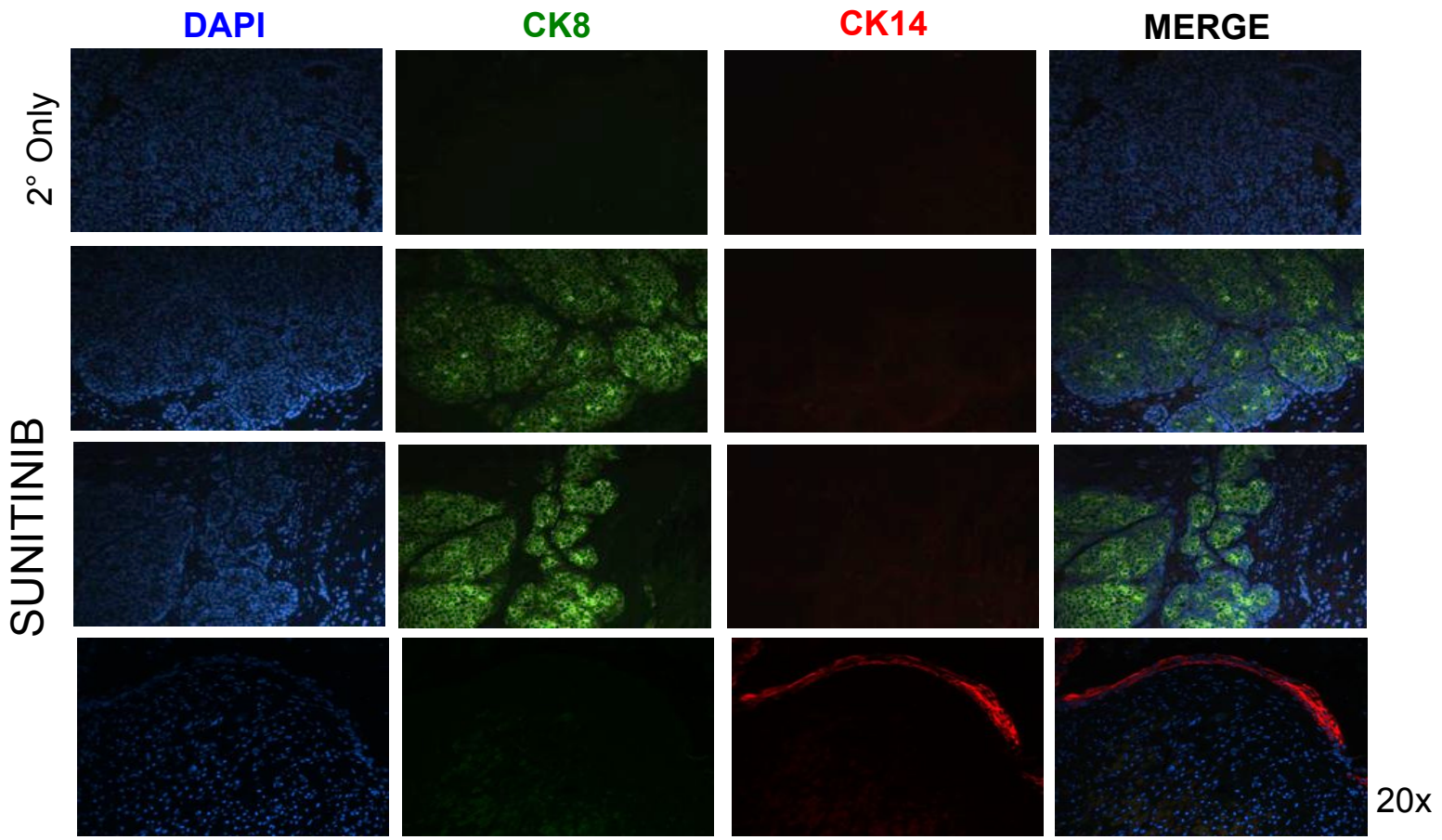


Figure 19 (C)

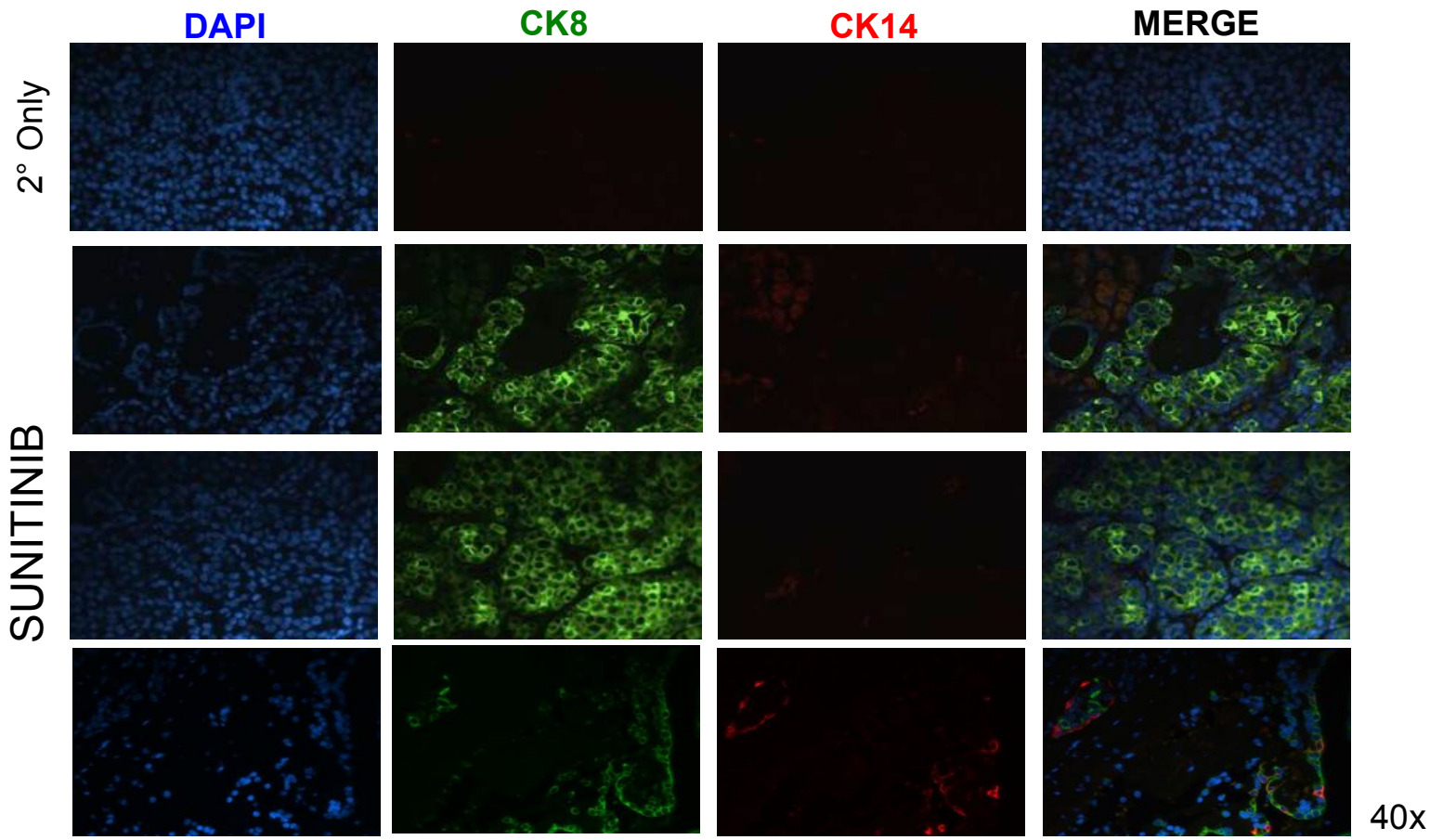


Figure 19 (D)

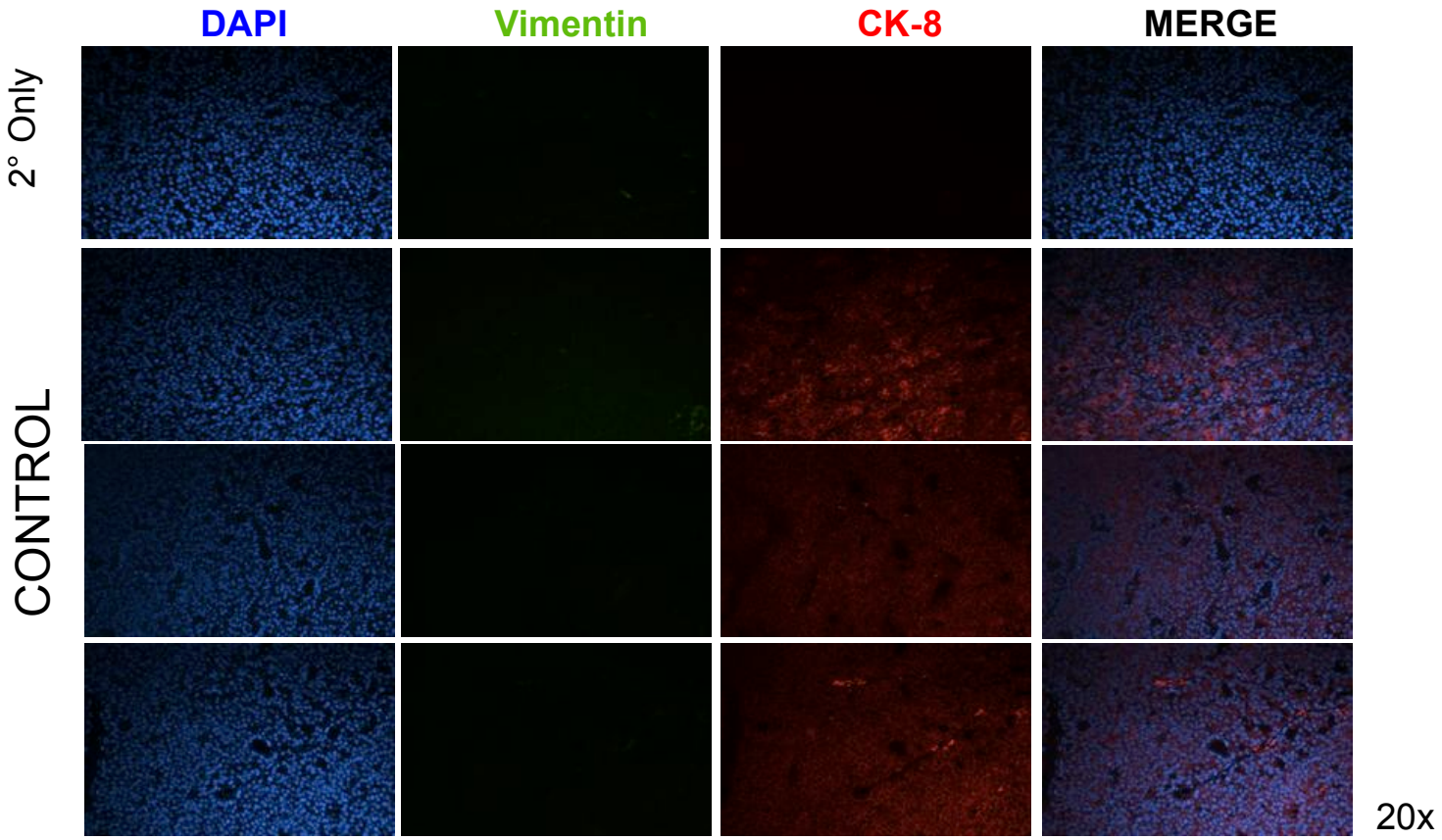


Figure 20- Control and Sunitinib treated tumor sections do not stain positive for vimentin- (A, B) Tumor section from control treated mice stained for CK8 and not for vimentin. (B, C) Similarly tumor sections from Sunitinib treated tumors stained for CK8 and not for vimentin.

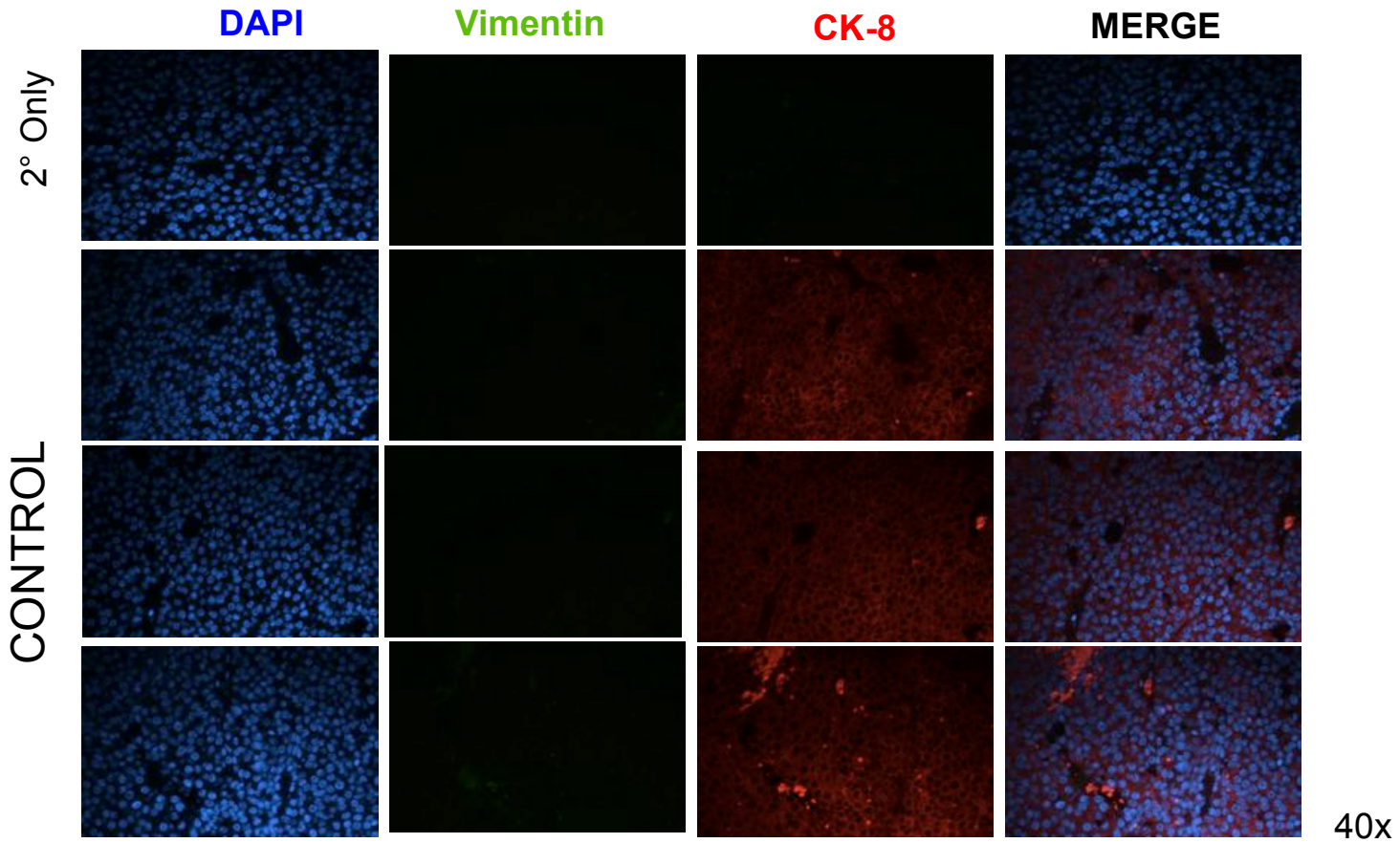


Figure 20 (B)

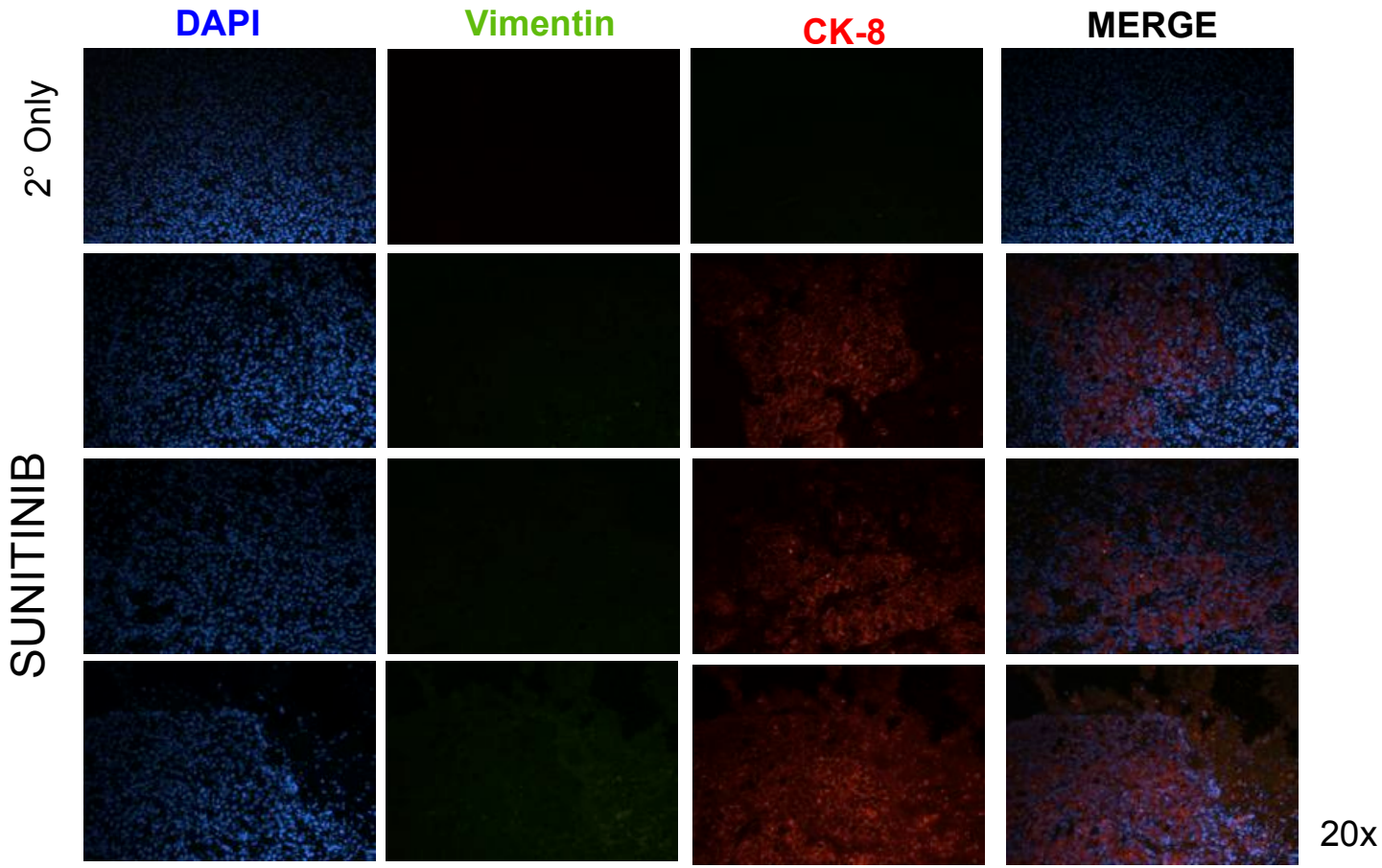


Figure 20 (C)

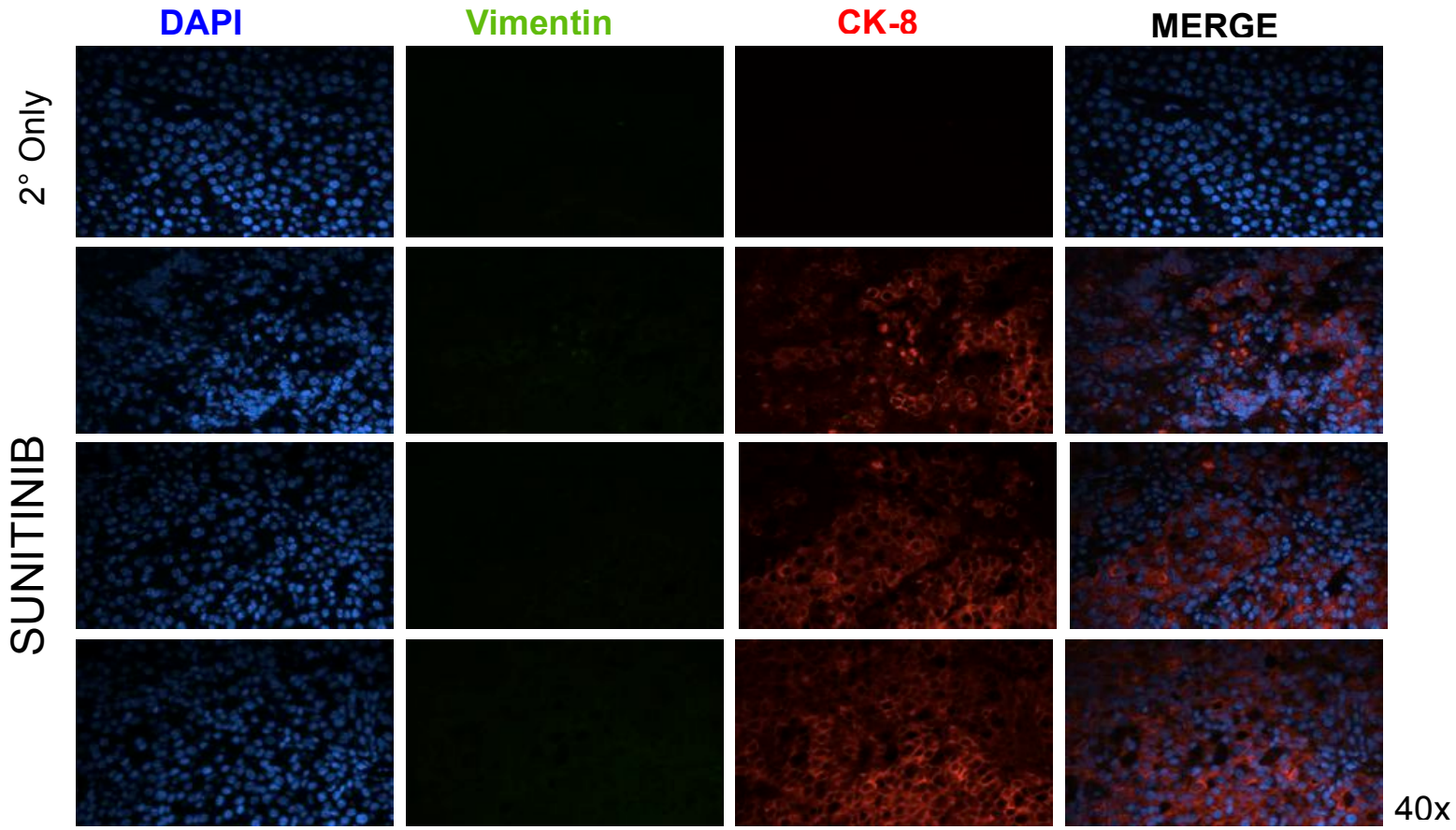


Figure 20 (D)