

**BO-264: HIGHLY POTENT TACC3 INHIBITOR AS A
NOVEL ANTICANCER DRUG CANDIDATE**

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By

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BO-264: Highly Potent TACC3 Inhibitor as a Novel Anticancer Drug Candidate

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January 2021

We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and in quality, as a dissertation for the degree of Doctor of Philosophy.

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ABSTRACT

BO-264: HIGHLY POTENT TACC3 INHIBITOR AS A NOVEL ANTICANCER

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Breast cancer has been consistently ranked to be the most common cancer and the second leading cause of cancer-related death in women worldwide for many years. Despite better understanding of tumor biology and the availability of plethora of anti-cancer therapeutics utilizing different strategies, complete response and/or long-term survival is achieved in only a small fraction of patients with aggressive disease. Since microtubule reorganization is an important step during cell division, drugs that interfere with this process have been a major focus of cancer research. Although anti-microtubule agents are widely used in clinic, cytotoxicity to non-tumorigenic cells and drug resistance are still the main obstacles. Therefore, development of alternative target molecules that selectively and efficiently target cancer cells, but restore normal cells are needed. Transforming acidic coiled-coil containing protein 3 (TACC3) is an important TACC family member, having both mitosis-related roles e.g. regulation of centrosomes and microtubule stability and interphase-related roles e.g. regulation of gene expression and cell migration. Being overexpressed in a broad spectrum of cancers and correlation of its expression with disease progression make TACC3 a highly attractive therapeutic target. Although the oncogenic role of TACC3 has been established albeit mostly in *in vitro* settings, there is

currently no TACC3 inhibitor being tested in clinics. Therefore, by combining rational drug design and screening, we aimed to identify and characterize a novel TACC3 inhibitor hit molecule with high potency and minimum toxicity in *in vitro* and *in vivo* systems that is amenable for future drug development.

BO-264 was identified as a novel inhibitor targeting TACC3 by direct binding validated by using several biochemical methods, including drug affinity responsive target stability, cellular thermal shift assay, and isothermal titration calorimetry. Compared to two other available TACC3 inhibitors, it showed superior inhibition of mitotic progression and cell viability, especially in aggressive basal and HER2+ breast cancer cell lines. Notably, BO-264 had remarkable cytotoxicity effect on several cancer cell lines in NCI-60 human cancer cell line panel ($\geq 90\%$ have less than 1 μM GI50 value) and inhibited the proliferation of FGFR3-TACC3 fusion protein-harboring cells, an oncogenic driver in several malignancies. Importantly, BO-264 did not cause any cytotoxicity to non-cancerous cell lines. Noteworthy, its oral administration significantly suppressed tumor growth in both breast and colon cancer syngeneic and xenograft models, and prolonged survival with no major toxicity. Finally, TACC3 expression level has been identified as a strong independent prognostic factor in breast cancer. Collectively, our preclinical findings suggest that BO-264 is a potent and non-toxic anti-cancer agent targeting TACC3 in breast and colon cancer and can be developed further to obtain better drug-like properties.

Keywords: Breast cancer, colon cancer, targeted therapy, TACC3, inhibitor, BO-264, FGFR3-TACC3



ÖZET

BO-264: ÖZGÜN ANTI-KANSER İLAÇ ADAYI OLARAK YÜKSEK ETKİ
POTANSİYELİNE SAHİP TACC3 İNHİBİTÖRÜ

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Meme kanseri kadınlarda en sık görülen kanser türü olup kanser kaynaklı ölümlerde ikinci sırada yer almaktadır. Tümör biyolojisinin daha iyi anlaşılmasına ve farklı yaklaşımlar içeren kanser tedavileri mevcut olmasına rağmen, agresif kansere sahip hastaların sadece bir kısmında tedaviye tam yanıt ve/veya uzun yaşam süreleri gözlemlenmiştir. Mikrotübüllerin yeniden düzenlenme ve dağılımı hücre bölünmesi sırasında önemli bir adım olduğundan, bu ve benzeri süreçleri bozan ilaçlar kanser çalışmalarının ana odaklarından biri olmuştur. Anti-mikrotübül ajanları klinikte sıkça kullanılmasına rağmen, normal hücrelerde gözlenen sitotoksite ve ilaç direnç gelişimi halen önemli sorun teşkil etmektedir. Bu yüzden, normal hücrelere zarar vermeden, seçici ve etkili bir şekilde kanser hücrelerini hedefleyen alternatif tedavi seçeneklerinin geliştirilmesine ihtiyaç duyulmaktadır. TACC3 geni, dönüştürücü asidik sarmalanmış sarmal (TACC) gen ailesine mensup olup mitoz sırasında sentrozomal adaptör olarak görev yapıp mikrotübül stabilizasyonunu sağlarken interfaz sırasında ise gen transkripsiyonu ve hücre göçünü düzenler. TACC3 seviyesinin birçok farklı kanser türünde artış göstermesi ve kanserin ilerlemesiyle olan ilişkisi, TACC3'ü cazip bir terapötik hedef yapmaktadır. TACC3'ün onkojenik aktivitesi çoğunlukla *in vitro* çalışmalarıyla belirlenmiş olup, klinikte test edilebilecek etkinlikte bir TACC3 inhibitörü bulunmamaktadır. Bu nedenle, bu tez

alışmasında rasyonel ilaç tasarım ve tarama yaklaşımlarının birleşimiyle *in vitro* ve *in vivo* sistemlerde yüksek etki ve düşük toksisite ile TACC3'yi hedefleyen ve gelecekte ilaç geliştirilmesine uygun özgün bir aktif molekül saptamayı ve karakterize etmeyi amaçladık.

BO-264, TACC3 ile doğrudan etkileşimi hedef bağlanma yöntemi, ilaç-eğilim duyarlı hedef dengesi yöntemi ve izotermal kalorimetri metotları gibi birçok biyokimyasal metot ile doğrulanmış bir TACC3 inhibitörü olarak saptanmıştır. BO-264, mevcut diğer iki TACC3 inhibitörüne kıyasla, özellikle bazal ve HER2+ meme kanseri hücre hatlarında mitotik ilerleme ve hücre canlılığını baskılamada daha üstün etki göstermiştir. Ayrıca, birçok NCI-60 insan kanser hücre hattı ile (hücrelerin %90'ından fazlası 1 µM hücre büyüme inhibisyonu değeri göstermiştir) ve birçok kanser türünde onkojenik etkiye sahip FGFR3-TACC3 füzyon proteinini içeren hücre hatlarında nanomolar seviyede yüksek etki göstermiştir. En önemlisi, BO-264 normal hücrelerde herhangi bir sitotoksosite yaratmamıştır. Bununla beraber, BO-264'ün oral kullanımı hem meme hem de kolon kanseri sinjenik ve zenograft fare modellerinde belirgin hiçbir organ toksisitesi oluşturmada tümör büyümesini baskılamış ve hayatta kalım süresini arttırmıştır. Son olarak, TACC3 seviyesi meme kanseri hastalarında güçlü bağımsız bir prognostik faktör olarak belirlenmiştir. Özetle, klinik öncesi bulgularımız BO-264'ü meme ve kolon kanserinde TACC3'yi hedefleyen, etki potansiyeli yüksek ve toksisitesi olmayan bir anti-kanser ajanı olarak sunmakta ve bu molekül daha iyi ilaç-benzeri özellikler kazandırmak adına geliştirilebilecektir.

Anahtar sözcükler: Meme kanseri, kolon kanseri, hedefli terapi, TACC3, inhibitor, BO-264, FGFR3-TACC3



I dedicate this work to my

MOTHER

who made me who I am today,

who greatly supports me at every stage of my life no matter what it takes and

who is always there for me with an endless love



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Abbreviations

ADME	Absorption, distribution, metabolism and excretion
APS	Ammonium peroxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Equiv	Equivalent
ER- α	Estrogen receptor alpha
FBS	Fetal bovine serum
FGFR3	Fibroblast growth factor receptor 3
FFPE	Formalin fixed paraffin embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GI ₅₀	50% Growth inhibition
GSEA	Gene set enrichment analysis
HER2	human epidermal growth factor receptor 2
H&E	Hematoxylin and eosin
HPRT	Hypoxanthine guanine phosphoribosyl transferase

HR+	Hormone receptor positive
HRP	Horseradish peroxidase
IC ₅₀	Inhibitory concentration 50%
IF	Immunofluorescence
IHC	Immunohistochemistry
iv.	Intravenous
IVIS	In vivo imaging system
kDa	Kilo dalton
KM	Kaplan Meier
LC ₅₀	Lethal dose concentration inducing 50% cell death
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
MFP	Mammary fat pad
MTA	Microtubule targeting agent
MTD	Maximum tolerated dose
NCI	National Cancer Institute
OS	Overall survival
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PFS	Progression free survival
PD	Pharmacodynamic
PK	Pharmacokinetic
PR	Progesterone receptor

PTM	Post-translational modifications
po.	Per os, by mouth
qRT-PCR	Quantitative real time polymerase chain reaction
RT	Room temperature
SAC	Spindle assembly checkpoint
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short/small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SPL-B	Spindlactone B
SRB	Sulforhodamine B
TACC3	Transforming Acidic Coiled-Coil Containing Protein
TBST	Tris buffer saline Tween20
TCA	Trichloroacetic acid
TCGA	The cancer genome atlas
TGI	Total growth inhibition
TNBC	Triple negative breast cancer
v/v	Volume/volume
w/v	Weight/volume

1. INTRODUCTION

Breast cancer

Breast cancer has been consistently ranked to be the most common cancer and the second leading cause of cancer-related death in women worldwide for many years [1, 2]. It has been estimated that approximately 280,000 women will be diagnosed with breast cancer in United States and around 64,000 will die from breast cancer in 2020 [2]. Although early detection, chemotherapy, radiation therapy and advanced targeted therapies significantly improves patient survival, there is still dire need of better detection methods as well as treatment options for cancer patients.

Biologic inter- and intratumoral heterogeneity of breast cancer is extensively studied [3]. Breast cancer is classified into three clinical subtypes based on protein expression status of estrogen receptor alpha (ER- α), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/ErbB2). Broadly, these subtypes include hormone receptor positive (HR+) which express higher levels of ER and PR, HER2-amplified and triple-negative breast cancer (TNBC), lacking the expression of all three receptors [4]. While this clinical stratification is still mainly used, its heterogeneous nature has been further classified into five distinct molecular subtypes based on gene expression; Luminal A, Luminal B, HER-2 enriched, Basal-like and a normal breast-like group (**Figure 1.1**) [5, 6]. Each subtype has its own heterogeneous pathologies and clinical outcomes, affecting the disease progression and guiding therapeutic decisions.

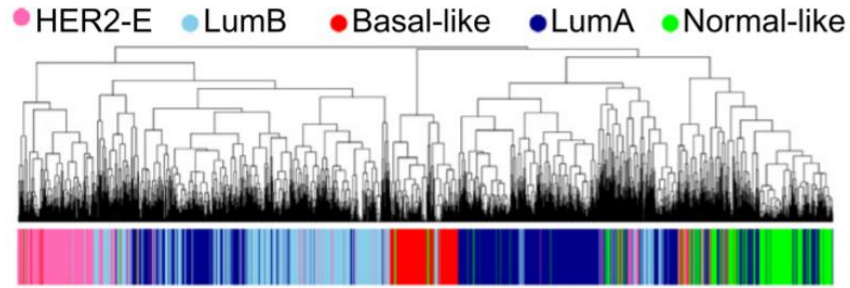


Figure 1.1. Molecular subtype classification of breast cancer according to the gene expression profiles of 1,834 breast cancer patients [5].

Several studies about breast cancer development and progression contributed to our understanding of disease heterogeneity, which in turn led to identification of novel therapies. However, there is still large patient population who need targeted therapies which could improve their survival.

Therapy options for breast cancer

Breast cancer is staged I-IV, where stage I denotes the cancer including only breast and no spread to the axillary lymph nodes, stage IV indicates distant metastatic disease [7]. Depending on the breast cancer type and how advanced it is, treatment options might change. Their mechanism of action might act through signaling pathways, growth factors, nuclear receptors or surface antigens, regulating cell proliferation, apoptosis, metastasis and angiogenesis [27, 28] (**Figure 1.2**). There are two major pillars to manage breast cancer, namely locoregional treatment and systemic therapy [8]. While local therapy for early stages consists of surgical resection or removal of axillary lymph nodes followed by postoperative radiation therapy, systemic therapy includes neoadjuvant (preoperative) and/or adjuvant (postoperative) therapies [7]. Neoadjuvant is mainly used to downstage

disease progression in breast and axilla which then allows to conserve breast and avoid lymph node dissection. On the other hand, after having breast surgery to remove primary tissue, patients receive adjuvant therapies to eliminate any remaining tumor cells completely which otherwise may recur and/or develop metastases [9, 10].

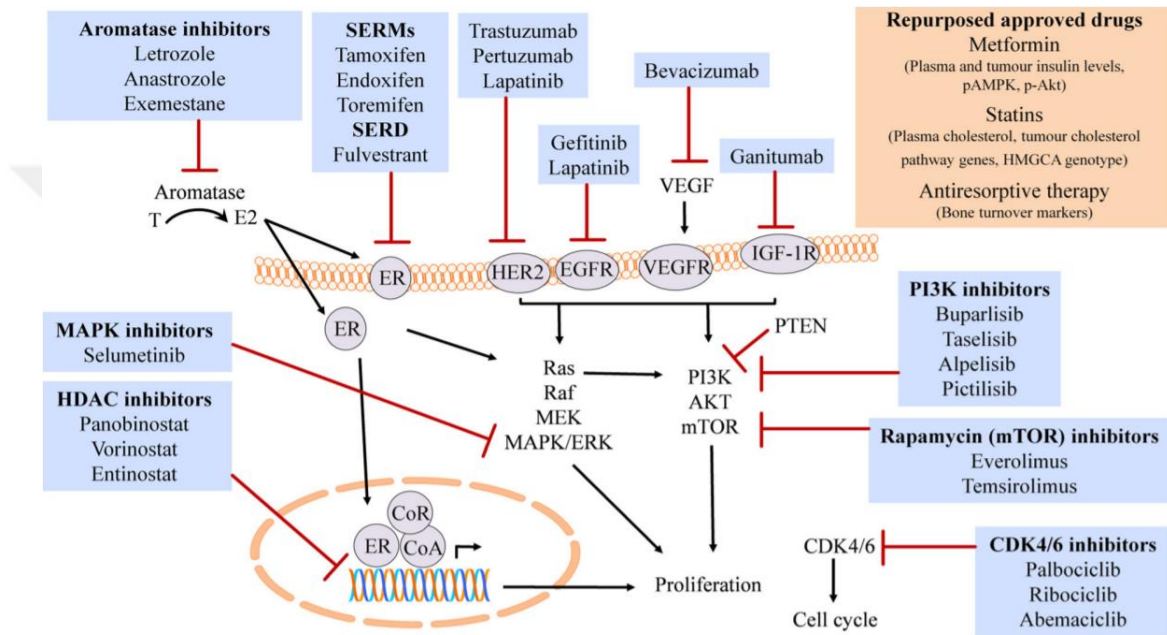


Figure 1.2. Treatment options for breast cancer. Schematic diagram of drugs used in clinics or clinical trials together with their target molecules are summarized [11].

ER α and/or PR are expressed in approximately 70% of invasive breast cancers. Targeting ER signaling with endocrine agents is the primary systemic therapy for patients belonging to hormone receptor positive breast cancer subtype [7]. Standard adjuvant endocrine therapy includes 5 years with antiestrogen medication like tamoxifen or aromatase inhibitors (anastrozole, exemestane and letrozole) [12] (**Figure 1.2**). Tamoxifen, a selective estrogen receptor modulator (SERM) that competitively inhibits estrogen binding to its receptor, is the oldest molecular targeted therapy used for ER-

positive breast cancer patients [13]. On the other hand, aromatase inhibitors reduce the circulating estrogen levels in blood through inhibition of conversion of androgens to estrogen [14]. Selective estrogen receptor degrader (SERDs), such as fulvestrant are used for the treatment of postmenopausal ER+ patients with metastatic breast cancer after progression on tamoxifen or aromatase inhibitors [11]. While existing endocrine therapies achieved remarkable success initially, they tend to be short lived due to resistance development with interference in the downstream signaling pathway. However, combination therapies, including CDK4/6 inhibitors (palbociclib, abemaciclib) [15], and α isoform-specific (p110- α) PI3K inhibitor (alpelisib) [11, 16] are commonly used to treat hormone therapy resistant aggressive breast cancers.

HER2 as a transmembrane receptor tyrosine kinase in the EGFR family is another main molecular target, which is amplified or overexpressed in approximately 20% of breast cancer patients. Trastuzumab, first therapeutic humanized monoclonal antibody targeting extracellular domain of ERBB2, have been used in a neo-adjuvant setting for the treatment of HER2-positive breast cancer [17] (**Figure 1.2**). Drugs like pertuzumab and neratinib are further used in patients with higher risk HER2+ breast cancer to reduce the recurrence risk [18]. However, due to *de novo* or acquired drug resistance, only one-third of patients with HER2-positive subtype benefit from HER2 targeted therapies which include trastuzumab and pertuzumab as first-line, trastuzumab-emtansine (T-DM1) as second-line [19] and lapatinib plus trastuzumab as third-line or greater therapy options [20]. To overcome this problem, drug combination strategies are generally preferred. For example, lapatinib has been shown to be effective on patients who progress on trastuzumab or combination treatment of trastuzumab and chemotherapy (capecitabine)

upon trastuzumab resistance associates with a better progression-free survival (PFS) [21, 22].

Compared to patients in HR+ and HER2+ breast cancer subtypes, TNBC patients, which makes up approximately 15% of breast cancer, have a relatively poorer outcome due to lack of known molecular targets for therapy. Chemotherapy is the primary therapeutic approach for these patients [23]. In TNBC setting, major chemotherapy options include anthracyclines, taxanes and platinum agents [24, 25]. In an advanced metastatic TNBC, patients have a relatively shorter median survival of 7-13 months and limited duration of response to chemotherapy [26, 27]. That is why, it is very important to define the suitable treatment option that patients can benefit most. Treatment landscape for TNBC patients started to change with the development of immune checkpoint inhibitors (ICIs). Recently, a programmed cell death ligand 1 (PD-L1) inhibitor (Keytruda) has been approved for TNBC [28, 29]. Furthermore, the FDA approved an antibody-drug conjugate (Trodelvy) targeting Trop-2 protein, which is overexpressed in 95% TNBC patients, and it is linked to the topoisomerase-I inhibitor, SN-38 to maximize the therapeutic potential for TNBC patients [30]. It showed an overall response rate of 33.3% with a median duration of response of 7.7 months [30].

Mitosis selective strategies against cancer

Since cancer is characterized by uncontrolled cell growth, drugs targeting molecules responsible for cell division or survival are widely developed [31]. Anti-mitotic chemotherapy agents which interfere mitotic progression are among the first-line

chemotherapy options being extensively used for a wide spectrum of cancer types [32, 33]. However, detrimental effects on non-tumor cells, drug resistance and mitotic slippage together with failure in triggering apoptosis are the main obstacles decreasing benefit of the treatment [34]. Therefore, alternative strategies to identify new mitosis-based cancer specific therapies which spare normal cells are in great need to improve patient survival.

Anti-microtubule agents

Microtubules are dynamic filamentous cytoskeletal proteins which are composed of tubulin polymers. They provide structure and shape to the cells, and they are important therapeutic targets in cancer cells [35]. Microtubule-targeting agents (MTAs) like taxanes (docetaxel, paclitaxel etc.) act as microtubule stabilizing agents while vinca alkaloids (vindesine, vinblastine etc.) act as microtubule destabilizing agents through different mechanism of actions. They lead to perturbation of microtubule assembly causing spindle assembly check point (SAC) activation; hence, mitotic arrest induction leading to apoptosis [36, 37]. They are mainly used as front-line therapies for different metastatic cancers, such as breast, ovarian and prostate [36]. For example, Taxol (paclitaxel) has long been used as a blockbuster chemotherapy agent which binds to β -tubulin with high affinity and induces conformational change in the tubulin structure, causing an increase and stabilization of its interaction with neighboring tubulin molecules [38]. Even though, it is efficiently used, most patients ultimately develop secondary resistance after long-term therapy. Meanwhile, the effects of SAC induction due to MTA treatment may not be persistent since cancer cells may exit mitosis through mitotic slippage where they start a new cell cycle by slipping into a tetraploid G1 state. This, later, causes inhibition of MTAs and limit their usage in cancer therapy [39]. Although MTAs target specifically dividing

malignant cells characterized by intense turnover, they may also target dividing normal cells and interphase cells since microtubules are prevalent throughout the cell cycle. Therefore, many patients suffer from toxic side effects such as neutropenia and neurotoxicity, which impairs the effectiveness of these agents and limits their therapeutic use [40, 41].

Mitosis-specific therapies (mitotic blockers)

To overcome unspecific targeting and toxicity problems and to improve chemotherapy response, anti-mitotic therapies targeting mitosis-specific kinases and microtubule-motor proteins were developed as second-generation compounds, often referred as “mitotic blockers” [41]. They are mainly aimed to interfere with the processes like chromosome segregation, proper spindle formation and/or mitotic exit [39] (**Figure 1.3**). Importantly, as phosphorylation is a critical step in cell cycle regulation and spindle assembly, kinases having roles in these processes have been extensively studied as potential targets. Among these, specific inhibitors against cyclin-dependent kinases (CDKs) [42], Aurora kinases [43] and Polo-like kinases (PLKs) [44] have been developed and clinically tested.

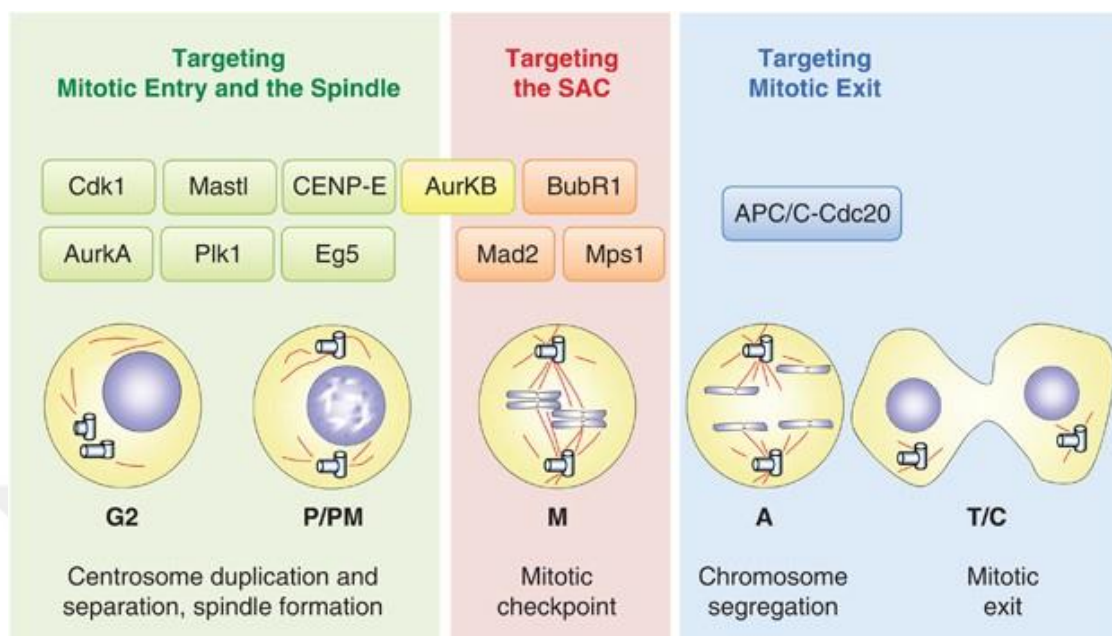


Figure 1.3. Targeting mitosis as an anti-cancer strategy. Mitosis can be interfered with targeting mitotic entry and spindles, SAC or mitotic exit. For a proper chromosome segregation in a normal cell, centrosome duplication and separation together with bipolar spindle formation are the required processes, where different major protein kinases and motor proteins actively enroll. P: prophase; M: metaphase; A: anaphase; T: telophase; C: cytokinesis [45]. *See copyright permission in Appendix Section.*

CDKs are important cell cycle enzymes that drives and controls the cell cycle transitions to ensure successful division [46] while PLKs [47] and Aurora kinase family (A, B and C) [43] regulates centrosome maturation and function, and spindle assembly during mitosis [41]. Based on their functions in mitosis, it was assumed that the efficient way to fight with cancer and increase survival is through inhibition of these kinases. To date, many inhibitors targeting Aurora kinases (pan-AURK inhibitors or specific inhibitors) have been reported and tested in clinical studies. Although, pan-AURK

inhibitors initially stabilized the disease in some solid tumor types, later they showed limited response rates [41]. For example, Alisertib, selective Aurora A inhibitor, is now in phase III clinical trial (NCT01482962) but clinical response rates in a phase II study (NCT0104542) was recorded as 18% in breast cancer, 21% in small-cell lung cancer (SCLC) and around 4-9% in head-and-neck squamous cell carcinoma, gastro-esophageal adenocarcinoma and non-small-cell lung cancer (NSCLC) [48]. Moreover, response rates were also observed low in hematologic malignancies. Similarly, for PLK1 inhibitors such as BI2536 reached to stage II/III in several cancer types which were refractory to conventional treatments (i.e. NCT00526149, NCT00710710); however, due to low maximum tolerated doses, more potent compounds were needed [49, 50]. Although, phase III clinical trial of Volasertib (BI627), a small molecule PLK1 inhibitor, with cytarabine in AML patients (NCT01721876), are still ongoing, there are several other clinical trials with volasertib which were terminated due to failure to improve patient survival or severe side effects, including hematological adverse reactions [51].

Eg5 is a plus end-directed motor protein belonging to kinesin family (KIF) microtubule motor proteins, another group of mitosis-specific targets [52]. Ispinesib, a most studied Eg5 inhibitor has reached to phase II clinical trials with patients having metastatic hepatocellular carcinoma, squamous cell carcinoma of head and neck, melanoma, ovarian cancer or renal cell carcinoma. However, none of these studies showed a conclusive evidence of benefit [53-55].

Compared to anti-microtubule agents, none of these anti-mitotic drugs demonstrated a spectacular clinical outcome [40, 56]. Narrow therapeutic window due to dose-limiting toxicities, insufficient specificity and sensitivity between cancer cells and non-malignant

cells, and poor compound uptake are the possible reasons why these drugs had not been licensed to clinics so far. [51, 57]. Thus, alternative target molecules that selectively and effectively target dividing cancer cells remain to be elucidated and developed.

TACC3 as a non-kinase microtubule binding protein

Transforming acidic coiled-coil containing protein 3 (TACC3) is an important TACC family member which is characterized by a highly conserved C-terminal coiled-coil domain (TACC domain) [58] (**Figure 1.4**). TACC domain is required for TACC proteins to interact with spindles and centrosome apparatus [59]. Vertebrates express 3 different TACCs (TACC1, TACC2, and TACC3) which play critical roles in gene regulation, cell growth and differentiation, mRNA processing, transcription and so on by interacting with different molecules involved in microtubule/centrosome dynamics [58, 60].

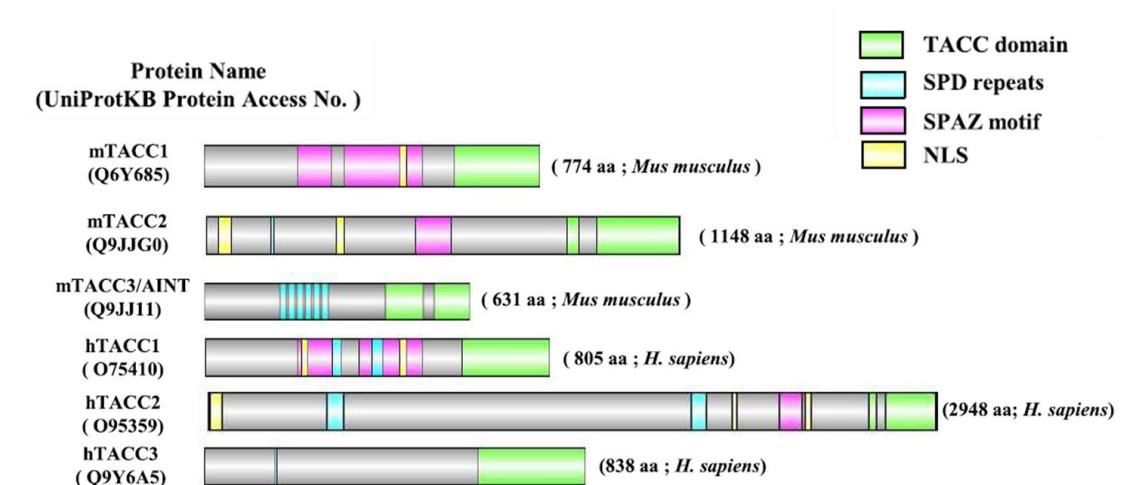


Figure 1.4. TACC family domains in mouse and human. TACC domain is highly conserved domain and shared in all TACC family members, which is approximately 200 amino acids. Modified from [60]. See copyright permission in Appendix Section.

Roles of TACC3 in cell cycle

TACC3, as a non-kinase microtubule binding protein, plays a key role in centrosome regulation, and ensures microtubule stability and efficient microtubule elongation during mitosis [58, 61]. TACC3 deficiency causes destabilization of microtubules, defects in chromosome alignment and reduction in microtubule content by impairing centrosome integrity [62, 63]. Accordingly, its shRNA-mediated silencing leads to cell proliferation inhibition [64]. On the other hand, ectopic TACC3 expression increases number and length of microtubules [65], supporting the function of TACC3 on microtubule stability.

In early mitosis, TACC3 is activated upon phosphorylation by Aurora A kinase at Ser588 residue and recruited to mitotic spindle apparatus [66]. TACC3 localization to spindles and recruitment of colonic, hepatic tumor overexpressed gene (ch-TOG) promotes microtubule assembly [67-69]. In addition, an endocytic and vesicle-trafficking protein, clathrin heavy chain (CHC) forms a ternary complex with TACC3 and ch-TOG (TACC3/ch-TOG/clathrin complex) and acts as an inter-microtubule bridge stabilizing kinetochore fibers of mitotic spindles and prevents mitotic catastrophe [67, 70, 71] (**Figure 1.5**). Binding of CHC to TACC3 is inhibited by importin β while GTP-bound Ran (RanGTP) can reverse the inhibition [70].

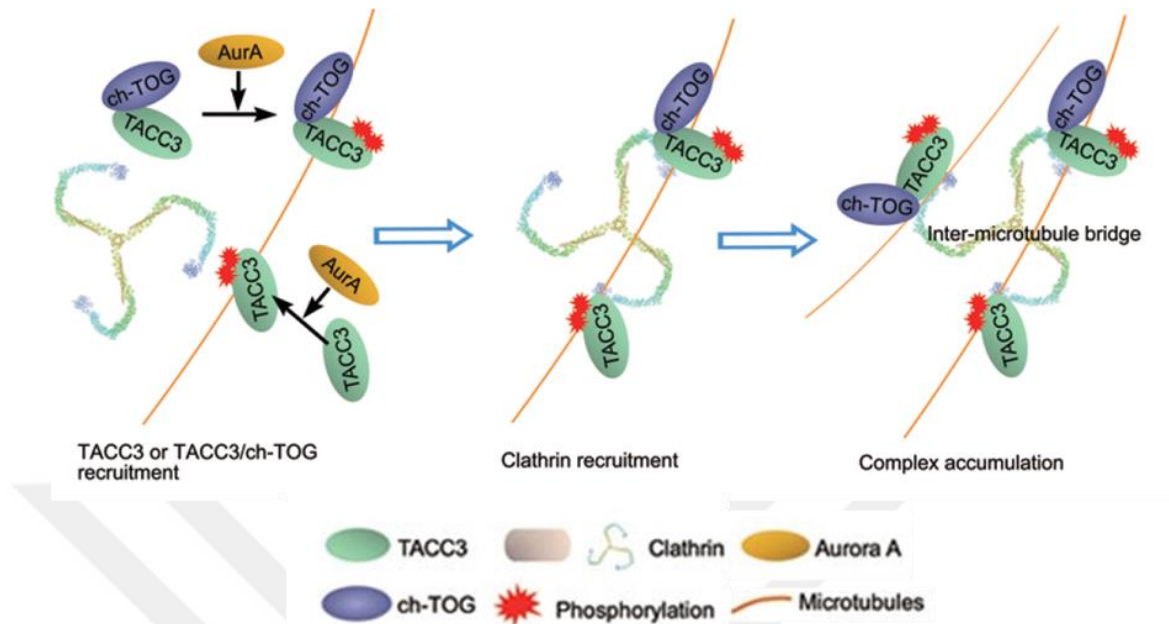


Figure 1.5 Schematic model summarizing the role of TACC3 in the regulation of microtubule dynamics at the mitotic spindle apparatus. Upon phosphorylation at Ser588 by Aurora A, TACC3 forms a complex with ch-TOG and localizes at the centrosome which then interacts with clathrin triskelion to mediate microtubule stability and growth kinetics. Modified from [72]. See copyright permission in Appendix Section.

Phosphorylated TACC3 is localized on mitotic spindle poles in pre-anaphase while it is localized on the poles and the central spindles during mitotic exit [73]. TACC3 stability is regulated with Cdh1, a well-known activator of anaphase-promoting complex/cyclosome during mitotic exit, where Cdh1 depletion prolonged TACC3 protein level [74]. Furthermore, another study described a link between TACC3 and regulation of mitotic exit through tuberous sclerosis (TSC2) and TACC3 interaction for the control of cytokinesis and maintenance of nuclear envelope structure, further supporting its important role in cell division [75].

In addition to mitotic functions, TACC3 has been shown to regulate several interphase related processes, such as gene expression and cell migration. TACC3 forms a complex with different histone acetyltransferases, including hGCN5L2 and pCAF showing its regulatory function in transcription [76]. Noticeably, TACC3 interacts with MBD2 (mCpG-binding domain 2) in the interphase nucleus where it facilitates the association of MBD2 with histone acetyltransferases to reactivate methylated promoters [77]. TACC3 has been also shown to promote G1/S transition by activating E2F1 transcription through reducing acetylation levels of histone H3 on E2F1 promoter, indicating that it can induce epigenetic changes by modulating chromatin remodeling complexes [78]. TACC3 depleted cells showed reduced cell migration speed *via* a change in cell adhesion [79]. This is supported by interaction of TACC3 with two modular proteins, ENAH and VASP, which are involved in actin polymerization and cell motility [69]. Moreover, it regulates transcription by a direct interaction with Aryl hydrocarbon receptor nuclear translocator (ARNT) in response to hypoxia [80].

TACC3 deregulation in cancer

TACC3 have been implicated in different human cancers including prostate cancer [81], non-small cell lung cancer [82], glioblastoma [83], ovarian cancer [84], colorectal cancer [85] and breast cancer [86] where their elevated levels correlated with poor prognosis in cancer patients. It has been reported that knockdown of TACC3 with specific a shRNA suppresses tumorigenesis and cell growth in renal cell carcinoma (RCC) [87] and induces G2/M arrest in cholangiocarcinoma [88]. TACC3 depletion using shRNAs also reduces mitogenic signaling in non-small cell lung cancer cell lines [89]. Moreover, perturbations

in TACC3 functions selectively inhibits the nucleation of centrosome microtubules in ovarian cancer cells [84]. Disruption of TACC3 function also causes a range of different phenotypes, including multi-polar spindle formation leading to mitotic arrest [90], chromosome misalignment resulting in caspase dependent apoptosis [63] and, in some cases, senescence [64]. Notably, TACC3 disruption in the thymic lymphomas by using TACC3 conditional knockout mice results in apoptosis which is accompanied by significant tumor regression [90]. Overall, these findings on the aberrant expression of TACC3 in many different cancer types and its critical roles in cell cycle suggests that it might be an important therapeutic target for cancer targeted therapy.

TACC3 was identified and mapped close to the fibroblast growth factor receptor 3 (FGFR3) at chromosomal region 4p16 [91]. In the recent years, an intrachromosomal FGFR3-TACC3 gene fusions were discovered mainly in a subset of Glioblastoma multiforme (GBM) and bladder urothelial tumors which are formed by tandem duplications on chromosome 4 [92-94] (**Figure 1.6**). Later, the presence of fusion gene is also detected in many other cancers [95] including cervical cancer [96] and breast cancer [97]. Fusion genes are chromosomal aberrations which are generally found in cancers and can be used as drug targets and prognostic markers. FGFR3-TACC3 fusion has a constitutively active tyrosine kinase domain, causing an induction in ERK and Akt signaling pathways [98] while TACC3 downregulation inactivates PI3K/Akt signaling in RCC and HCC [87, 99]. Therefore, FGFR3-TACC3 may be useful as a diagnostic marker and therapeutic target in different cancers. Even though there are different FGFR inhibitors in clinical development and some are recently approved by FDA (i.e. Erdafitinib [100], Pemigatinib [101]) for a subset of patients with advanced tumors, there

are still limited treatment options for patients with aggressive tumors with FGFR3-TACC3 fusion. Combined approach targeting both FGFR3 and TACC3 in those fusion protein harboring cancers may further enhance the efficacy of the treatment.

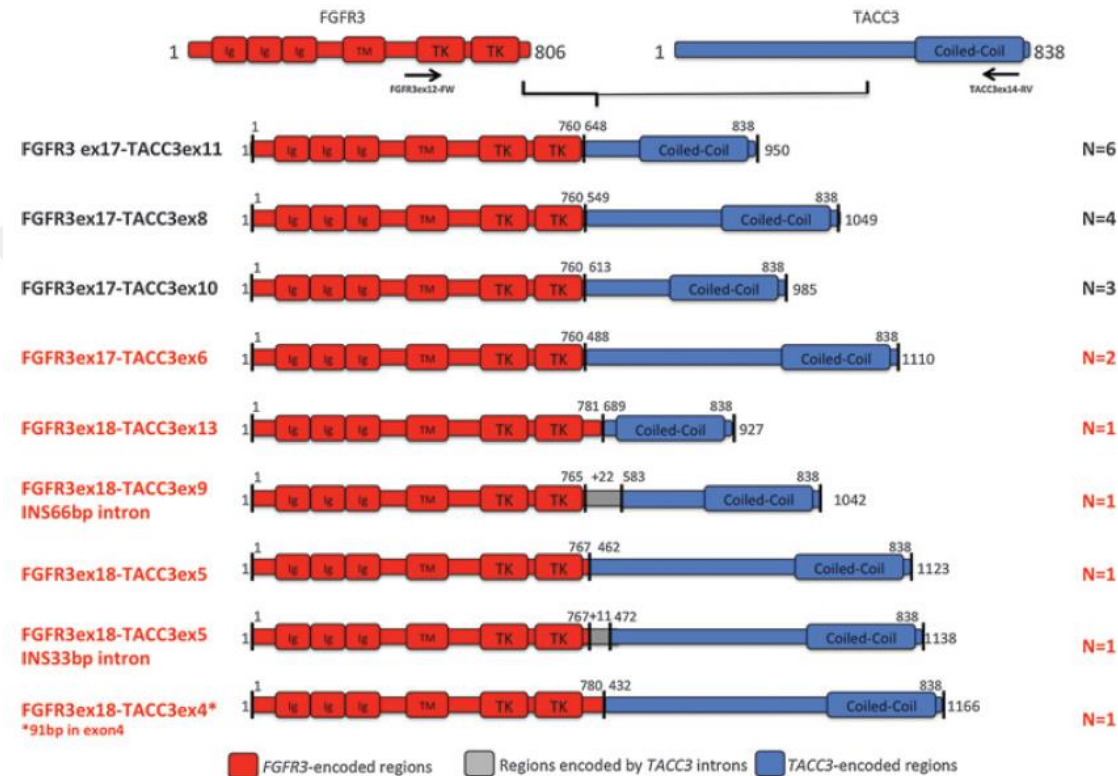


Figure 1.6. FGFR3-TACC3 gene fusions. Tyrosine kinase domain of FGFR3 and TACC domain of fuse together through tandem duplication and insertion to form FGFR3-TACC3 fusion gene at different exonic or intronic regions. Fusions were identified with RT-PCR Sanger sequencing. N indicates the number of patients harboring the corresponding fusion variant. Modified from [102]. See copyright permission in Appendix Section.

Targeting TACC3 with small molecule inhibitors

As mentioned before, phosphorylation of TACC3 by Aurora A kinase, one of the master regulators of mitotic progression, is required to recruit ternary TACC3/ch-TOG/clathrin

complex on spindles [71, 103]. Therefore, activity of Aurora A kinase is very important and any problems during this process might interfere with proper mitotic progression. Aberrant Aurora A kinase activity has been shown in many different cancers and associated with oncogenic transformation through chromosomal instability and tumor cell heterogeneity [104-107]. However, due to its pleiotropic functions, direct inhibition of its cell-essential kinase activity might result in various serious toxicities in proliferating normal cells [57, 108]. Therefore, based on relatively higher expression of TACC3 in cancer cells and strong dependency on TACC3 for cellular survival, targeting TACC3 as a critical downstream effector of Aurora A kinase might be a better option to reduce the off-target toxicities on normal tissues. Furthermore, TACC3 might have Aurora A kinase-independent key functions in cancer cells.

KHS101, a small molecule TACC3 inhibitor was first identified to promote neuronal differentiation in rats [109]. Later, it has been shown to reduce tumor growth of glioblastoma xenografts [110]. KHS101 is a useful chemical probe to study TACC3 functions; however, it cannot be translated into clinics due to its low systemic stability and high working doses [109]. Another TACC3 inhibitor, Spindlactone B (SPL-B), was demonstrated to inhibit the centrosome microtubule nucleation in ovarian cancer cells and suppress tumor growth in ovarian cancer xenografts [84]. However, like KHS101, SPL-B remains to be chemical probe to study TACC3 functions *in vitro* and needs further optimization for future drug development. Therefore, detailed understanding of TACC3 functions in cancer/normal cells and the limitations of the available TACC3 inhibitors may help develop better anti-cancer drugs which subsequently inhibit tumor growth but retain non-dividing or normal cells.

The rationale and the aims of the dissertation

Anti-microtubule agents that target molecules responsible for cell division or survival are widely used during cancer treatment [31]. However, unexpected side effects and systemic drug toxicity on normal cells become the main obstacles preventing any possible benefits of treatments [34]. Therefore, identifying new targets as cancer specific alternative therapies sparing normal cells are in great need to improve patient survival. This dissertation starts from the view to elucidate the importance of TACC3, which is an essential gene with different mitosis- and interphase-related functions, in cancer development and progression using online patient datasets and continues with identification of a novel TACC3 inhibitor hit molecule which is more potent and efficacious than commercially available inhibitors by utilizing *in vitro* and *in vivo* models.

In this dissertation, the aims are as following:

- To elucidate of the roles of TACC3 in tumor progression
- To identify and characterize a novel TACC3 inhibitor hit molecule which is more potent and efficacious in *in vitro* and *in vivo* systems and can be developed further to obtain better drug-like properties.

2. MATERIALS

2.1. Buffers

1X Anode Buffer I	300 mM Tris, 20% (v/v) methanol
1X Anode Buffer II	25 mM Tris, 20% (v/v) methanol
1X Cathode Buffer	40 mM 6-aminocaproic acid, 20% (v/v) methanol
1X PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 2 mM KH ₂ PO ₄ (pH: 7.4)
1X SDS-PAGE Running Buffer	25 mM Tris, 14.41 g/l glycine, 1% (v/v) SDS
1X TAE	40 mM Tris, 20 mM acetic acid, 1 mM EDTA
1X TBST	20 mM Tris, 137 mM NaCl, 0.2% (v/v) Tween20
DARTS Lysis Buffer	50 µl 20X protease inhibitor cocktail, 50 µl 1M NAF, 100 µl 100 mM β-glycerophosphate, 100 µl 50 mM Na ₄ P ₂ O ₇ , and 10 µl 200 mM Na ₃ VO ₄ , with 690 µl M-PER reagent for 1 ml buffer
RIPA Lysis Buffer	150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) Sodium DOC, 50 mM Tris-HCl (pH:8.0), 50 mM NAF, 1 mM Na ₃ VO ₄ , 4% (v/v) Protease inhibitor, 4% (v/v) Phosphatase inhibitor

2.2. Chemicals and reagents

4X Protein Loading Dye	250 mM Tris HCl (pH: 6.8), 10% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 50% Glycerol (v/v), 25% (v/v) β-mercaptoethanol
6-aminocaproic acid	Sigma Aldrich, St Louis, MO, USA
6X DNA loading dye	New England Biolabs, Ipswich, MA, USA
Acetic acid	Sigma Aldrich, St Louis, MO, USA
Acrylamide/bisacrylamide	Applchem, Darmstadt, Germany
Agar	Sigma Aldrich, St Louis, MO, USA
Agarose	Promega, Madison, WI, USA

Ammonium peroxisulfate	Carlo Erba, Cornaredu, Italy
Ampicillin	Sigma Aldrich, St Louis, MO, USA
Blasticidin	Thermo Fisher Scientific, Waltham, MA, USA
Bovine Serum Albumin	Santa Cruz, Dallas, TX, USA
Chloroform	Sigma Aldrich, St Louis, MO, USA
Crystal Violet	Merck, Darmstadt, Germany
D-Luciferin Potassium salt	Perkin Elmer, MA, USA
DAPI	Sigma Aldrich, St Louis, MO, USA
dNTPs	Thermo Fisher Scientific, Waltham, MA, USA
Doxycycline	Thermo Fisher Scientific, Waltham, MA, USA
Enhanced chemiluminescence (ECL)	Amersham Pharmacia Biotech, Amersham, UK Thermo Fisher Scientific, Waltham, MA, USA
Ethanol	Sigma Aldrich, St Louis, MO, USA
Ethidium Bromide	Thermo Fisher Scientific, Waltham, MA, USA
Formalin	Merck, Darmstadt, Germany
Gene Ruler 100bp DNA Ladder	New England Biolabs, Ipswich, MA, USA
Gene Ruler 1kb DNA Ladder	New England Biolabs, Ipswich, MA, USA
Glycine	Santa Cruz, Dallas, TX, USA
Isoflurane	Piramal Critical Care, Bethlehem, PA, USA
Isopropanol	Sigma Aldrich, St Louis, MO, USA
Kanamycin	Sigma Aldrich, St Louis, MO, USA
KHS101	Sigma Aldrich, St Louis, MO, USA
LightCycler 480 SYBR Green I Master	Roche Applied Science, Mannheim, Germany
Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
Matrigel	BD, Franklin Lakes, NJ, USA
Methanol	Sigma Aldrich, St Louis, MO, USA
Milk powder	Sigma Aldrich, St Louis, MO, USA
Nuclease free water	Applied Biosystems/Ambion, Austin, TX, USA
Page Ruler Protein Ladder	Thermo Fisher Scientific, Waltham, MA, USA
Paraformaldehyde	Sigma Aldrich, St Louis, MO, USA

PhosSTOP	Roche Applied Science, Mannheim, Germany
Polybrene	Santa Cruz, Dallas, TX, USA
Ponceu S	Sigma Aldrich, St Louis, MO, USA
Prolong Gold Antifade Reagent	Thermo Fisher Scientific, Waltham, MA, USA
Pronase	Sigma Aldrich, St Louis, MO, USA
Protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
Puromycin	Thermo Fisher Scientific, Waltham, MA, USA
Sodium Chloride	Merck, Darmstadt, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstadt, Germany
Spindlactone B (SPL-B)	Axon MedChem, Reston, VA, USA
Sulforhadamine B (SRB)	Sigma Aldrich, St Louis, MO, USA
TEMED	Serva, Heidelberg, Germany
Trichloroacetic acid (TCA)	Sigma Aldrich, St Louis, MO, USA
TRIsure	Bioline, Luckenwalde, Germany
Triton X-100	Sigma Aldrich, St Louis, MO, USA
Trizma base	Sigma Aldrich, St Louis, MO, USA
Trysin-EDTA 10X	Sigma Aldrich, St Louis, MO, USA
Trypton	Sigma Aldrich, St Louis, MO, USA
Tween-20	VWR, Radnor, PA, USA
Yeast Extract	Sigma Aldrich, St Louis, MO, USA

2.3. Media and supplements

Dulbecco's modified Eagle's medium (DMEM, with phenol red)	Lonza, Basel, Switzerland
DMEM (without phenol red)	Thermo Fisher Scientific, Waltham, MA, USA
Epidermal Growth Factor (EGF)	Sigma Aldrich, St Louis, MO, USA
Fetal bovine serum (FBS)	Biowest, Nuaille, France
Hydrocortisone	Sigma Aldrich, St Louis, MO, USA
Insulin	Sigma Aldrich, St Louis, MO, USA

L-glutamine	Sigma Aldrich, St Louis, MO, USA
Lysogeny Broth (LB) Agar	1.5% (w/v) agar, 10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl
LB Broth	10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl
Mccoy's 5A (Modified) Medium	Thermo Fisher Scientific, Waltham, MA, USA
Non-essential aminoacids (NEAA)	Lonza, Basel, Switzerland
optiMEM	Thermo Fisher Scientific, Waltham, MA, USA
Penicillin/streptomycin (P/S)	Lonza, Basel, Switzerland
RPMI-1640	Biowest, Nuaille, France

2.4. Kits

Apop Tag Peroxidase In Situ Apoptosis Detection kit	Milipore, Burlington, MA, USA
BCA Protein Assay kit	Pierce, Rockford, IL, USA
Cell Titer-Glo Luminescent Cell Viability Assay kit	Promega, Madison, WI, USA
First strand cDNA synthesis kit	Fermantas, St. Leon-Roth, Germany
FITC Annexin V Apoptosis Detection Kit with PI	Biolegend, San Diego, CA, USA
Trans-lentiviral Packaging kit	Dharmacon, Lafayette, Colorado, United States
MycoAlert detection kit	Lonza, Basel, Switzerland
Optiform® Select kit (formulation #2)	Catalent, San Diego, CA, USA
Plasmid isolation kit	MN, Duren, Germany
Plasmid Maxi kit	Qiagen, Hilden, Germany

2.5. Equipment

Accuri FACS	BD Biosciences, San Diego, CA, USA
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Amersham Imager 600	GE Healthcare, Chicago, Illinois, USA
Axiovision 4.3 microscopy	Carl Zeiss, Munich, Germany
LSM 700 Confocal Microscope	Carl Zeiss, Munich, Germany
Centrifuges	Thermo Fisher Scientific, Waltham, MA, USA
	Beckman, Pasadena, CA, USA
Cell culture hood	Nüve, Ankara, Turkey
Cell culture incubator	Nüve, Ankara, Turkey
Counting chamber	Marienfeld, Königshofen, Germany
Freezer (-80oC)	Hettich, Geldermansen, Holland
Freezer (-20oC) and Fridge (4oC)	Bosch, Stuttgart, Germany
Horizontal Shakers	Bellco, Vineland, NJ, USA
LightCycler 96	Roche Applied Science, Mannheim, Germany
Lumina III In Vivo Imaging System	Perkin Elmer, MA, USA
MicroCal™ iTC200	GE Healthcare, Little Chalfont, UK
Mini-PROTEAN Gel casting module	Biorad, Hercules, CA, USA
Mini-PROTEAN Tetra Cell	Biorad, Hercules, CA, USA
Multichannel Pipette	Thermo Fisher Scientific, Waltham, MA, USA
Nanodrop 1000	Thermo Fisher Scientific, Waltham, MA, USA
Nikon TS300 Inverted microscope	Nikon, Tokyo, Japan
Thermocycler	Thermo Fisher Scientific, Waltham, MA, USA
Power supplies for electrophoresis	Biorad, Hercules, CA, USA
Semidry Western Blot transfer unit	Biorad, Hercules, CA, USA
Synergy HT microplate reader	Biotech, Winooski, VT, USA
UV-Reader	Vilber Lourmat, Eberhardzell, Germany
Vortex	Isolab, Wertheim, Germany
Water bath	Nüve, Ankara, Turkey
X-ray cassette	Amersham Pharmacia Biotech, Amersham, UK
X-ray hyper processor	Amersham Pharmacia Biotech, Amersham, UK

2.6. Consumables

100 mm dishes	Greiner bio-one, Frickenhausen, Germany
145 mm dishes	Greiner bio-one, Frickenhausen, Germany
96-well plates	Greiner bio-one, Frickenhausen, Germany
6-well plates	Greiner bio-one, Frickenhausen, Germany
Cell scrapers	Greiner bio-one, Frickenhausen, Germany
Coverslips	Marienfeld, Königshofen, Germany
Cryovials	Greiner bio-one, Frickenhausen, Germany
Cuvettes	VWR, Radnor, PA, USA
Filtered pipette tips (10 ul, 20 ul, 200 ul, 1000 ul)	Greiner bio-one, Frickenhausen, Germany
LightCycler® 480 Multiwell Plate 96, white	Roche Applied Science, Mannheim, Germany
Microscope slides	Marienfeld, Königshofen, Germany
Parafilm	VWR, Radnor, PA, USA
PCR tubes	Axygen, Corning, NY, USA
Plastic pipettes (5 ml, 10 ml, 25 ml)	Corning Incorporated, Corning, NY, USA
PVDF Membrane	Biorad, Hercules, CA, USA
Reaction tubes (500 ul, 1.5 ml, 2 ml)	Axygen, Corning, NY, USA
Storage bottles (250 ml, 500 ml, 1 L)	Corning Incorporated, Corning, NY, USA
X-ray films	Kodak, Rochester, NY, USA
Whatmann paper	GE Healthcare, Little Chalfont, UK
White plates	Costar, Corning, NY, USA

3. METHODS

3.1. *In vitro* experiments

3.1.1. Culturing normal and cancer cell lines

Human breast cancer cell lines MDA-MB-231, MDA-MB-436, MDA-MB-157, BT-474, MCF-7, ZR-75-1 and T-47D, mouse breast cancer cell lines 4T1 and EMT6, human bladder cancer cell lines RT112 and RT4, mouse colorectal carcinoma cell line CT-26 and normal human breast epithelial cell line MCF-12A were purchased from ATCC (Manassas, VA, USA). JIMT-1, HCC1954, CAL51 and HCC1143 were kindly provided by Ali Osmay Güre from Bilkent University, Ankara, Turkey. Human colon carcinoma cell line, HCT-116, was a gift from Serkan Göktuna and mouse embryonic fibroblast cell line, MEF, was provided by Onur Çizmecioglu both from Bilkent University, Ankara, Turkey. Cells were cultured in DMEM, supplemented with 10% FBS, 1% non-essential amino acid, 2 mM L-glutamine and 50 U/ml penicillin/streptomycin. Additionally, BT-474 cells were grown in 0.1% insulin containing medium. MCF-12A cells were also supplemented with 20 ng/ml epidermal growth factor and 500 ng/ml hydrocortisone. T-47D and MCF-7 cells were cultured in phenol red-free DMEM with 10% FBS, 1% nonessential amino acids, 50 U/mL penicillin/streptomycin and 0.1% insulin. EMT6 and RT112 cells were maintained in RPMI-1640 while RT4 and HCT-116 cells were cultured in McCoy's 5A (modified) medium supplemented with L-glutamine, FBS and P/S. The cell lines were authenticated and tested for mycoplasma contamination regularly using MycoAlert mycoplasma detection kit. The cumulative length of the cells between thawing and use in the experiments was less than 20 passages.

3.1.2. Cell viability assay and inhibitor treatments

KHS101 and SPL-B were dissolved in 100% DMSO to yield a stock concentration of 50 mM. BO-264 was dissolved in 100% DMSO to yield a stock concentration of 10 mM.

For cell viability assays including IC₅₀ value calculation experiments, MCF-12A (5x10³ cells/well), MCF-7 (7x10³), T-47D (6x10³), ZR-75-1 (6x10³), BT-474 (5x10³), JIMT-1 (3x10³), HCC1954 (5x10³), MDA-MB-231 (5x10³), HCC1143 (5x10³), MDA-MB-436 (4x10³), CAL51 (6x10³), MDA-MB-157 (3x10³), RT112 (6x10³) and RT4 (6x10³) cells were seeded into 96-well plates. 24 hours after cell seeding inhibitor treatments were performed at different concentrations. Cell viability was measured 72 hours after drug treatment with Sulforhodamine B (SRB) assay. Briefly, cells were fixed with %25 trichloroacetic acid (TCA) for at least 1 hour at 4°C. After 5X washing with ddH₂O, fixed cells were stained with %0.4 SRB for 30 minutes at room temperature (RT). Excessive and unbound dye was removed by washing the plate with %1 acetic acid and left for drying. To dissolve the dye bound to proteins of living cells. Wells were incubated with 10 mM Tris-base solution for 10 min and the absorbance were measured at 554 nM. The analysis was done by calculating the percentage cell viability as the ratio of the signal in treated wells to the signal in vehicle treated wells multiplied by 100.

For western blotting, different concentrations of KHS101, SPL-B or BO-264 were given to JIMT-1 cells (1.5x10⁵ cells/well) or RT112 (2x10⁵) for 24 hours. HCT-116 (2x10⁵) and CT-26 (2x10⁵) cells were also treated with different doses of BO-264 for 24 hours. Annexin V/PI staining was performed according to manufacturer's instructions

using JIMT-1 cells treated with 500 nM BO-264 for 48 hours. For shRNA western blotting experiment, both control and TACC3 shRNA JIMT-1 cells were seeded (1.5×10^5) and induced with 2 $\mu\text{g/ml}$ doxycycline for 48 hours. Cells were then treated with BO-264 at different doses for 24 hours. For siRNA immunofluorescence experiment, JIMT-1 cells transfected with siRNAs targeting TACC3 were treated with BO-264 for 12 hours. To analyze the effect of TACC3 pharmacological inhibition on TACC3 levels in centrosomes during mitosis and interphase, JIMT-1 cells were treated with 300 nM BO-264 for 24 hours.

3.1.3. Transient transfection with siRNAs and overexpression (OE) vectors

For siRNA transfected cell viability experiments, JIMT-1 (3×10^3 cells/well), MDA-MB-436 (4×10^3) and MDA-MB-157 (3×10^3), cells were seeded into 96-well plates in 80 μl P/S-free growth medium. 24 hours after seeding, cells were transfected with two different siRNAs targeting TACC3 at a final concentration of 20 nM using Lipofectamine 2000™ transfection reagent in a volume of 40 μl (**Table 3.1**). 72 hours following transfection, cell viability was measured with SRB assay. To assess the knockdown level with siRNAs, JIMT-1 (1.5×10^5 cells/well), MDA-MB-436 (1.5×10^5) and MDA-MB-157 (1.5×10^5) cells were transfected with both siTACC3s for 48 hours. Knockdown efficiency at TACC3 mRNA and protein level was analyzed by quantitative real-time PCR (qRT-PCR) and western blotting, respectively. For immunofluorescence experiments, JIMT-1 cells (3×10^5 cells/well) were seeded into 6-well plates, and next day transfected with two different siRNAs targeting TACC3 at a final concentration of 40 nM.

Table 3.1. List of siRNAs and OE vectors

siRNA/OE vectors	Catalog number	Company
siAllstar	1027280	Qiagen, Hilden, Germany
siTACC3#1	D-004155-03	Dharmacon, CO, USA
siTACC3#2	D-004155-02	Dharmacon, CO, USA
TACC3 cDNA ORF clone (NM_006342.3)	OHu21751	Genscript, Piscataway, NJ, USA

For transient TACC3 overexpression, MCF-12A cells were transfected with 250 ng of empty or TACC3 vector for 48 hours. Overexpression efficiency at TACC3 protein level was assessed by western blotting.

For both siRNA and OE experiments, siRNA or OE vectors were diluted in either 20 μ l (for 96-well plate) or 250 μ l (for 6-well plate) optiMEM (mix A) depending on the well size and vortexed for 10 seconds. In the meantime, 0.2 or 2 μ l lipofectamine was diluted in 20 or 250 μ l optiMEM (mix B) by vortexing for 20 seconds. After 5 minutes incubation of both mixtures, they were mixed in 1:1 (v/v) ratio and further incubated for 20 minutes. For 6-well plates, cells were washed with PBS and then media was replaced with 1 ml fresh P/S free media and 500 μ l of transfection mixture was added on top of the cells. Cells were incubated at 37°C, 5% CO₂ for further experiments.

3.1.4. Colony formation assay

For monolayer culture, single-cell suspensions of JIMT-1 cells (3×10^3 cells/well) were plated in a 12-well plate in 500 μ l media. After 6 hours incubation, cells were treated with

different doses of BO-264, SPL-B and KHS101. In order to test the colony formation capacity of MCF-12A cells during TACC3 overexpression, MCF-12A cells (2×10^5 cells/well) were seeded into 6-well plates and TACC3 transfection was performed next day. 48 hours following transfection, cells were counted and 1×10^3 cells/well were plated into 12-well plates. For both experimental setups, the media were refreshed every 4 days and cells were incubated for 12 days. Cells were then fixed with 2% paraformaldehyde for 15 minutes and stained with 1% crystal violet for 15 minutes at RT. Surviving colonies (composed of at least 50 cells) were counted with ImageJ software (NIH).

3.1.5. Generation of stable cell lines with lentiviral vector-based transfections

pTRIPZ doxycycline-inducible lentiviral shRNA vectors were obtained from Dharmacon (**Table 3.2**). Packaging and infection of the lentiviral vector was carried out using trans-lentiviral packaging system. Briefly, HEK293FT cells, acting as host, were transfected with shRNA vectors by calcium phosphate method. The supernatant containing virus particles were cleared through a 0.45- μ m filter, collected and later used to infect JIMT-1 cells for 48 hours. shRNA expressing cells were selected with 2 μ g/ml puromycin for at least two weeks. Resistant cells were induced with doxycycline (2 μ g/ml) for 48 hours for TACC3 for both immunofluorescence and western blotting assays.

Table 3.2. List of shRNA vectors

shRNA vectors	Catalog number	Company
shControl	RHS4743	Dharmacon, CO, USA
shTACC3	RHS4696-200764244	Dharmacon, CO, USA

3.1.6. Doubling time assessment

To assess doubling time, normal breast epithelial cell line MCF-12A and breast cancer cell lines were plated (3×10^4 cells/well) in 6-well plates. Cells were collected by trypsinization and cell number was counted every 24 hours for one week. Growth curves for these cells were drawn as number of cells/cm² versus days after seeding. The doubling time was calculated using the following formula;

$$\text{Doubling time (DT)} = \frac{t \times \ln 2}{\ln\left(\frac{X_e}{X_b}\right)},$$

where t is the time spent in the logarithmic phase of cell growth, X_e and X_b are cell numbers at the end and beginning of the logarithmic phase, respectively.

3.1.7. Annexin V/PI staining

Annexin V/PI staining was performed according to manufacturer's instructions using JIMT-1 cells treated with 500 nM BO-264 for 48 hours. Briefly, cell supernatant was centrifuged at 4000 rpm for 5 minutes. Then cells were trypsinized and added on top of the centrifuged supernatant and the cell number was counted. Next, cell suspension was centrifuged at 2000 rpm for 5 minutes and washed once with PBS. 100 µl of Annexin V binding buffer and 1.5 µL of FITC-conjugated Annexin V were added and cells were incubated for 15 minutes at RT in dark followed by incubation with DAPI for an additional 5 minutes. Then cell solution was diluted in 400 µl of buffer and flow cytometry was performed with BD FACS Diva software (BD, NJ, USA) and analyzed with De Novo FCS Express software.

3.1.8. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To assess the TACC3 knockdown efficiency, total RNA isolation, cDNA synthesis and qRT-PCR analysis was performed, respectively.

3.1.8.1 Total RNA isolation

Cells were seeded, transfected as previously described in **Section 3.1.3**. After indicated times, cell pellets were collected through trypsinization. RNA isolation was performed using TRIsure reagent according to manufacturer's instructions.

3.1.8.2 cDNA synthesis

RevertAid RT Reverse Transcription kit was used to synthesize cDNAs from 2 µg of total RNA for each sample according to the manufacturer's instructions. The protocol was given at **Table 3.3** and the reaction was carried out in thermocycler using the program given in **Table 3.4**. After the reaction was completed, cDNA samples were diluted to 1:20 with nuclease-free H₂O and proceeded with qRT-PCR experiments.

Table 3.3. Reverse transcription reaction components

Reagent	Concentration / Volume
RNA	2 µg (X µL)
oligoDT	1 µL
5x Revert Aid reaction buffer	4 µL
Ribolock Ribonuclease Inhibitor	1 µL

dNTPs (10mM)	2 μ L
Revert Aid H Minus M-MuLV RT	1 μ L
Nuclease-free H ₂ O	(20-X) μ L

Table 3.4. Reverse transcription reaction conditions

Temperature	Time
37°	5 minutes
42°C	60 minutes
70°C	10 minutes
4°C	∞

3.1.8.3 qRT-PCR

SYBR Green Master mix was used for qRT-PCR together with the gene specific primers provided in **Table 3.5**. qRT-PCR reaction was carried out in 96-well white plate and a reaction master mix was prepared for each primer pair according to the **Table 3.6**.

Table 3.5. Primer sequences of genes used in qRT-PCR analysis

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
TACC3	GACAGTGGAGCAGAAGACTAAA	GTCTGTCTGTCCTGTCTGATTC
HPRT	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTTCAGTCCT
GAPDH	GCCCAATACGACCAAATCC	AGCCACATCGCTCAGACAC

Table 3.6. qRT-PCR reaction master mix

Reagent	Volume
SYBR Green	5 μ L
Forward Primer (20 μ M)	0.25 μ L
Reverse Primer (20 μ M)	0.25 μ L
Nuclease-free H ₂ O	2.5 μ L

Eight μ l of master mix were mixed with 2 μ l of cDNA (final concentration of 20 ng) in each well. After sealing the plate tightly, it was centrifuged at 1000 rpm for 1 minute at 4°C and ran on LightCycler 96 qRT-PCR Thermocycler following the program shown in **Table 3.7**. $\Delta\Delta$ CT method was used for the analysis of the data [111].

Table 3.7. qRT-PCR program

Pre-incubation							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:05:00	4.4	5	0	0	0
Amplification							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:10	4.4	5	0	0	0
58	Single	00:00:20	2.2	5	0	0	0
72	None	00:00:20	4.4	5	0	0	0
Melting Curve							

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Seconds Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:05	4.4	5	0	0	0
55	None	00:01:00	2.2	5	0	0	0
95	Continuous	00:00:00	0.11	5	0	0	0

Cooling

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Seconds Target (°C)	Step Size (°C)	Step Delay (cycles)
40	None	00:00:30	2.2	5	0	0	0

3.1.9. Protein biochemistry

3.1.9.1 Protein isolation

Cells were seeded, transfected as previously described in **Sections 3.1.2** and **3.1.3**. After indicated times, culture media was collected and centrifuged at 5000 rpm for 5 minutes at 4°C to collect the apoptotic bodies (in case of drug treatments). Then the cells were trypsinized and collected through centrifugation at 1500 rpm. To isolate the proteins, 30-100 µl RIPA lysis buffer was added to cell pellets and vortexed every 5 minutes for 5-10 seconds for a total of 30 minutes. The components of RIPA buffer were given in **Table 3.8**. Cell suspension were later centrifuged at 13000 rpm for 30 minutes at 4°C. Supernatant containing proteins was transferred into a new eppendorf and stored at -80°C for further experiments.

Table 3.8. Components of RIPA lysis buffer

Ingredients	Stock Concentration	Final Concentration	For 1 ml
NaCl	2 M	150 mM	75 µl
NP-40	10%	1%	100 µl
Sodium DOC	10%	0.5%	50 µl
SDS	10%	0.1%	10 µl
Tris HCl (pH 8.0)	2 M	50 mM	25 µl
NAF	1 M	50 mM	50 µl
NaVO ₄	100 mM	1mM	10 µl
Protease Inhibitor	25X	4%	40 µl
Phosphatase Inhibitor	10X	4%	40 µl
ddH ₂ O			600 µl

3.1.9.2 Protein quantification

Isolated proteins were quantified using BCA protein assay kit. Firstly, standard solutions ranging from 0 to 2 µg/µl BSA in PBS were prepared according to manufacturer's instructions and 24 µl of each standard were added into 96-well plate while 3 µl of protein solutions were used, where dilution factor between protein and standard solutions were 8. Then, solution A and B were mixed in 50:1 ration to prepare working solution which was pipetted into 96-well plate at an amount of 200 µl/well. Plate was incubated at 37°C for 30 minutes and absorbance was measured on SynergyHT microplate reader at 562 nm. Depending on the absorbance of BSA standards were used to draw a standard calibration curve where sample concentrations were quantified from.

3.1.9.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

After calculating required protein amount, protein samples were mixed with 4X protein loading dye (**Table 3.9**) and heated at 95°C for 5 minutes.

Table 3.9. 4X loading dye preparation protocol

Components	Stock concentration	Final concentration	For 10 ml
Glycerol	100%	40%	4 ml
Tris HCL (pH 6.8)	1 M	240 mM	2.4 ml
SDS		8%	0.8 g
Bromophenol blue		0.04%	4 mg
Beta-mercaptoethanol	100%	5%	0.5 ml
ddH ₂ O			Up to 10 ml

Then polyacrylamide gels composed of resolving and stacking gels were prepared according to **Table 3.10** using Mini-Protean Gel casting module. After pouring resolving gel solution, isopropanol was added on top of the gel to make a linear line on top, which was then removed when the resolving gel was polymerized. Then stacking gel was added on top of the resolving gel and a 10- or 15-well comb was inserted on top of the casting system. After it was polymerized, too, the comb was removed, and protein samples were loaded into each well. Electrophoresis was performed at 100-120V for 0-90 minutes.

Table 3.10. Components of resolving and stacking gels

Reagent	Resolving gel (10%)	Stacking gel (5%)
ddH ₂ O	1.9 ml	1.36 ml
30% acrylamide mix	1.7 ml	340 µl
1 M Tris	(pH=8.8) 1.3 ml	(pH=6.8) 260 µl
10% SDS	50 µl	20 µl
10% APS	50 µl	20 µl
TEMED	5 µl	2 µl
Total volume	5 ml	2 ml

3.1.9.4 Western blotting

After the gel ran to the end, semi-dry transfer was performed by using three of 3 mm thick Whatman papers (7x9 cm²) which were moistened in anode buffer-I, two in anode buffer-II and 5 in cathode buffer. For transfer, Biorad Semi-Dry Turbo Blot machine was used. PVDF membrane was first dipped in 100% methanol and then in anode buffer-I. The Whatman paper, gel and the membrane were placed into the system in the following order: Whatman papers soaked into anode buffer-I, then the ones in anode buffer-II, PVDF membrane, gel and finally the ones in cathode buffer. Transfer was performed at 25V for 30minutes. To visualize the bands before proceeding with antibody incubations, membrane was soaked into Ponceu S dye for a couple of minutes and then washed with ddH₂O until the stain was completely removed. Then the membrane was cut at specific molecular weight (kDa) of interest. Cut membranes were blocked with 5% (w/v) milk:1X TBS-T or 5% (w/v) BSA:1X TBS-T for 1 hour at room temperature on slow shaking. Then the membranes were incubated with primary antibody (**Table 3.11**) overnight at 4°C.

Table 3.11. List of primary and secondary antibodies used in Western blotting

Antibody	Company	Catalog number	Dilution
Beta-actin	MP Biomedicals	69100	1:5000
Cdk4	Abcam	68266	1:1000
Cleaved Caspase 3	Cell Signaling Technology	9664	1:1000
Cleaved PARP	Cell Signaling Technology	5625S	1:1000
GAPDH	Santa Cruz	47724	1:2000
HRP-coupled anti-mouse IgG	Cell Signaling Technology	7076	1:10000
HRP-coupled anti-rabbit IgG	Cell Signaling Technology	7074	1:10000
p-ERK (Thr202/Tyr204)	Cell Signaling Technology	4376	1:1000
p-H2AX (Ser139)	Cell Signaling Technology	9718	1:1000
p-Histone H3 (Ser10)	Cell Signaling Technology	4056	1:1000
TACC3	Santa Cruz	376883	1:1000

After primary antibody incubation was done, membranes were washed 3X with TBS-T for 10 minutes each to remove unspecific binding of antibody and then incubated with secondary antibodies (**Table 3.11**) for either 2 hours at RT or overnight at 4°C. Membranes were later washed again with TBS-T for 30 minutes and Amersham or Thermo ECL reagent were applied onto membranes to visualize the bands using Amersham imager for different exposure times.

3.1.10. Immunofluorescence

To visualize the changes in spindle formations during mitosis upon drug treatment or the TACC3 levels in centrosomes of JIMT-1 cells, immunofluorescence methods were used. JIMT-1 cells/well were seeded on glass coverslip in 6-well plates. Next day, the cells were treated with either vehicle or different doses of BO-264 for 12-24 hours as described previously (**Section 3.1.2**). For the siRNA immunofluorescence experiments, 1.5×10^5 cells/well were seeded on glass coverslip in 6-well plates and transfected with two different siTACC3s. Cells were then treated with 0.2 μ M BO-264 for 12 hours. After drug treatment, cells are fixed with ice-cold methanol for 10 min at -20°C. Cells were then blocked with 3% BSA in PBS solution for 1 hour at RT and incubated with primary and secondary antibodies for 1 hour at RT (**Table 3.12**). Cells were counter stained with DAPI for 5 min (0.01 μ g/ml). Lastly, cover slides were mounted using Prolong Gold Antifade Reagent. Images were taken with an upright fluorescent microscope equipped with DIC prisms or LSM 700 Confocal Microscope.

Table 3.12. List of primary and secondary antibodies used in Immunofluorescence

Antibody	Company	Catalog number	Dilution
Alexa Fluor® 488 anti-rabbit	Life Technologies	A11034	1:1000
Alexa Fluor® 647 anti-mouse	Life Technologies	A31573	1:500
Alpha-tubulin	Santa Cruz	32293	1:500
Gamma-tubulin	Sigma Aldrich	T3195	1:200
BuBR1	Abcam	54894	1:200
TACC3	Santa Cruz	376883	1:400

3.1.11. Drug affinity responsive target stability (DARTS)

To analyze the interaction between BO-264 and their target proteins in intact cells, DARTS assay was performed. Briefly, JIMT-1 cells were grown to 70-80% confluency and lysed using DARTS lysis buffer. Cell lysate-drug mixtures were incubated at RT on a shaker for 20 minutes to allow binding, and each sample was mixed with 2 μ l of 8 ng/ μ l pronase solution or buffer only (undigested) and incubated at RT. Protein digestion was stopped by adding 2 μ l of protease inhibitor cocktail and incubating on ice for 10 minutes. Then lysates were mixed with 8 μ l of 4X SDS loading buffer and heated at 70°C for 10 minutes. SDS-PAGE was performed with anti-TACC3 and anti-CDK4 antibody as negative control.

3.1.12. Cellular thermal shift assay (CETSA)

To validate the binding of BO-264 to TACC3, CETSA was performed. Briefly, JIMT-1 cells were incubated with vehicle or BO-264 (1 μ M) for 6 hours. After treatment, cell pellets were resuspended in TBS buffer containing protease and phosphatase inhibitors (1X). The cell suspension was divided into 6 PCR tubes and heated for 5 minutes to 45, 46, 47, 48, 49 and 50°C. Subsequently, cells were lysed by three repeated freeze-thaw cycles with liquid nitrogen. Soluble proteins were collected with centrifugation at 13000 rpm for 20 minutes at 4°C and analyzed by SDS-PAGE followed by western blot analysis.

3.1.13. Isothermal calorimetry titration (ITC)

Purified TACC3 recombinant protein (TP310754; Origene, MD, USA) and BO-264 were prepared in 25 mM Tris.HCl, pH 7.3, 100 mM glycine, 10% glycerol solution. BO-264 was loaded and titrated with TACC3 protein in duplicate experiments. Titrations were carried out using MicroCal™ iTC₂₀₀ equipment at 25°C. For each titration, 10 injections were made with minutes spacing. The reference power was set at 2 µcal/sec, and the sample cell was continuously stirred at 500 rpm. The data was analyzed using Origin 7 Software provided along with the ITC200, and binding parameters, such as association constant (K_a), number of binding sites (N) and enthalpy (ΔH), were calculated.

3.1.14. NCI-60 anticancer drug screening

BO-264 was submitted to the National Cancer Institute (NCI number S807620) for screening in the NCI-60 panel of human tumor cell lines. BO-264 was tested twice in the five-dose NCI-60 screen at doses ranging from 10 nM to 100 µM to determine three end points, such as GI50 (50% growth inhibition), TGI (total growth inhibition) and LC50 (lethal dose concentration inducing 50% cell death) values for all 60 cell lines. Briefly, 24 hours after cell seeding into 96-well plates, cells were treated with 5-logM concentration range of the compound for 2 days. Cytotoxicity was assessed using SRB assay. The data presented in the figures are the average values of both experiments.

3.2.Synthesis of BO-264

- ***Ethyl 4-methoxybenzoate (1)***

Thionyl chloride (SOCl₂) (34.4 mmol, 2.27 equivalent) was added dropwise to a solution of 4-methoxybenzoic acid (15.15 mmol, 1 equiv) in absolute ethanol (25 ml) at room temperature (RT). The reaction was stirred at 80°C for 4 hours. After the reaction was complete, the resulting mixture was concentrated under reduced pressure to give colorless liquid (86% yield). HRMS (m/z): [M+H]⁺ calculated for C₁₀H₁₃O₃: 181.0865; found:181.0858. CAS: 94-30-4

- ***3-(4-Methoxyphenyl)-3-oxopropanenitrile (2)***

Sodium hydride (NaH) (60% dispersion in mineral oil) (27.501 mmol, 3 equiv) and acetonitrile (MeCN) (27.501 mmol, 3 equiv) were added to a solution of **1** (9.167 mmol, 1 equiv) in dry toluene at RT and then refluxed under nitrogen atmosphere for 2 hours. The resulting mixture was cooled to RT. The salt obtained was filtered and washed with petroleum ether. The salt was dissolved in water and then acidified with concentrated hydrochloric acid (HCl). The resulting solid was filtered and triturated with a solution of NaHCO₃, and then filtered to give light yellow solid. (77% yield). MP: 127.5-129.4°C [71] HRMS (m/z): [M-H]⁻ calculated for C₁₀H₈NO₂: 174.0561; found: 174.0564. CAS: 3672-47-7

- ***3-(4-Methoxyphenyl)isoxazol-5-amine (3)***

Compound **2** (2.8571 mmol, 1 equiv) and hydroxylamine hydrochloride (H₂NOH.HCl) (2.8856 mmol, 1.05 equiv) were added to solution of sodium hydroxide (NaOH) (5.8570 mmol, 2.05 equiv) in water. The reaction mixture was refluxed for 4 hours. After the

resulting mixture cooled, it was diluted with water and extracted with dichloromethane (DCM). The organic layer was dried, filtered and evaporated to give the crude product which was then purified by automated-flash chromatography on silica gel (24 g) eluting with a gradient of 0–60% ethyl acetate (EtOAc) in hexane (59% yield). MP: 135.4–137.2°C. [112] HRMS (m/z): $[M+H]^+$ calculated for $C_{10}H_{11}N_2O_2$: 191.0821; found: 191.0812 CAS: 86685-98-5

- ***N*-(2-Chloropyrimidin-4-yl)-3-(4-methoxyphenyl)isoxazol-5-amine (4)**

Potassium tert-butoxide (t-BuOK) (2.4025 mmol, 2.5 equiv) was added to a solution of **3** (0.961 mmol, 1 equiv) in tert-butanol and the mixture was stirred for 1 hour at RT. 2,4-Dichloropyrimidine (1.441 mmol, 1.5 equiv) was added to the reaction mixture and it was stirred for 24 hours at RT. After the reaction was complete, the mixture was quenched by aqueous ammonium chloride (NH_4Cl) solution and then extracted with EtOAc. The organic layer was dried, filtered and evaporated to give the crude product which was purified by automated-flash chromatography on silica gel (24 g), eluting with a gradient of 0–60% EtOAc in DCM (42% yield). MP: 197–198.8 °C HRMS (m/z): $[M+H]^+$ calculated for $C_{14}H_{12}N_4O_2Cl$: 303.0649; found: 303.0645. ^1H NMR (400 MHz, DMSO): δ 3.82 (3H, s), 6.68 (1H, s), 7.01 (1H, d, $J=5.8$ Hz), 7.07 (2H, d, $J=8.6$ Hz), 7.77 (2H, d, $J=8.6$ Hz), 8.40 (1H, d, $J=5.8$ Hz), 11.73 (1H, s). ^{13}C NMR (100 MHz, DMSO): δ 55.22, 85.54, 106.48, 114.42, 121.06, 127.86, 158.60, 158.72, 159.40, 160.69, 161.51, 162.26.

- ***3*-(4-Methoxyphenyl)-*N*-(2-morpholinopyrimidin-4-yl)isoxazol-5-amine (5) (BO-264)**

Morpholine (1.0746 mmol, 3 equiv) was added to a solution of **4** (0.3582 mmol, 1 equiv) in n-butanol. The reaction mixture was refluxed under nitrogen atmosphere for 5 hours.

After the reaction was complete, the mixture cooled down to RT, and then quenched by ice water to give light yellow solid. The resulting solid was filtered, dried to give crude product which was purified by automated-flash chromatography on silica gel (24 g), eluting with a gradient of 0–60% EtOAc in DCM (70% yield). MP: 192.5-194.2°C HRMS (m/z): [M+H]⁺ calculated for C₁₈H₂₀N₅O₃: 354.156; found:354.1572. ¹H NMR (400 MHz, DMSO): δ 3.68-3.70 (8H, m), 3.80 (3H, s), 6.24 (1H, d, *J*=5.6 Hz), 6.54 (1H, s), 7.04 (2H, d, *J*=9.0 Hz), 7.74 (2H, d, *J*=9.0 Hz), 8.11 (1H, d, *J*=5.6 Hz), 10.98 (1H, s). ¹³C NMR (100 MHz, DMSO): δ 44.10, 55.24, 65.99, 84.03, 97.00, 114.43, 121.43, 127.87, 157.38, 157.45, 160.61, 161.37, 162.13 162.61.

3.3.*In vivo* experiments

Six-to-eight-week-old female athymic nude and Balb/c mice were housed with a temperature-controlled and 12-hour light/12-hour dark cycle environment. This study was carried out in accordance with Institutional Animal Care and Use Committee of Bilkent University and performed according to the institution's guidelines and animal research principles.

3.3.1. Primary tumor xenografts and syngeneic models

For *in vivo* breast primary tumor growth, 4x10⁶ JIMT-1 cells were prepared in 150 µl of 1:1 DMEM and Matrigel (v/v) and injected into mammary fat pads (MFP) of female nude mice. Mouse weight and tumor volume were measured daily using calipers. Tumor volumes were calculated as length x width² x 0.5. Once the tumor volume had reached

about 90-100 mm³, xenografts were randomized into groups (3-5 mice per group). Animals were treated with vehicle or different doses and administration ways of BO-264 (every other day 2 mg/kg oral gavage (po.) or iv, or 5 mg/kg po.). *In vivo* tumor growth inhibitory effect of BO-264 was also compared with SPL-B (every other day 5 mg/kg po.). The vehicle for these drugs includes 4% DMSO, 4% HCl in 0.9% NaCl. In a separate experiment, animals were also tested for a higher dose of BO-264 (25 mg/kg) dissolved in 0.05% (g/ml) HPMC (hydroxypropyl methylcellulose) and 2% Tween-80 in ddH₂O. Mice were sacrificed 20-30 days after initiation of the treatment and the tumors were collected and stored for subsequent analyses. For the survival analysis, 1x10⁶ EMT6 cells were prepared in PBS and injected into MFPS of immunocompetent female Balb/c mice. Similarly, once the tumor volume had reach to 90-100 mm³, mice were randomized into two groups and received either vehicle or 25 mg/kg BO-264 every day. Survival was calculated using a predefined tumor volume cut-off of 1500 mm³.

For the *in vivo* colon cancer studies, female nude and Balb/c mice were inoculated in the right flank with human colorectal carcinoma cell line, HCT-116, and murine colorectal carcinoma cell line, CT-26, respectively. 5x10⁶ HCT-116 cells were prepared in 150 µl Matrigel:PBS (1:1) while 5x10⁵ CT-26 cells were prepared in 100 µl PBS. Once tumor volumes reached 100 mm³, animals were given vehicle (Optiform® Select kit, formulation #2) or 50 mg/kg BO-264 daily by oral gavage. Twenty days after the treatment, mice were sacrificed.

3.3.2. In vivo toxicity studies

To test the in vivo toxicity of BO-264 we performed two different experiments. Nude female mice received 100 mg/kg BO-264 daily for 7 days and body weight changes were recorded. In a separate experiment, mice were given 500 mg/kg BO-264 only once and monitored for 1-3 days. Organs were collected at the end of the experiment to for toxicity assignment.

3.4. Immunohistochemistry (IHC)

Immunohistochemistry was performed on 5- μ m-thick formalin-fixed paraffin-embedded (FFPE) tissue sections of major organs isolated from nude mice bearing JIMT-1 and Balb/c mice bearing EMT6 tumors. Tissue sections mounted on slides were deparaffinized at high temperature (60°C) overnight, and the paraffin was removed with xylene. Sections were stained with hematoxylin and eosin (H&E) and Ki-67 staining as previously described [113]. Apoptosis was detected by the TUNEL assay with the Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Millipore).

3.5. Physicochemical and Pharmacokinetic analysis

3.5.1. Physicochemical – ADME studies

BO-264 was also evaluated for its physicochemical properties and metabolic stability provided by Eurofins Advinus Company (Bengaluru, India). It was tested in terms of Absorption, Distribution, Metabolism and Excretion (ADME) properties which includes

solubility, lipophilicity, stability in liver microsomes, plasma protein binding, Caco-2 permeability and cytochrome P450 inhibition profiles.

3.5.2. Pharmacokinetic analysis

Female CD-1 mice were used to determine pharmacokinetic parameters of BO-264 following intravenous and oral gavage administration of BO-264 dose formulations. Mice received a single dose of 5 mg/kg iv. (n=25) or a single dose of 25 mg/kg po. (n=25). IV formulation was 0.05% HPMC, 2N HCl, pH 1-2 while po. formulation was 0.05% HPMC, 0.3 g Tween 80, 2N HCl, pH 1-2. Blood was collected from 3 mice per time-point by terminal bleeds. The concentration of BO-264 in mouse plasma was determined using high performance liquid chromatographer coupled to triple quad mass spectrometer (Shimadzu LC-MS 8050, Shimadzu Corp., Kyoto, Japan). PK parameters (including but not limited to AUC, T_{1/2}, C_{max}, Cl and V_d) were determined using Phoenix WinNonlin (version 6.2) on a validated computer system.

3.6. Bioinformatics and statistical analysis

3.6.1. Patient dataset analysis

TACC3 differential plots between different tumor and normal tissues was constructed using The Cancer Genome Atlas (TCGA) patients [114] and data was downloaded from <http://firebrowse.org/>. The microarray dataset GSE39582 including mRNA expression profiles of colorectal cancer and normal colorectal mucosa patients was used for TACC3

expression level analysis [115]. For survival analysis and prognostic significance of TACC3, two independent publicly available cancer datasets were used. The first one is the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset [116]. Here, TACC3 expression levels of METABRIC Discovery and Validation set composed of 1992 tumors was used for tumor grade analysis. These data were accessed through European Genome-phenome Archive. TACC3 expression pattern on survival was also analyzed with Kaplan Meier plotter database which includes information on overall survival of 1402 breast cancer [117], 1926 lung cancer [118] and 876 gastric cancer [119] patients.

3.6.2. Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed using gene sets related to mitosis and DNA damage available at the Broad Institute website (<http://software.broadinstitute.org/gsea/index.jsp>). METABRIC patient gene expression profiles were used in GSEA analysis. Patients from 25th and 75th quartiles of TACC3 levels were used and defined as low and high TACC3, respectively.

3.6.3. Dependency score analysis

For the analysis of TACC3 dependency of NCI-60 cell lines, gene dependency data obtained through combined RNAi screens were downloaded from Broad Institute, Novartis and Marcotte et al. [120, 121]. The data is available in <https://depmap.org/portal/>.

3.6.4. Multivariant analysis

Multivariate Cox regression analysis was done using METABRIC dataset in SPSS software. TACC3 level, tumor grade, tumor stage, ER, PR and HER2 status were selected as covariates. TACC3 expression was separated based on 25th percentiles.

3.6.5. Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc) and expressed as mean $\pm$ standard deviation from three independent experiments unless otherwise indicated. Statistical significance of two groups comparisons was determined by two-tailed Student's t-test. To compare the doubling time curves of different cell lines one-way ANOVA was used. A multiple t-test was used to determine pair-wise significances between the treatment groups of tumor volumes of EMT6 tumor bearing mice. A *p* and an adjusted *p* (*q*) value of less than 0.05 were considered to be statistically significant. Kaplan-Meier survival curve analysis was performed using the Log-rank (Mantel-Cox) test.

4. RESULTS

4.1. TACC3 is frequently overexpressed in different cancer types and associated with worse overall survival

To demonstrate the clinical relevance of targeting TACC3 in different cancers, we first analyzed the mRNA expression of TACC3 in various tumor types and their normal tissue counterparts using The Cancer Genome Atlas (TCGA) data (**Figure 4.1 A**). We found that TACC3 is significantly upregulated in a broad spectrum of cancer types compared to their normal counterparts, which highlights the potential of TACC3 as a cancer specific therapeutic target. However, differential plots of other TACC family members, TACC1 and TACC2, either showed a higher expression in normal tissue, compared to tumor or the difference was not significant (**Figure 4.1 B and C**). Oncomine database analysis also showed that TACC3 is mostly upregulated in cancer while other two members were upregulated in normal, further supporting the oncogenic profile of TACC3 [60].

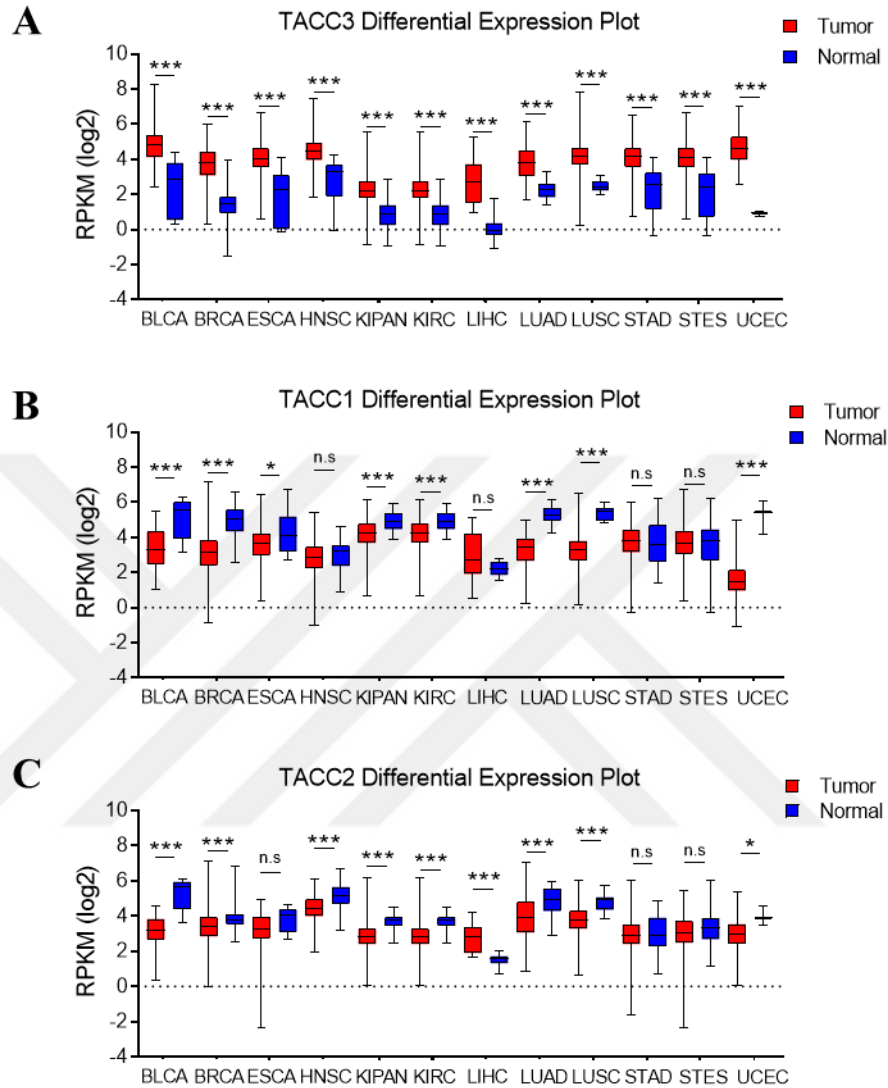


Figure 4.1. TACC3 is significantly overexpressed in many different cancer types unlike other TACC family members; TACC1 and TACC2. Differential expression plots of TACC3 (A), TACC1 (B) and TACC2 (C) between tumor and normal tissues of TCGA patients represented as reads per kilobase million (RPKM; log2) values. *: $P < 0.05$; ***: $P < 0.001$; n.s: not significant. BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; ESCA, esophageal carcinoma; HNSC, head and neck squamous cell carcinoma; KIPAN, pan-kidney cohort (KICH+KIRC+KIRP); KIRC, kidney renal clear cell carcinoma; LIHC, liver hepatocellular carcinoma; LAUD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; STAD, stomach adenocarcinoma; STES, stomach and esophageal carcinoma; UCEC, uterine corpus endometrial carcinoma.

To investigate the role of TACC3 level in patient survival outcome, we analyzed different patient datasets/databases, Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) [116] and Kaplan-Meier (KM) plotter [117-119]. We found that higher TACC3 levels are significantly correlated with worse overall survival in breast, lung and gastric cancer (**Figure 4.2 A-D**). Clinical relevance of TACC3 in breast cancer has been further elucidated by analyzing the METABRIC patient dataset where we also observed a higher TACC3 expression in higher grade tumors (**Figure 4.2 E**), suggesting a strong positive correlation between TACC3 levels and tumor aggressiveness.

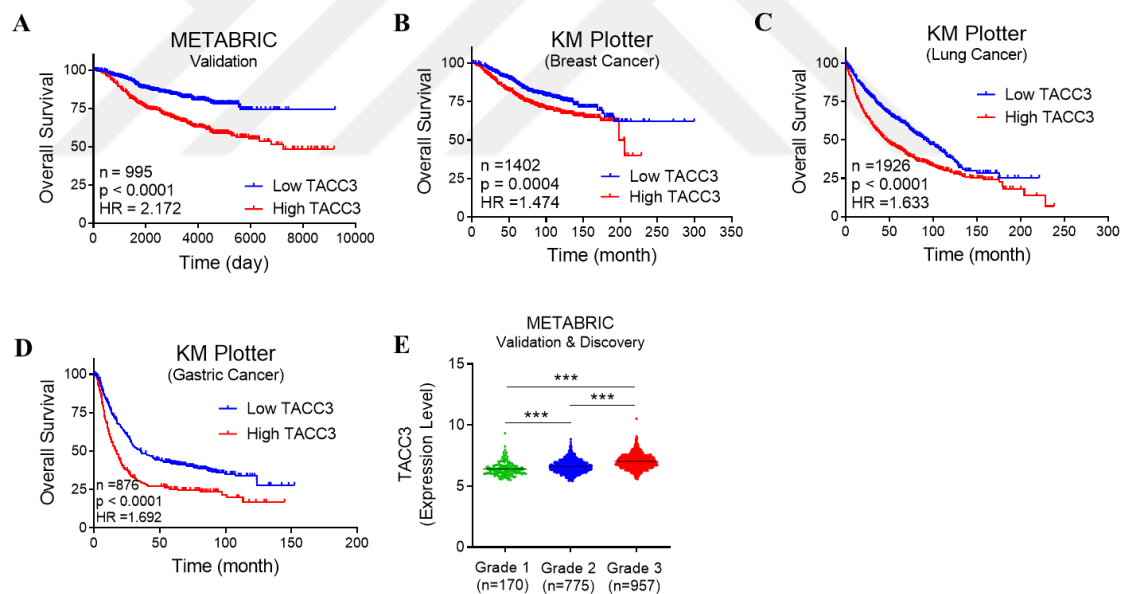


Figure 4.2. TACC3 overexpression is correlated with worse overall survival and higher tumor grade. Effect of TACC3 level on overall survival of breast cancer patients retrieved from METABRIC Validation dataset (A). Effect of TACC3 level on overall survival of patients with breast (B), lung (C), and gastric cancer (D) retrieved from Kaplan–Meier (KM) plotter database. Log-rank test was used for survival statistical analyses. (E) TACC3 expression levels according to the tumor grade in METABRIC Validation and Discovery datasets. Student t-test was used for statistical analysis. ***: p < 0.001.

To test if TACC3 is an independent prognostic factor in breast cancer, we performed a multivariate analysis of TACC3 in breast cancer patients. For this purpose, a Cox proportional hazard model, which describes the relation between event incidences and relates several risk factors to survival time, was used [122]. Importantly, high TACC3 expression was found to be an independent prognostic factor for poor prognosis in breast cancer (**Table 4.1**). Overall, our data suggest that TACC3 expression is increased in tumors compared to normal tissues and its expression is significantly associated with disease progression in several cancers, including breast cancer that make it highly attractive, clinically-relevant target.

Table 4.1. TACC3 is an independent prognostic factor for breast cancer.

Parameter	n	HR	P
TACC3 expression		2.727	7.65E-09
Low	476		
High	476		
Tumor Grade		1.079	0.44
1	102		
2	353		
3	455		
Tumor Stage		1.038	0.594
0	200		
1	186		
2	299		
3	36		
4	5		
ER Status		0.904	0.57
Negative	235		
Positive	717		
Her2 Status		1.14	0.499
Negative	854		
Positive	98		
PR Status		0.953	0.779
Negative	455		

Abbreviations: PR, progesterone receptor; ER, estrogen receptor; n, number of patients; HR, hazard ratio

4.2. BO-264 is identified as a novel TACC3 inhibitor

As mentioned above, none of the available TACC3 inhibitors, such as KHS101 [109] and SPL-B [84] has yet advanced to the clinical studies. Having demonstrated the clinical relevance of targeting TACC3, we aimed to identify a novel and more potent TACC3 inhibitor than currently available ones that effectively eliminates cancer cells and preserves non-tumorous cells. To this end, we performed an in-house screening of a series of small molecules obtained by rational drug design where we tested the *in vitro* cytotoxic potential of the molecules followed by functional assays to demonstrate the mechanism of action of our hit molecule targeting TACC3.

We first screened our in-house compound collection designed and synthesized by our collaborators Drs. Erden Banoğlu and Burcu Çalışkan, Gazi University to test their effects on cell viability of breast cancer cell line JIMT-1 cells. We have chosen JIMT-1 cells due to its high TACC3 protein levels and its high tumorigenic capacity in mice [123, 124], which will be discussed in detail in **Figure 4.7 A**, **Figure 4.21 A** and **Figure 4.22 A**. Our in-house screening efforts led to the identification of BO-264 as a potential TACC3 inhibitor having an IC₅₀ of 0.19 µM. Detailed synthesis of BO-264 is summarized in **Figure 4.3**.

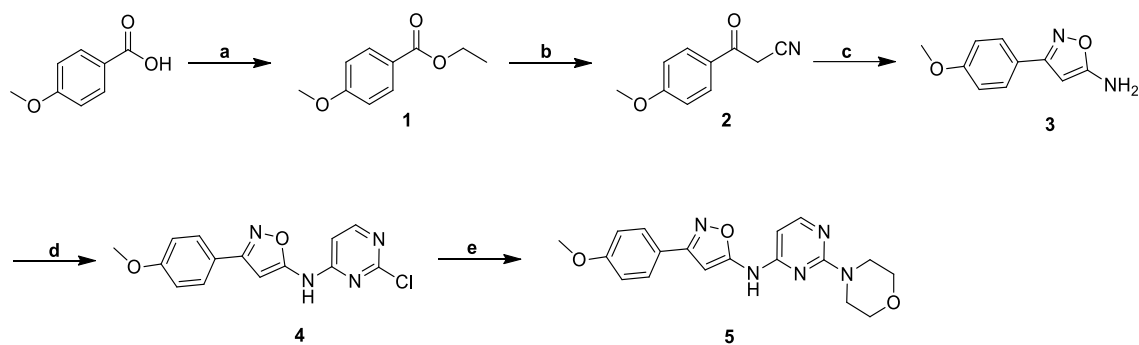


Figure 4.3. The synthesis of BO-264. (A) Chemical structure of BO-264 and modifications applied from KHS101. (B) The reagents and the conditions during BO-264 synthesis: (a) SOCl_2 , EtOH, reflux, 4 hours; (b) NaH, MeCN, toluene, reflux, 2 hours; (c) $\text{H}_2\text{N.OH HCl}$, NaOH, H_2O , reflux, 4 hours; (d) t-BuOK, 2,4-dichloropyrimidine, t-BuOH, rt, 24 hours; (e) Morpholine, n-butanol, reflux, 5 hours.

We next determined whether BO-264 interacts with TACC3 by utilizing three different protein-drug binding assays; drug affinity responsive target stability (DARTS), cellular thermal shift assay (CETSA) and isothermal titration calorimetry (ITC). DARTS is a label-free method which is based on stabilization and protection of protein targets from proteolysis upon binding to small molecules [125]. As shown in **Figure 4.4 A** and **B**, the presence of BO-264 prevented pronase-mediated digestion of TACC3 which was even stronger than that of the known TACC3 inhibitor, SPL-B, which was used as a positive control [84]. Binding of BO-264 to TACC3 was further validated with CETSA, which is based on stabilization and protection of target proteins from aggregation and/or precipitation at high temperatures upon binding to small molecules [126]. Treatment of JIMT-1 cells with BO-264 stabilized cellular TACC3 protein levels upon increased temperature as indicated by a shift of thermal melting curve of TACC3 in the presence of BO-264 compared to control (**Figure 4.4 C and D**). Consistent with DARTS and CETSA experiments, we also utilized isothermal titration calorimetry (ITC) method to directly

probe the strength of the physical interaction between TACC3 and BO-264 in collaboration with Dr. Urartu Şeker, Bilkent UNAM. ITC is an analytical method which directly measures the heat generation or absorption during a bimolecular reaction between two molecules [127]. A titration of TACC3 recombinant protein into BO-264 was carried out and monitored by recording discharged or consumed heat (**Figure 4.4 E**). The thermodynamic parameters of BO-264 binding to TACC3 at 25°C was measured as dissociation constant (KD): 1.5 nM; enthalpy (ΔH): 4.929E7 cal/mol; entropy (ΔS): 1.65E5 cal/mol/deg, and number of binding sites (N): 0.704, confirming the interaction between these two molecules. These data suggest that, our novel small-molecule inhibitor, BO-264 directly interacts with TACC3 and therefore acts as a novel TACC3 inhibitor.

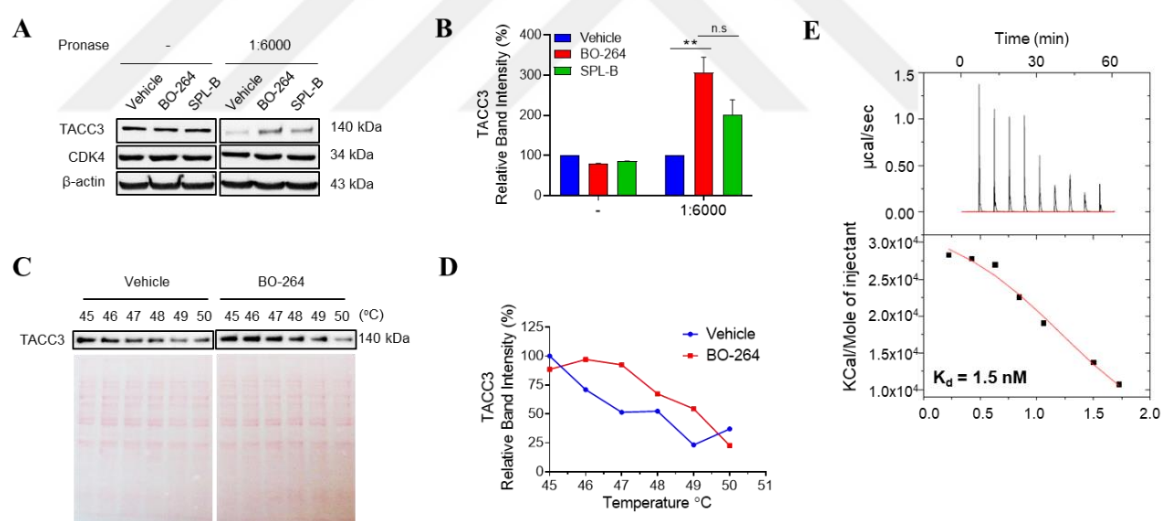


Figure 4.4. BO-264 is identified as a novel TACC3 inhibitor binding to TACC3. (A) Western blot results of DARTS assay using JIMT-1 cell protein extracts to confirm the interaction between BO-264 and TACC3. CDK4 was used as a non-target protein whose level unchanged between treatment groups. 10 μ M of drugs were used. (B) Graph showing the quantification of the TACC3 relative band intensity between the treatment groups normalized to β -actin and are representative of at two independent experiments. Data is presented as means $\pm$ SD. ** p < 0.01, n.s: not significant. (C) Target engagement of BO-264 to TACC3 protein in intact JIMT-1 cells. JIMT-1

cells were treated with vehicle or BO-264 (1 μ M) for 6 h, and cells were collected, heated at the indicated temperatures and then lysed. Soluble proteins in supernatant were subjected to western blot, and TACC3 protein levels were detected. Ponceu staining was used as loading control. (D) CETSA curves indicate TACC3 % relative band intensity and show the shift between treatment groups. (E) Determination of the association constant for the TACC3 and BO-264 interaction using isothermal titration calorimetry (ITC). Upper trace shows raw data while lower trace shows integrated data from a titration of TACC3 into BO-264. Model fitting of a single interaction model was applied using the Origin 7 software provided along with the ITC200 instrument.

4.3. BO-264 inhibits the viability of breast cancer cell lines and is superior to currently available TACC3 inhibitors

After validating the interaction between TACC3 and BO-264, we analyzed its anti-cancer potential and compared it with currently available TACC3 inhibitors, KHS101 and SPL-B. BO-264 inhibited cell growth at lower doses in JIMT-1 breast cancer cells giving an IC_{50} value of 0.19 μ M compared to other two inhibitors (SPL-B: IC_{50} : 1.15 μ M and KHS101: IC_{50} : 1.79 μ M) (**Figure 4.5 A**). The superior anticancer activity of BO-264 in JIMT-1 cells has also been validated with a colony formation assay (**Figure 4.5 B**). We showed a significantly lower average colony number of JIMT-1 cells upon treatment with BO-264 compared to other two inhibitors in a dose-dependent manner (**Figure 4.5 C**).

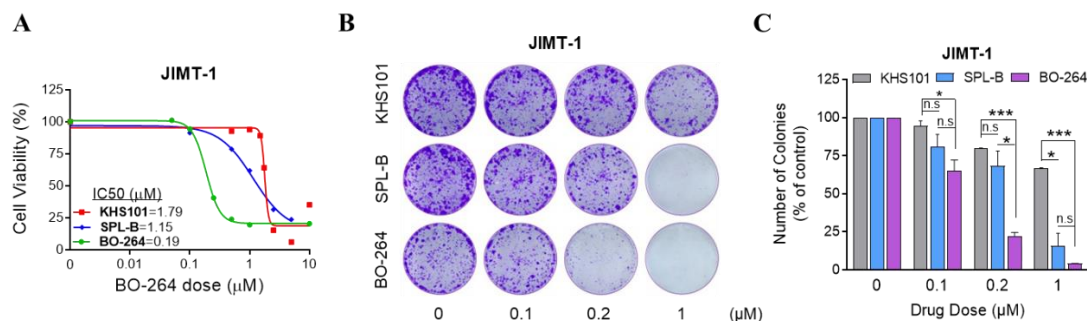


Figure 4.5. BO-264 is more effectively inhibits cell viability and colony formation than other available TACC3 inhibitors, SPL-B and KHS101. (A) Pharmacological inhibition of TACC3 with three different inhibitors and their effect on cell viability of breast cancer cell line, JIMT-1 (IC_{50} : inhibitory concentration 50%). Cell viability was measured by SRB assay as triplicates in all the following cell viability experiments, unless otherwise indicated. (B) Colony formation assay of JIMT-1 cells treated with three inhibitors for 12 days. Colonies were stained with crystal violet. (C) Quantification of the colonies was done and analyzed using ImageJ software. Data is presented as means $\pm$ SD. n.s: not significant, *: $p < 0.05$; ***: $p < 0.001$.

Next, we tested other breast cancer cell line models from different subtypes. Similarly, BO-264 inhibited the viability of basal (CAL51 (**Figure 4.6 A**), MDA-MB-231 and MDA-MB-436), and HER2+, HCC1954 cell lines at lower doses than SPL-B and KHS101 (**Figure 4.6 C**), while it demonstrated no toxicity in normal breast epithelium cell line, MCF-12A (**Figure 4.6 B and C**), showing that BO-264 specifically targets tumor cells while it spares normal cells. IC_{50} range for BO-264 in cancer cell lines were found to be 0.12-0.36 μ M while it is 0.79-3.67 μ M for SPL-B and 1.79-17.40 μ M for KHS101, suggesting its remarkably potent cytotoxic activity against cancer cells (**Figure 4.6 C**).

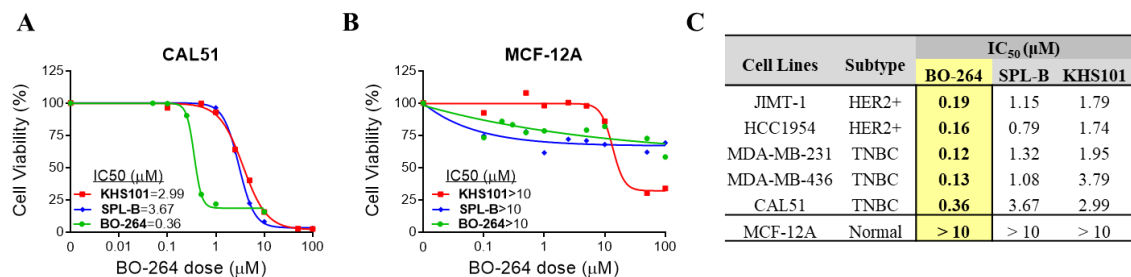


Figure 4.6. BO-264 specifically and efficiently target cancer cells, but not normal cells. Pharmacological inhibition of TACC3 with three different inhibitors and their effect on cell viability of breast cancer cell line, CAL51 (A) and normal breast epithelial cell line, MCF-12A (B) (IC₅₀: inhibitory concentration 50%). Cell viability was measured by SRB assay as triplicates. (C) Summary table of the breast cell lines (cancer or normal) tested for responses to three inhibitors.

To identify the cancer specificity of BO-264, we analyzed TACC3 protein levels in cell line models from different subtypes of breast cancer as well as normal breast cell line, MCF-12A. As shown in **Figure 4.7 A**, MCF-12A has the lowest TACC3 protein levels while breast cancer cells from different subtypes have relatively higher TACC3 protein levels, supporting the cancer-specific high levels of TACC3. In addition to the small cell line screen that we performed (**Figure 4.6 C**), we tested more cell lines from different subtypes and observed a trend for increased TACC3 expression in relatively more aggressive breast cancer subtypes, basal, HER2+ and luminal B as compared with luminal A cell lines (**Figure 4.7 B**). This further supports the notion that TACC3 expression might be associated with cancer aggressiveness. Similar to the breast cancer cell lines shown in (**Figure 4.6 C**), BO-264 was found to be more toxic also in other breast cancer cell lines with IC₅₀ values less than 1 μM (**Figure 4.7 C**).

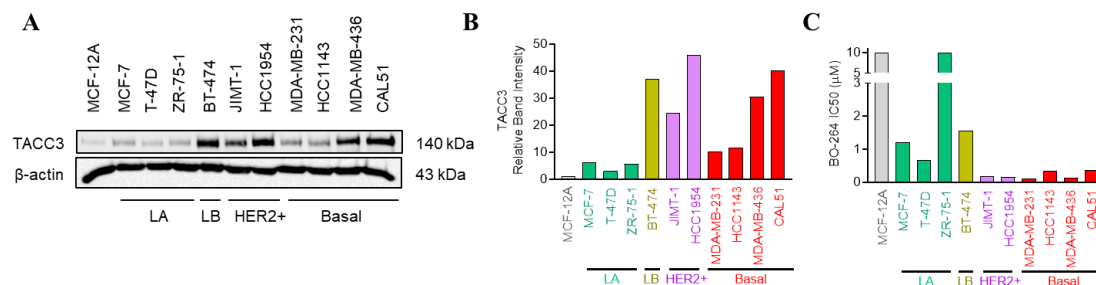


Figure 4.7. TACC3 level is critical for BO-264 response. TACC3 protein levels of breast normal and cancer cell lines. TACC3 protein level was analyzed by Western blot. β -actin was used as loading control. (B) Band intensities were quantified by normalizing TACC3 intensities to the loading control β -actin using Image J software. (C) BO-264 IC₅₀ levels of breast normal epithelial cell line and cancer cell line separated according to their subtypes. IC₅₀ levels of MCF-12A and ZR-75-1 are larger than 10 μ M. LA: Luminal A, LB: Luminal B subtypes.

Next, to test whether cells are dependent on TACC3 to survive, we first overexpressed TACC3 in low TACC3 expressing normal breast cells, MCF-12A (**Figure 4.8 A**) which were then used for a clonogenic growth assay. As shown in **Figure 4.8 B** and **C**, TACC3 overexpression significantly increased the colony formation capacity and cell survival of MCF-12A cells, which in return renders them sensitive to BO-264 (**Figure 4.8 D**). This further demonstrates the dependency of cells to TACC3 and the specificity of our inhibitor.

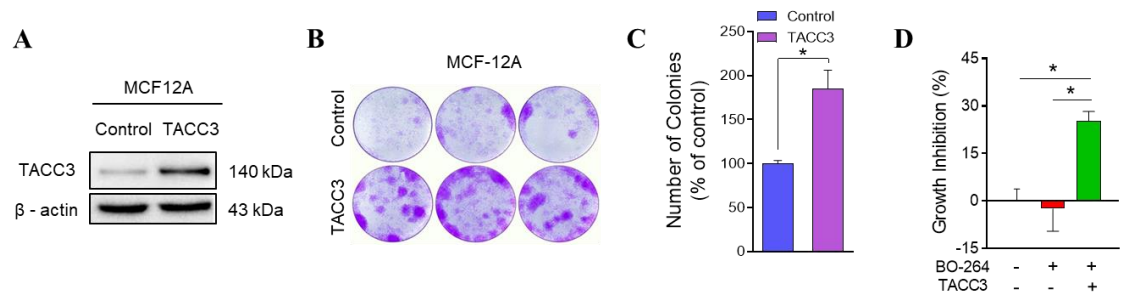


Figure 4.8. Overexpression of TACC3 induces BO-264 sensitivity. (A) Western blot analysis of MCF-12A cells transiently transfected with either control or TACC3 overexpression vector (250 ng) for 48 hours. Beta actin was used as loading control. (B) Colony formation assay of MCF12A cells. Cells were transiently transfected with either control or TACC3 vector (250 ng) for 48 hours as triplicates. Cells were cultured for 13 days and stained with crystal violet. (C) Number of colonies was counted and analyzed using ImageJ software. Data is presented as means $\pm$ SD. *: $p < 0.05$. (D) Cell viability assay upon BO-264 treatment of TACC3 overexpressing MCF-12A cells.

Division times, also called duplication times, varies from one cell type to another for fundamental biological reasons [128]. Therefore, to exclude the possibility that the differences we observed in BO-264 responses of cells lines are not due to the differences in the duplication times, we calculated their proliferation rates, represented as duplication times. We observed no significant difference between MCF-12A and breast cancer cell lines, indicating that responsiveness of BO-264 may not be dictated by increased rates of cell divisions (**Figure 4.9**). Taken together, these results suggest that BO-264 specifically targets breast cancer cells while it spares normal cells.

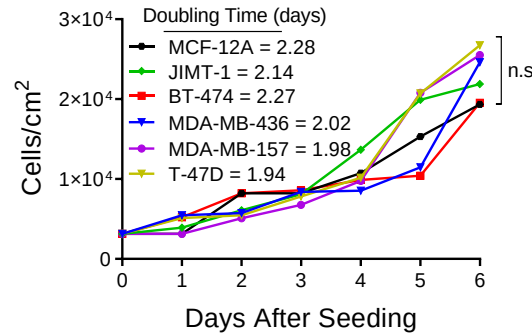


Figure 4.9. Responsiveness to BO-264 is not related with doubling time. Doubling time assessment of MCF-12A and breast cancer cell line models. Cells were plated at low density and grown for 1 week. They were then counted every day. One-way ANOVA test was used. n.s.: not significant.

4.4. BO-264 inhibits the viability of cancer cells with FGFR3-TACC3 fusion and is highly potent in NCI-60 cancer cell line panel

FGFR3-TACC3 activating fusions, involving tyrosine kinase domain of FGFR3 gene and the coiled coil domain of TACC3 gene, is observed in various type of cancers [95] promoting malignant transformation through activating the downstream MAPK signaling pathway [98] and inducing mitotic defects, such as formation of lagging chromosomes in anaphase and misaligned chromosomes in metaphase [129]. Therefore, FGFR3-TACC3 oncogenic fusion proteins are important candidates for targeted therapy for patients with solid tumors [95]. We sought to identify whether BO-264 is also cytotoxic to the cell lines harboring fusion protein where we used two human urinary bladder cancer cell lines, RT112 and RT4 which have FGFR3-TACC3 fusion protein [93]. Western blot analysis of TACC3 revealed a higher protein expression in RT112 cells compared to RT4 cells (**Figure 4.10 A**). Similar to our previous observation that higher TACC3 levels defines

BO-264 sensitivity (**Figure 4.7**), we also observed a stronger response to BO-264 in RT112 cells as indicated by a lower IC₅₀ (**Figure 4.10 B and C**). Although RT4 showed a lower response to BO-264 than RT112, which is potentially due to lower TACC3 levels, BO-264 yielded the lowest IC₅₀ value in both models as compared to SPL-B and KHS101. This suggests that our novel inhibitor is also very effective in FGFR3-TACC3 fusion harboring cells and could potentially be used as a highly relevant therapeutic to target FGFR3-TACC3 fusion harboring tumors. Moreover, BO-264 reduced ERK1/2 phosphorylation, which is a marker for activated FGFR signaling [98], along with strong mitotic arrest indicated with Histone H3 phosphorylation [130] (**Figure 4.10 D**) at 10X lower dose than other two inhibitors (SPL-B and KHS101). These data suggest that BO-264 specifically blocks the function of FGFR3-TACC3 fusion protein.

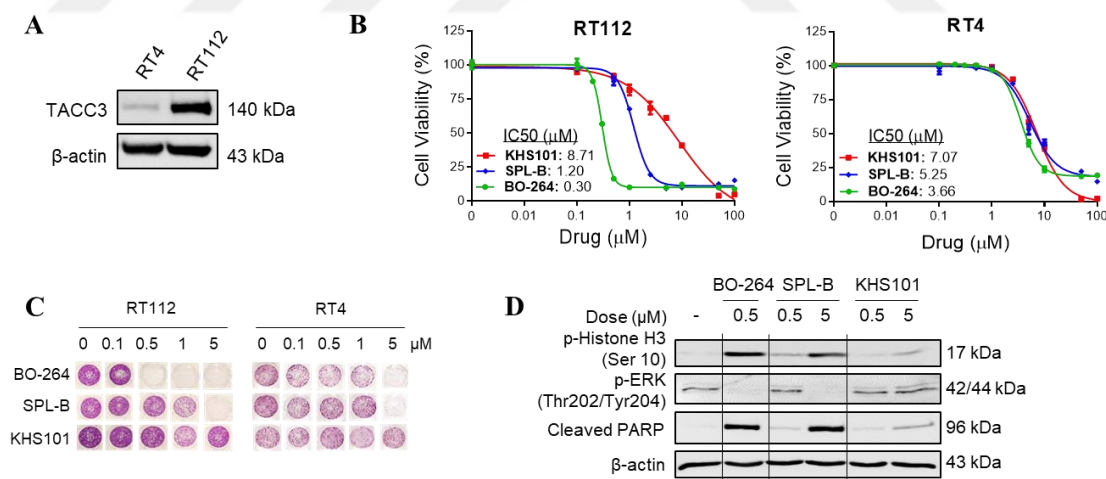


Figure 4.10. BO-264 inhibits the viability of cancer cells harboring FGFR3-TACC3 fusion protein. (A) Western blot analysis of TACC3 protein levels in RT112 and RT4 cells. β-actin was used as loading control. (B) Pharmacological inhibition of TACC3 with three different inhibitors and their effect on cell viability of FGFR3-TACC3 fusion expressing cell lines, RT112 and RT4. Cell viability was tested upon 3 days of drug incubation and measured by SRB assay. (C) Images showing SRB stained plates after treatment with three inhibitors. (D) Western blot analysis of

mitotic arrest marker, p-Histone H3 as well as the ERK phosphorylation (Thr202/Tyr204) upon treatment with TACC3 inhibitors at different doses for 24 hours.

The strong cytotoxic effects obtained in breast cancer cell lines as well as FGFR3-TACC3 fusion protein harboring cell lines, and the upregulation of TACC3 in several different cancers further prompted us to test our novel inhibitor in other cancer cell lines. To this end, we carried out a five dose (0.01 μ M-100 μ M) antiproliferative and cytotoxicity analysis of BO-264 in NCI-60 human cancer cell line panel which is an ideal tool to further understanding of the disease and molecular targets of new agents [131]. BO-264 showed a remarkable anti-cancer activity against more than 90% of the cell lines representing nine different cancer types with GI₅₀ (growth inhibition 50%) values less than 1 μ M (**Figure 4.11 A**). Moreover, a heat map was constructed to visualize each cell line specifically and their GI₅₀ values in the NCI-60 panel (**Figure 4.11 B**). This further supports the notion that TACC3 might also be relevant target in cancers other than breast cancer.

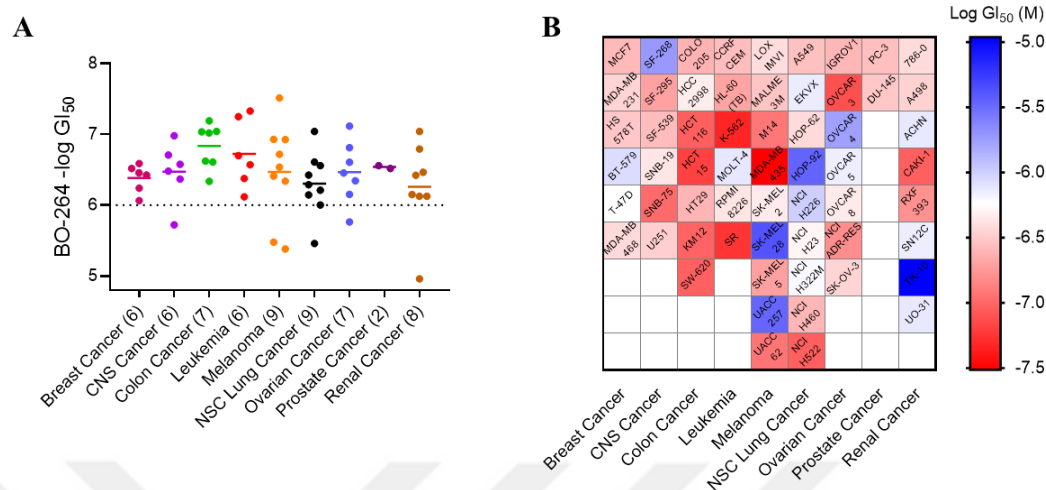


Figure 4.11. NCI-60 five-dose screening reveals remarkable anti-cancer activity of BO-264.

(A) Average GI_{50} values (M) determined from two independent NCI-60 five-dose screens for BO-264. Black dotted line indicates $1 \mu M$ threshold. (B) The heat map representing GI_{50} concentrations of BO-264 for each cell line across the NCI-60 human cancer cell line panel.

Moreover, we found that GI_{50} values obtained from NCI-60 screen for BO-264 show a positive correlation with the TACC3 dependency score which is obtained from DepMap.org [121] (**Figure 4.12**). TACC3 dependency score was retrieved from a combined RNAi screen. Here, a lower TACC3 dependency score means that cells being more dependent on TACC3 are more sensitivity to BO-264. Overall, these data suggest the strong anti-cancer activity of BO-264, highlighting its potential applications also in other cancer types.

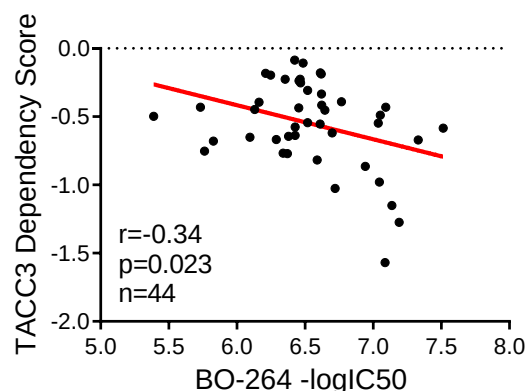


Figure 4.12. TACC3 dependency defines BO-264 sensitivity. The correlation between GI_{50} values of BO-264 obtained from NCI-60 screen and TACC3 dependency score retrieved from DepMap portal. A lower score means that TACC3 is more likely to be dependent in a given cell line.

4.5. TACC3 inhibition with BO-264 induces mitotic arrest, apoptosis and DNA damage, causes aberrant spindle formation and reduces centrosomal localization of TACC3

Depletion of TACC3 in HeLa cells has been previously shown to induce caspase-dependent apoptosis [132] and mitotic arrest [63]. Moreover, our lab has recently showed that blockage of PLK1 activity, another mitotic gene, in T-DM1 resistant breast cancer cells causes mitotic arrest and caspase activation, leading to apoptosis and DNA damage [123]. Like TACC3, PLK1 is also enrolled in mitotic cell division and conducts important functions in a cell-cycle dependent manner [133]. Therefore, we investigated whether a similar effect is observed upon TACC3 suppression in breast cancer. We first performed gene set enrichment analysis (GSEA) using two different mitotic progression and DNA repair-related gene sets in breast cancer patient datasets where we were able to

demonstrate that mitotic (**Figure 4.13 A**) and DNA repair (**Figure 4.13 B**) gene sets were significantly enriched among patients with higher TACC3 levels.

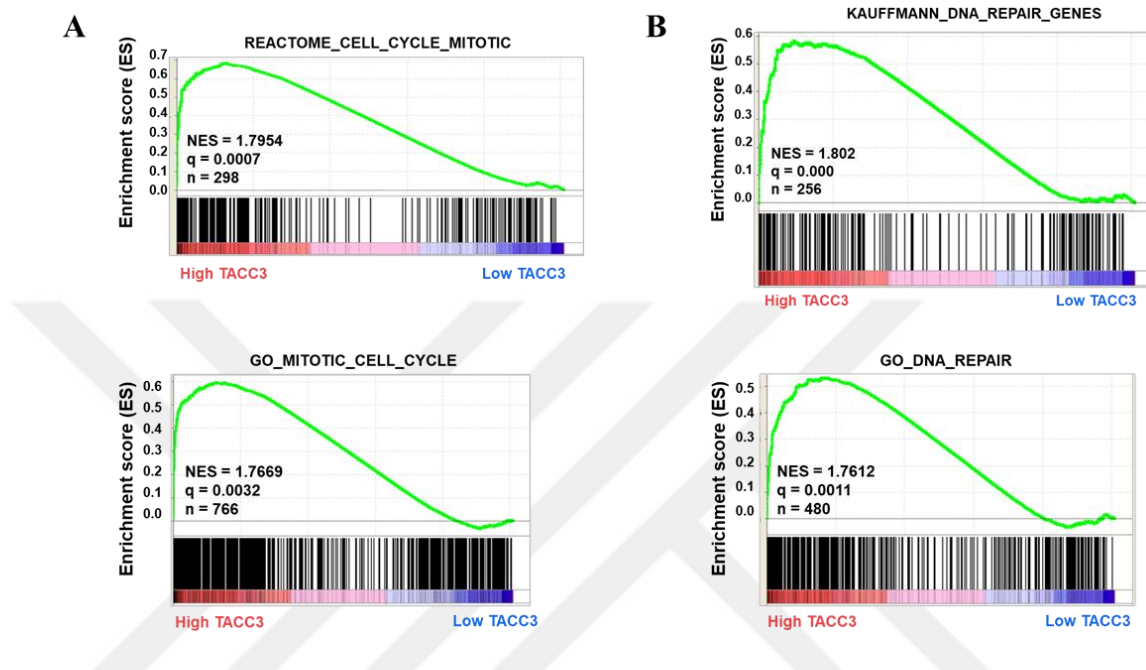


Figure 4.13. Gene set enrichment analysis identifies TACC3 associated mitotic progression and DNA repair pathways Enrichment plot from GSEA conducted with mitosis (A) and DNA repair-related (B) Reactome, Kauffmann and GO gene sets in METABRIC patients separated according to their TACC3 expression level. Data significance is presented as Normalized Enrichment Score (NES) and FDR (q) value. N indicates total number of the genes used in the analysis.

To test the effects of TACC3 on these processes, we utilized small interfering RNAs (siRNAs) and pharmacological inhibitors against TACC3. First, we tested the efficiency of two different siRNAs targeting TACC3 in JIMT-1 cells by qRT-PCR where we observed at least 60% knockdown (**Figure 4.14 A**). Consistent with this, inhibition of TACC3 with siRNAs caused significant growth inhibition (**Figure 4.14 B**), which is also in line with the induction of p-Histone H3 (Ser10), showing mitotic arrest [130], cleaved

PARP, showing apoptosis [134], and p-H2AX (Ser139), showing DNA damage [135] in JIMT-1 cells (**Figure 4.14 C**).

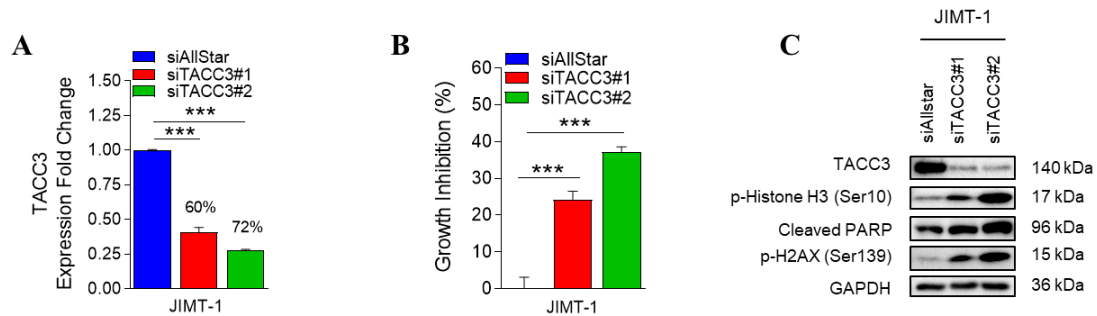


Figure 4.14. Inhibition of TACC3 with siRNAs causes mitotic arrest, apoptosis and DNA damage in JIMT-1 cells. (A) qRT-PCR showing knockdown efficiency of TACC3-specific siRNAs in breast cancer cell line, JIMT-1. Cells were transfected with 20 nM siRNAs targeting TACC3 and TACC3 mRNA level was checked after 48 h of transfection. (B) Growth inhibition of these cells upon TACC3 knockdown. Cells were transfected with siTACC3s, and cell viability was checked after 72 hours of transfection. Student t-test, ***: $p < 0.001$. (C) Western blot analysis of mitotic arrest, DNA damage, and apoptosis markers in JIMT-1 cells upon TACC3 knockdown with two different siRNAs. Cells were transfected with 20 nM siRNAs for 48 hours. GAPDH was used as a loading control.

To eliminate cell or subtype specific effects, we also tested two breast cancer cell lines from basal subtype, MDA-MB-436 and MDA-MB-157. Knockdown efficiency was confirmed with qRT-PCR (**Figure 4.15 A**). Similar phenotypes like growth inhibition (**Figure 4.15 B**) and induction of mitotic arrest, apoptosis and DNA damage pathways upon TACC3 knockdown (**Figure 4.15 C**) were also observed in these cells supporting the importance of TACC3 in cell survival.

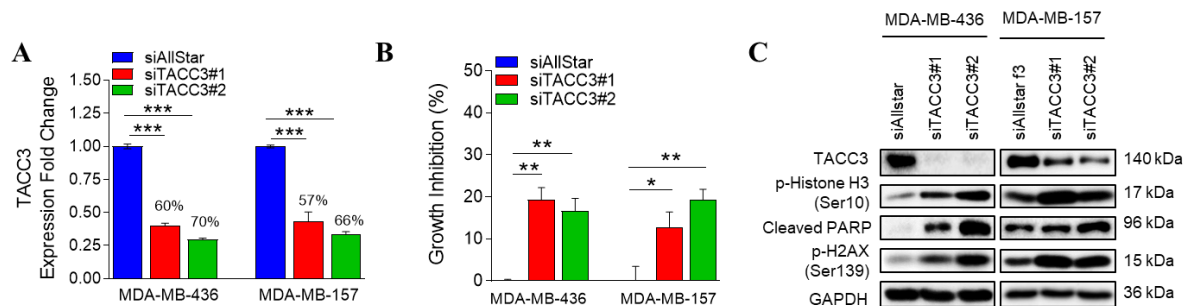


Figure 4.15. Inhibition of TACC3 with siRNAs causes mitotic arrest, apoptosis and DNA damage in TNBC cell lines. (A) qRT-PCR showing knockdown efficiency of TACC3-specific siRNAs in breast cancer cell lines, MDA-MB-436 and MDA-MB-157. Cells were transfected with 20 nM siRNAs targeting TACC3 and TACC3 mRNA level was checked after 48 h of transfection. (B) Growth inhibition of these cells upon TACC3 knockdown. Cells were transfected with siTACC3s, and cell viability was checked after 72 hours of transfection. Student t-test, ***: $p < 0.001$. (C) Western blot analysis of mitotic arrest, DNA damage, and apoptosis markers in MDA-MB-436 and MDA-MB-157 cells upon TACC3 knockdown with two different siRNAs. Cells were transfected with 20 nM siRNAs for 48 hours. GAPDH was used as a loading control.

We next analyzed the expression of mitotic arrest, apoptosis and DNA damage markers in JIMT-1 cells after treatment with increasing doses of BO-264, SPL-B and KHS101 to test whether superior IC_{50} value of BO-264 as compared to other inhibitors is also reflected to TACC3 downstream signaling. As shown in **Figure 4.14 C**, siTACC3-induced mitotic arrest, DNA damage and apoptosis were fully recapitulated by BO-264 in a dose-dependent manner (**Figure 4.16 A**) while the markers were induced at considerably higher doses of SPL-B and KHS101. We even included higher KHS101 doses which were used by others [136] and observed an induction of these markers only at the highest doses. Notably, these results are consistent with the cell viability data and the IC_{50} values obtained by three inhibitors (**Figure 4.5 A**). These results further validate the high potency of BO-264 with a working range at nM scale and its specificity to TACC3

as it generates similar levels of induction of the molecular markers as obtained by siRNA-mediated TACC3 downregulation. Moreover, activation of apoptosis upon BO-264 treatment in JIMT-1 cells was further validated with Annexin V/propidium iodide (PI) staining indicated by a prominent increase in the fraction of apoptotic cells (from 4.1% to 45.6%) (**Figure 4.16 B**). The induction of mitotic arrest (p-Histone H3, Ser10) and DNA damage (p-H2AX, Ser139) forced by siRNA knockdown and BO-264 treatment was further supported by immunofluorescence staining (**Figure 4.16 C-E**). We demonstrated that BO-264 significantly induced mitotic arrest and DNA damage marker fluorescence intensity in JIMT-1 cells. Overall, these data suggest that BO-264 recapitulates the molecular and phenotypical alterations caused by TACC3 inhibition with siRNAs at notably lower doses compared to SPL-B and KHS101.

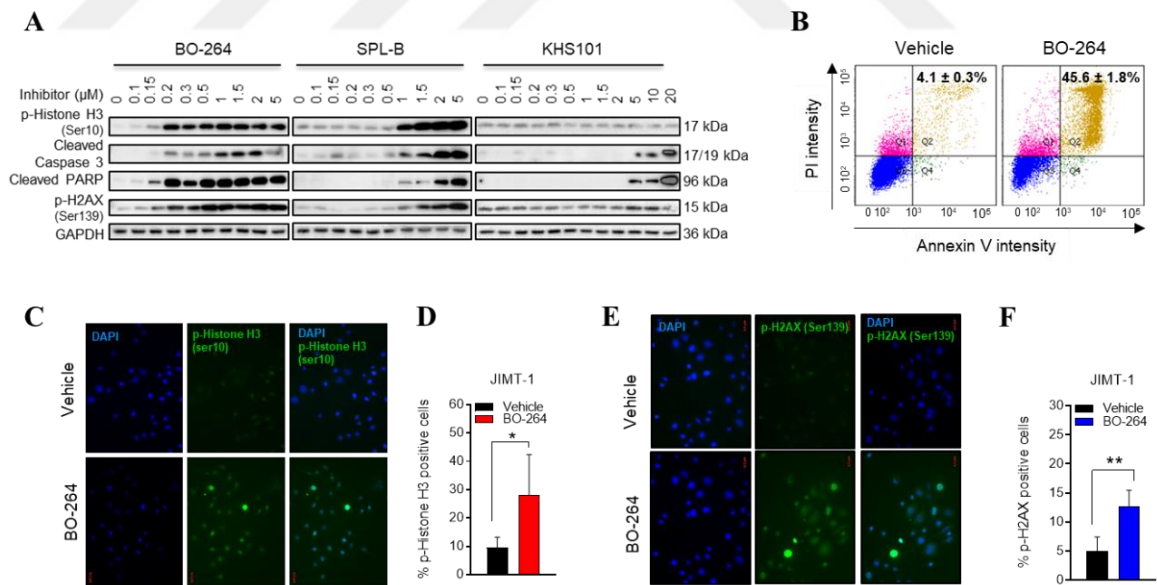


Figure 4.16. TACC3 inhibition induces mitotic arrest, apoptosis and DNA damage in JIMT-1 cells. (A) Western blot analysis of JIMT-1 cells treated with several doses of BO-264, SPL-B or KHS101 for 24 hours to test the effect on mitotic arrest, DNA damage and apoptosis markers. The same amount of protein and the same exposure time were used to compare three drugs. (B) AnnexinV/PI staining of BO-264 (500 nM) treated JIMT-1 cells. The percentage of Annexin V/PI

double-positive cells are provided on the graph. (C) Immunofluorescence staining of p-Histone H3 (Ser10) in JIMT-1 cells treated with vehicle or BO-264 (150 nM) for 24 hours and (D) its quantification of positively stained cells. (E) Immunofluorescence staining of p-H2AX (Ser139) in JIMT-1 cells treated with vehicle or BO-264 (150 nM) for 24 hours and (F) its quantification of positively stained cells. Last panels in both staining show the merged images of DAPI and specified marker stainins. For visualization, Alexa Fluor 488 secondary antibody was used. Scale bar: 5 μ m. For each condition, 400 cells were counted. *: $p < 0.05$; **: $p < 0.01$.

TACC3 inhibition has been shown to cause severe spindle abnormalities [63]; therefore, we next tested a potential disruption of microtubules upon BO-264 treatment. Suppression of TACC3 function with BO-264 caused formation of aberrant spindle structures in a dose-dependent manner (**Figure 4.17 A**). The most prominent phenotype seen upon BO-264 treatment was the formation of multipolar, disorganized and monopolar spindles which were characterized by improperly aligned chromosomes at the metaphase plate (73.4%, 24.5%, and 1.5% frequency, respectively, upon treatment with the highest dose) in JIMT-1 cells (**Figure 4.17 B**).

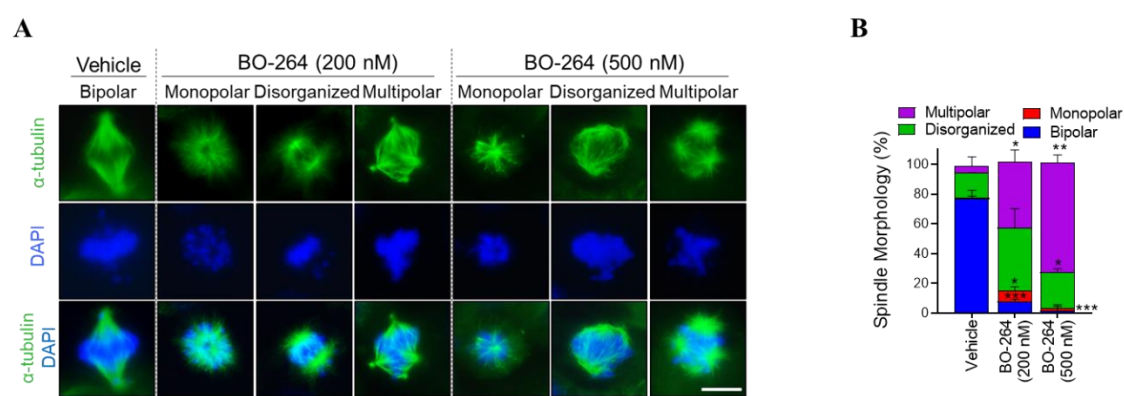


Figure 4.17. TACC3 inhibition by BO-264 causes aberrant spindle formation. (A) Induction of aberrant spindle formations in BO-264-treated JIMT-1 cells. Cells were treated with two different doses of BO-264 for 12 hours, fixed in methanol, and then stained with anti-a-tubulin

(green) antibody. DNA was stained with DAPI (blue). Scale bar, 10 μ m. (B) Quantification of spindle abnormalities as seen in A. The data represent the mean values and the SDs of three independent experiments (t test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Significance was determined by comparison with vehicle group.

To eliminate the possibility that the molecular alterations and phenotypical changes induced upon BO-264 treatment are not due to any off-target effects but due to TACC3 inhibition, we created JIMT-1 cells inducibly expressing short hairpin RNA (shRNA) targeting TACC3. Inducible knockdown of TACC3 also caused mitotic arrest, DNA damage and apoptosis in which treating shTACC3 cells with BO-264 did not further induce these changes compared with untreated shTACC3 cells, supporting the specificity of our TACC3 inhibitor (**Figure 4.18 A**). Similarly, TACC3 knockdown using two different siRNAs recapitulated the effects of BO-264 on mitotic spindles. However, treatment with BO-264 did not further induce these aberrant spindle formations compared with vehicle-treated siTACC3 cells, further strengthening the on-target effect of BO-264 (**Figure 4.18 B and C**). Collectively, these results clearly show that observed sensitivity of breast cancer cell lines to BO-264 is potentially due to a stronger induction of the downstream mitotic cell death pathways upon specific TACC3 inhibition with BO-264 treatment.

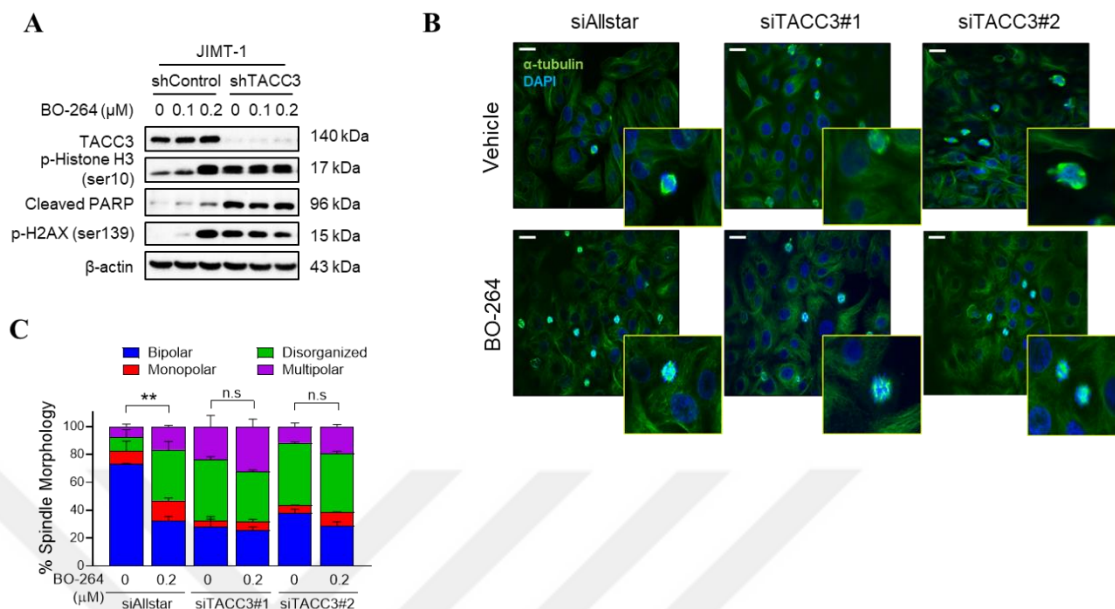


Figure 4.18. Molecular alterations and phenotypic changes induced by BO-264 is a result of TACC3 inhibition. (A) Western blot analysis of mitotic arrest, DNA damage and apoptosis markers in JIMT-1 cells which were stably knocked down with an inducible TACC3 shRNA. JIMT-1 shControl/shTACC3 cells were induced with 2 μg/ml doxycycline for 48 hours and then treated with BO-264 for 24 hours. (B) Immunofluorescence analysis of aberrant spindle formations in JIMT-1 cells transfected with two different siRNAs upon BO-264 treatment. Cells were transfected with siRNAs for 24 hours and then treated with 0.2 μM BO-264 for 12 hours. Cells were fixed in methanol and then stained with anti-α-tubulin (green) antibody. DNA was stained with DAPI (blue). Scale bar: 20 μm. (C) Quantification of spindle abnormalities as seen in B. The data represent the mean values and the SD's of two independent experiments (t-test). Significance was determined by comparison of all aberrant spindle formations (monopolar, disorganized and multipolar) with bipolar formations of vehicle and BO-264 treated groups. n.s: not significant. **: $p < 0.01$

Disrupting microtubule dynamics activates the spindle assembly checkpoint (SAC), which provides time to the cells to repair spindle defects; otherwise it induces mitotic cell death [137, 138]. Since we observed disrupted microtubule structures upon

BO-264 treatment, we asked if SAC is activated. Treatment of JIMT-1 cells with BO-264 increased chromosomal localization of BuBR1, which is a core component of SAC and a marker of SAC activation [139], as assessed by immunofluorescence staining (**Figure 4.19 A**). We then tested the contribution of an active SAC signaling to BO-264-induced mitotic arrest, apoptosis and DNA damage by inhibiting SAC kinase, Mps1 using a specific inhibitor (TC Mps1) [140]. Inhibition of Mps1 kinase reversed the molecular alterations induced by BO-264 treatment where mitotic arrest, DNA damage and apoptosis induction were lost (**Figure 4.19 B**). This suggests that BO-264 may function by activating SAC upon inducing severe spindle defects that further causes prolonged mitosis, damaged DNA and apoptotic cell death.

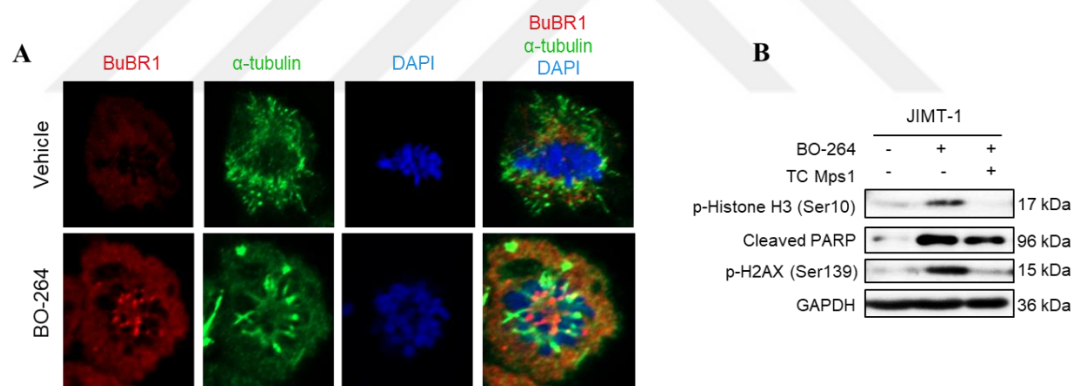


Figure 4.19. Inhibition of TACC3 function causes spindle assembly checkpoint activation.

(A) Immunofluorescence staining of BubR1 (red) and α -tubulin (green) in vehicle versus BO-264 (500 nM) treated JIMT-1 cells. (B) Western blot analysis of mitotic arrest, DNA damage and apoptosis markers showing mitotic slippage in BO-264-treated JIMT-1 cells upon treatment with a spindle checkpoint kinase (Mps1) inhibitor, TC Mps1. JIMT-1 cells were treated with 200 nM BO-264 for 24 hours followed by 12 hours treatment with 1 μ M TC Mps1. GAPDH was used as a loading control.

As mentioned before, TACC3 is a centrosomal adaptor protein which plays a crucial role in centrosome-driven mitotic spindle assembly [141]. In addition to centrosome localization during mitosis [142], TACC3 has been also shown to localize to centrosomes during interphase [143]. To test whether BO-264 also interferes centrosome localization of TACC3 during mitosis and/or interphase, JIMT-1 cells were treated with BO-264 and stained with antibodies for TACC3 and γ -tubulin, a centrosome marker [144]. Centrosomal localization of TACC3 during mitosis was greatly reduced upon BO-264 treatment (**Figure 4.20 A and B**). Similarly, a significant decrease in TACC3 localization during interphase was also recorded, indicating loss of microtubule nucleation (**Figure 4.20 C and D**). Overall, these data demonstrate that BO-264 alters intracellular localization of TACC3 and interferes its cellular functions.

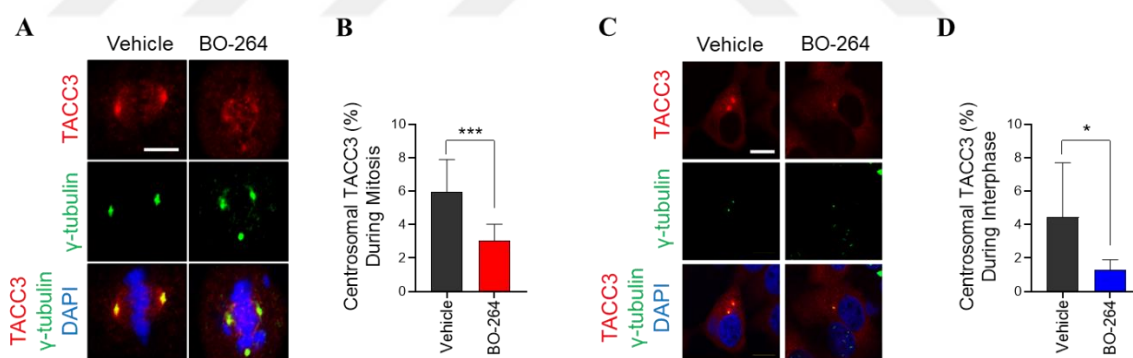


Figure 4.20. BO-264 reduces centrosomal localization of TACC3. (A) Immunofluorescence images of JIMT-1 cells during mitosis treated with either vehicle or BO-264 (300 nM) for 24 hours, followed by staining with anti-TACC3, anti- γ -tubulin antibody, and DAPI. (B) Quantification of the relative centrosomal localization of TACC3 during mitosis in these treatment groups as seen in A. (C) Immunofluorescence images of JIMT-1 cells during interphase treated with either vehicle or BO-264 (300 nM) for 24 hours, followed by staining with anti-TACC3, anti- γ -tubulin antibody, and DAPI. (D) Quantification of the relative centrosomal localization of TACC3 during interphase in these treatment groups as seen in C. Scale bar, 10 μ m. *: $p < 0.05$; ***: $p < 0.001$.

4.6. BO-264 significantly impairs tumor growth and improves survival of tumor-bearing mice without major toxicity

The results described above demonstrated that breast cancer cells expressing high TACC3 levels are more sensitive to our novel TACC3 inhibitor, BO-264, *in vitro*. Moreover, having important oncogenic functions and being upregulated in different cancers encouraged us to test the anti-tumorigenic potential of TACC3 inhibition by BO-264 *in vivo* as well. We, therefore, performed several *in vivo* experiments with xenografts and syngeneic mouse models and studied the effects of TACC3 inhibition on tumor growth. Firstly, we utilized HER2+ JIMT-1 xenografts that are known to be highly tumorigenic [123, 124, 145]. To this end, JIMT-1 cells were injected into mammary fat pad (MFP) of female nude mice, and mice were subsequently treated with vehicle or low doses of BO-264 via different administration routes or doses (2 or 5 mg/kg oral, po. or 2 mg/kg intravenous, i.v.) for 30 days (**Figure 4.21**). Compared to the vehicle-treated group, three different dosing/administration of BO-264 significantly reduced tumor growth rate (**Figure 4.21 A**). Intravenous administration of BO-264 did not significantly differ from the oral application, suggesting its oral use as a treatment option, which is more convenient and pharmaco-economic for the patients [146]. BO-264 was well-tolerated since treatment did not affect the body weight of mice (**Figure 4.21 B**). Reduction in tumor volume with BO-264 treatment is also reflected in tumor weight (**Figure 4.21 C**).

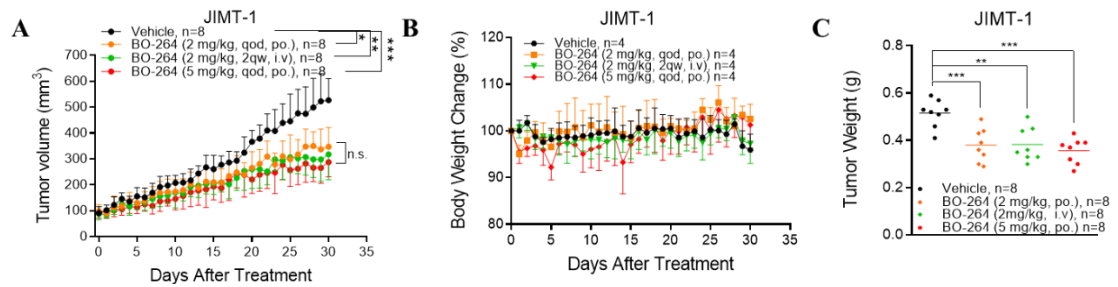


Figure 4.21. Different doses and administration routes of BO-264 inhibits tumor growth in *in vivo* JIMT-1 xenografts without affecting mouse body weight. (A) Tumor volume change of JIMT-1 xenografts for 30 days after the treatment with vehicle or BO-264 at low dose and different administration ways. Treatments were started when tumors reached to 90-100 mm³. (B) Mice body weight change (%) of JIMT-1 xenografts for 30 days. (C) Tumor weights of JIMT-1 xenografts isolated from mice treated with vehicle or BO-264. n.s.: not significant, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Since we observed significant suppression of tumor growth rate in low dose BO-264-treated mice, we next wanted to test the tolerability of mice to BO-264 and whether we obtain a better and dose-dependent anti-tumorigenic effect. To this end, JIMT-1 cells were injected into MFPs of female nude mice and this time they were treated with vehicle or 25 mg/kg BO-264 orally for 23 days. BO-264-treated mice showed a significant suppression of tumor growth compared to vehicle-treated mice (**Figure 4.22 A**), which is also reflected with tumor weights (**Figure 4.22 B**). Importantly, higher BO-264 doses compared to previous experiment (**Figure 4.21**) was again well tolerated since treatment did not affect the mice weight (**Figure 4.22 C**) and did not cause any organ toxicity (**Figure 4.22 D**). Furthermore, H&E and Ki-67 staining of tumors showed a reduction in proliferation after 25 mg/kg BO264 administration. Furthermore, increased peroxidase-based TUNEL assay suggest a higher level of apoptosis induction in BO-264-treated tumors (**Figure 4.22 E**). Moreover, when tumor volumes of 5 mg/kg (**Figure 4.21 A**) and

25 mg/kg (**Figure 4.22 A**) BO-264-treated groups were compared, we see a clear dose-dependent suppression of tumor growth.

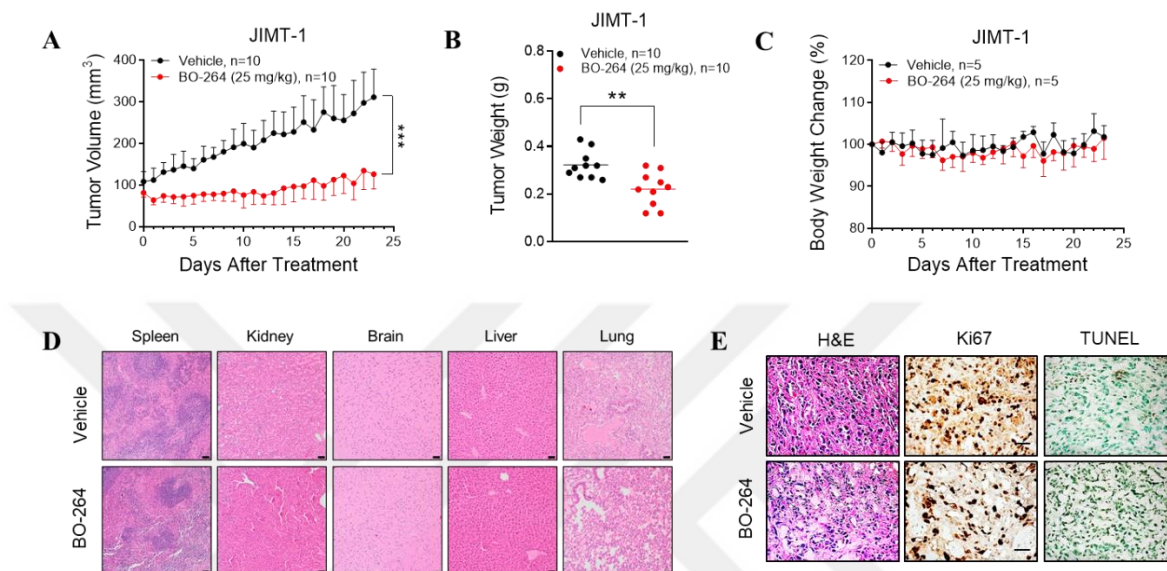


Figure 4.22. BO-264 significantly impairs tumor growth in *in vivo* JIMT-1 xenografts without any major toxicity. (A) Tumor volume change of JIMT-1 xenografts after the treatment with vehicle or 25 mg/kg BO-264 in female nude mice. Treatments were started when tumors reached to 90-100 mm³. (B) Tumor weights of JIMT-1 xenografts isolated from mice treated with vehicle or BO-264. (C) Mice body weight change (%) of JIMT-1 xenografts. (D) Histologic analysis of major organs (spleen, kidney, brain, liver, and lungs) of JIMT-1 xenografts by H&E staining. Representative images for each organ are presented. Scale bar, 50 µm. (E) H&E, Ki67 and TUNEL staining of the tumors from the treatment groups of JIMT-1 xenografts. Representative pictures of IHC staining from each group were taken at 40X magnification. Scale bar: 40 µm.

We further tested the effects of TACC3 inhibition *in vivo* using an immunocompetent model. EMT6 murine triple-negative breast cancer cell line, which is a fast-growing and highly aggressive model [147], and has higher TACC3 levels compared to normal mouse embryonic fibroblast (MEF) cells (will be discussed in **Figure**

4.26), was injected into MFPs of syngeneic BALB/c mice. Pairwise comparison of treatment days between vehicle and BO-264 treated groups revealed a significant delay in tumor growth in BO-264-treated mice (**Figure 4.23 A**). We also observed 57.1% increased lifespan [148] in BO-264-treated mice (median survival 22 days) compared to vehicle-treated mice (median survival 14 days) (**Figure 4.23 B**). Similar to xenograft model, BO-264 was well-tolerated also in the syngeneic mouse model which was determined with % body weight change (**Figure 4.23 C**) and organ H&E staining (**Figure 4.23 D**).

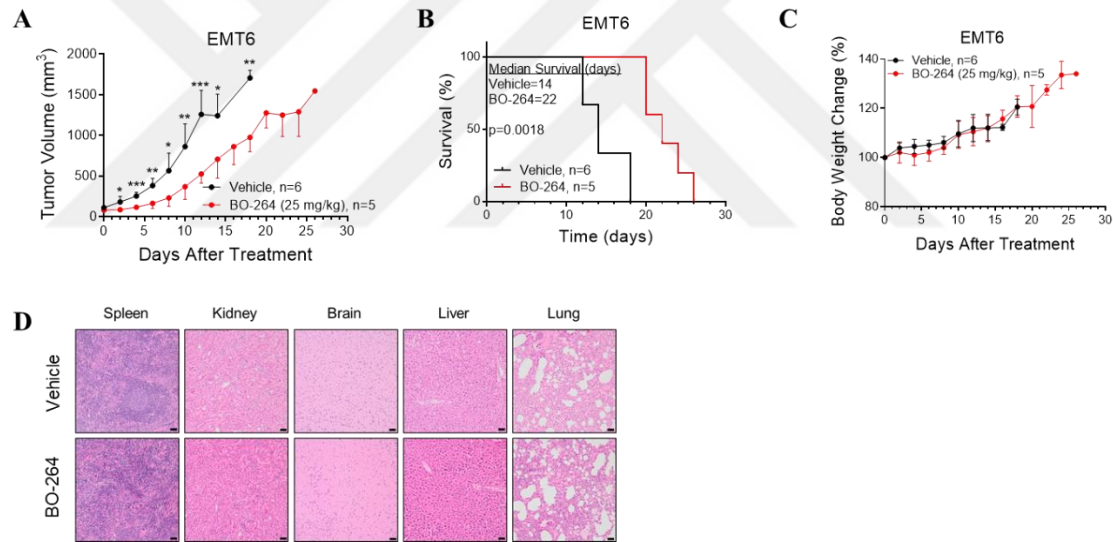


Figure 4.23. BO-264 significantly impairs tumor growth in immunocompetent mouse model of breast cancer and improves survival without any major toxicity. (A) Tumor volume measurements of the effect of BO-264 on a highly aggressive breast cancer mouse model with EMT6 cells. Significance was calculated using multiple t test for each data point for each day compared with vehicle control. (B) Kaplan–Meier survival curve of EMT6 syngeneic tumors treated with vehicle (median survival 14 days) or BO-264 (median survival 22 days). P value was calculated using the log rank test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. (C) Body weight change (%) of Balb/c mice bearing EMT6 syngeneic tumors. (D) H&E staining of major organs in EMT6 syngeneic model. Representative images of for each organ are presented. Scale bar, 50 μm .

Oral administration of SPL-B has been shown to suppress tumor growth of ovarian cancer xenografts [84]. Since our inhibitor is also very effective for oral administration both in immunocompromised and immunocompetent mice models, we further compared the efficacy of SPL-B and BO-264 side by side for tumor growth suppression using JIMT-1 xenografts in nude mice. As shown in **Figure 4.24 A**, 5 mg/kg SPL-B dosing failed to suppress tumor growth while BO-264 was significantly more effective in delaying tumor growth. Importantly, BO-264 or SPL-B treatment did not cause any significant body loss and they were well tolerated by the mice (**Figure 4.24 B**), suggesting a better in vivo efficacy of BO-264 compared to SPL-B. Altogether, these data show that oral administration of BO-264 significantly and efficiently inhibits tumor growth and improves survival in different mice models with breast cancer.

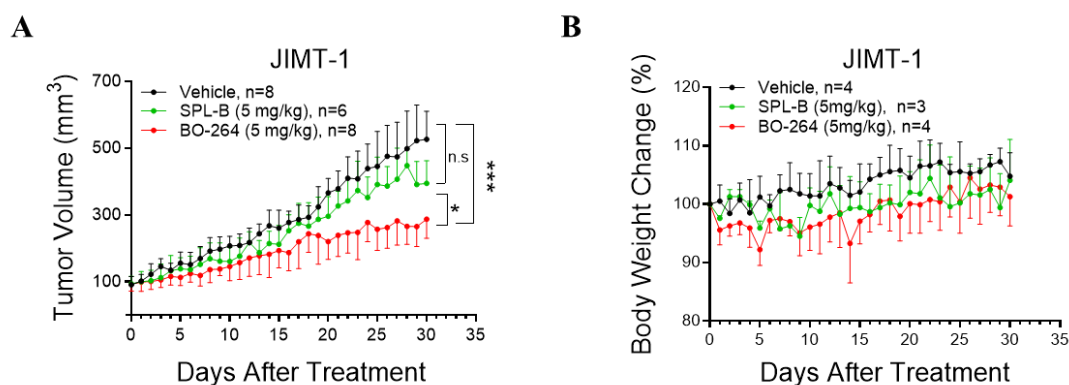


Figure 4.24. Low dose BO-264 impairs tumor growth significantly while SPL-B has no significant effect (A) Tumor volume change of JIMT-1 xenografts treated with vehicle, 5 mg/kg BO-264 or SPL-B orally for 30 days. Treatments were started when tumors reached to 90-100 mm³. (B) Percent body weight changes of JIMT-1 xenografts followed for 30 days. *: $p < 0.05$; ***: $p < 0.001$; *n.s.*: not significant.

In addition to breast cancer, TACC3 has been also shown to be deregulated in colon cancer, in which its levels play an important role for disease progression [85]. We have also showed that BO-264 is greatly cytotoxic for colon cancer cell lines in NCI-60 panel at very low doses (**Figure 4.11**). For this reason, we aimed to investigate the effect of TACC3 inhibition with BO-264 in colon cancer *in vitro* and *in vivo* models. TACC3 has been found to be upregulated in colon cancer tissues compared with normal tissues obtained from two independent datasets, TCGA Colon Adenocarcinoma (COAD) (**Figure 4.25 A**) and GSE39582 (**Figure 4.25 B**), validating its oncogenic profile also in colon cancer.

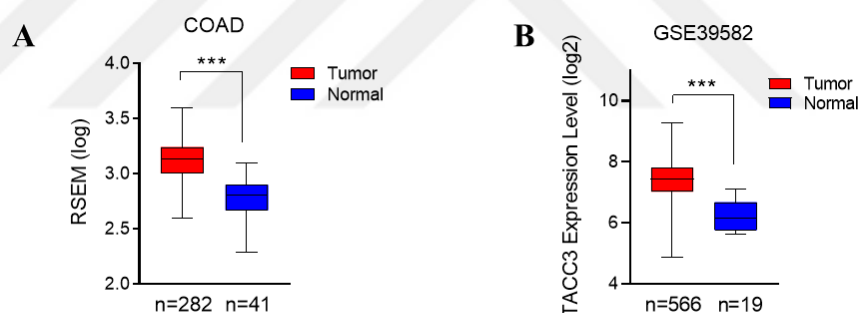


Figure 4.25. TACC3 is upregulated in colon cancer. (A) Differential expression plots of TACC3 between tumor and normal tissues of TCGA Colon Adenocarcinoma (COAD) patients represented as RNA-Seq by Expectation-Maximization (RSEM) (log) values. (B) TACC3 expression data in colon tissues obtained from GEO dataset in accession GSE39582. ***: $p < 0.001$.

As we wanted to test the effect of BO-264 on tumor growth of colon cancer models, we first tested the TACC3 expression levels of colon cancer cell lines and compared them with breast cancer cell lines used for *in vivo* studies. HCT-116, a human

colorectal carcinoma cell line, and CT-26 (a murine colon carcinoma cell line) have higher TACC3 protein levels compared to breast cancer models (JIMT-1 and EMT6, respectively) (**Figure 4.26 A**). We next tested whether downstream mechanism of action of BO-264 is also preserved in colon cancer models. TACC3 inhibition by BO-264 caused mitotic arrest, apoptosis and DNA damage in HCT-116 and CT-26 cells, which is in line with our previous data with breast cancer (**Figure 4.16 A**).

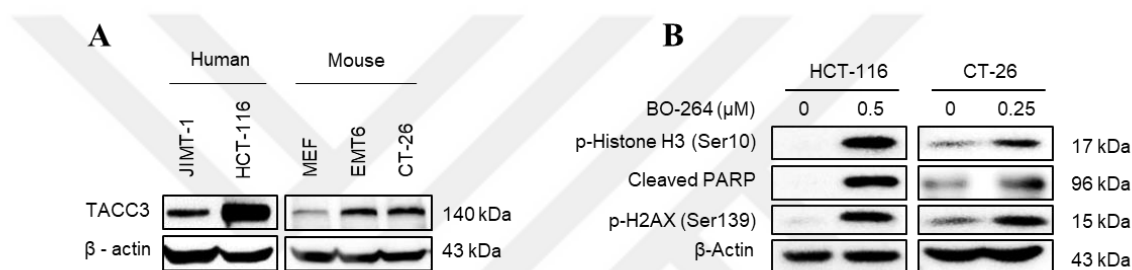


Figure 4.26. Downstream mechanism of TACC3 inhibition is conserved in colon cancer. (A) TACC3 protein levels of human and mouse normal and cancer cell lines. TACC3 protein level was analyzed by Western blot. β-actin was used as loading control. (B) Western blot analysis of mitotic arrest, DNA damage and apoptosis markers in HCT-116 and CT-26 cells treated with BO-264 for 24 hours. β-actin was used as a loading control.

Based on the efficient slow down/suppression of tumor growth in BO-264-treated xenograft and syngeneic breast cancer models, we next tested its anti-tumorigenic activity on colon cancer xenograft and syngeneic tumor models. Female nude mice were injected with HCT-116 cells into flank region of mice subcutaneously and treated with 50 mg/kg BO-264 for 20 days. BO-264 treatment significantly impaired tumor growth in these models (**Figure 4.27 A**) without any toxicity assessed by mice body weight change (**Figure 4.27 B**).

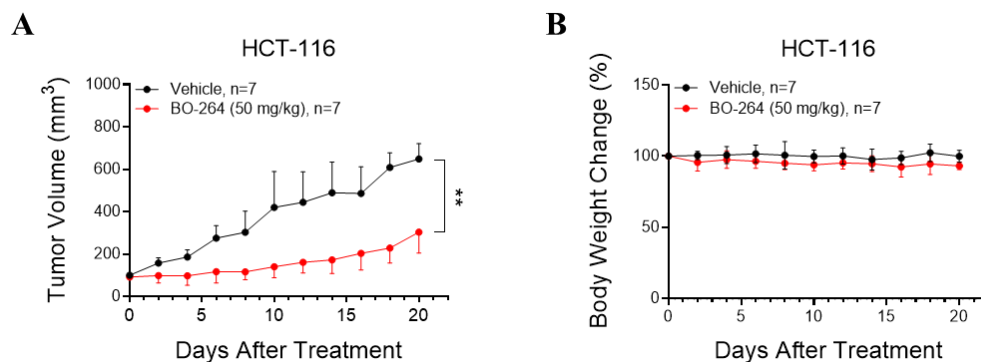


Figure 4.27. Targeting TACC3 with BO-264 significantly impairs tumor growth in immunocompromised colorectal carcinoma mouse model. (A) Tumor volume change of HCT-116 xenografts after the treatment with vehicle or 50 mg/kg BO-264 in female nude mice. Treatments were started when tumors reached to 90-100 mm³ and performed daily *via* oral gavage. **: $p < 0.01$. (B) Body weight change (%) of nude mice bearing HCT-116 tumors treated with vehicle or BO-264 (50 mg/kg).

Similar to immunocompromised models, we also injected murine colon carcinoma cell line, CT-26, into flank region of female immunocompetent Balb/c mice and performed drug treatment for 20 days orally. In line with previous data, TACC3 inhibition with BO-264 inhibited tumor growth significantly (**Figure 4.28 A**). Oral administration of BO-264 at 50 mg/kg dose did not cause any significant toxicity determined with mice body weight change (**Figure 4.28 B**).

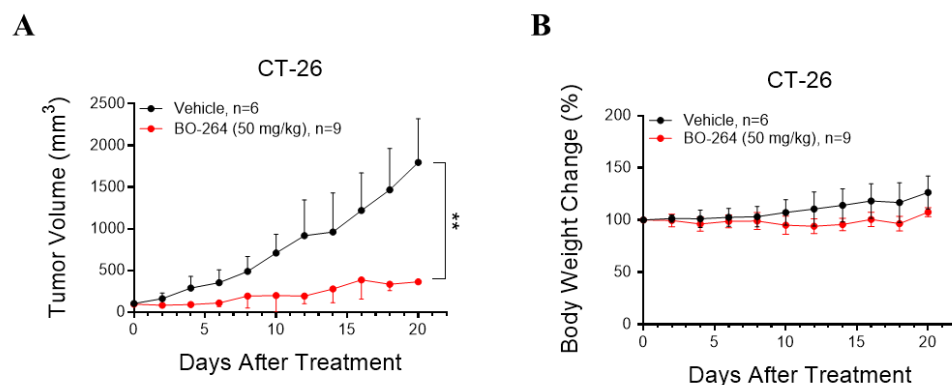


Figure 4.28. Targeting TACC3 with BO-264 significantly impairs tumor growth in immunocompetent colorectal carcinoma mouse model. (A) Tumor volume change of CT-26 syngeneic mice after the treatment with vehicle or 50 mg/kg BO-264 in female balb/c mice. Treatments were started when tumors reached to 90-100 mm³ and performed daily *via* oral gavage. **: $p < 0.01$. (B) Body weight change (%) of nude mice bearing CT-26 tumors treated with vehicle or BO-264 (50 mg/kg).

Lastly, we aimed to determine the toxicity of higher doses of BO-264. For this purpose, firstly female nude mice received 100 mg/kg BO-264 orally and daily for 7 days, and body weights are recorded (**Figure 4.29 A**). BO-264 did not change body weight significantly and did not create any observable toxicity at that high dose. Secondly, female nude mice were given 500 mg/kg of BO-264 orally only once and monitored for 1-3 days. Mice receiving BO-264 had a 10% loss of their body weight 24 h after drug treatment, but their general condition was stabilized (**Figure 4.29 B**). Organs were collected at the end of the experiment, and we did not observe any organ toxicity, indicating that BO-264 was well-tolerated at this high dose (**Figure 4.29 C**).

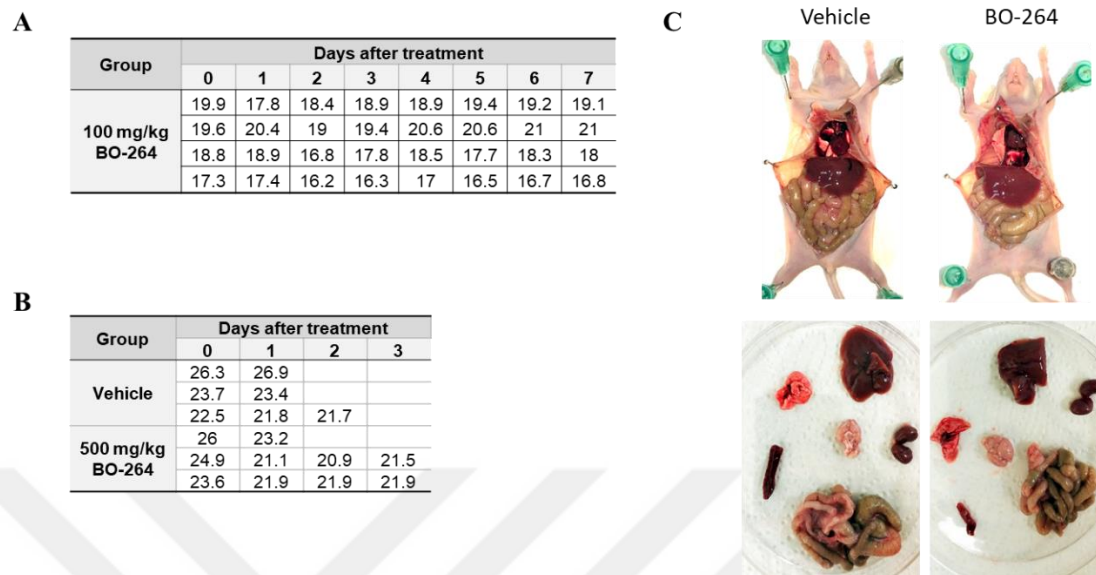


Figure 4.29. In vivo toxicity analysis upon BO-264 administration. (A) Mice body weight (g) after daily oral administration of 100 mg/kg BO-264 for 7 days. (B) Mice body weight (g) after a single-dose administration of BO-264 (500 mg/kg). Mice were sacrificed at different days. (C) Photos of mice and their organs (lower panel) treated with either vehicle or 500 mg/kg BO-264. They were sacrificed 48 hours after drug treatment.

Collectively, these data clearly show that oral administration of BO-264 significantly inhibited the tumor growth in both immunodeficient and immunocompetent mice and supports its potential development as a novel therapeutic agent not only for breast cancer, but also for colon cancer treatment.

4.7. BO-264 has favorable but optimization-needed preclinical ADME and pharmacokinetic profile

Based on overall profile of BO-264 in *in vitro* and *in vivo* studies, it was further evaluated for its ADME (absorption, distribution, metabolism and excretion) and pharmacokinetic

properties, which are important for selection and administration of a new drug candidate during drug discovery and development [149].

BO-264 was first tested in terms of its physiochemical properties and metabolic stability. It was found to have a medium lipophilicity with a log $D_{7.4}$ of 2.3 and demonstrated low solubility as well as low microsomal stability in both human and mouse liver microsomes, and high plasma protein binding (unbound fraction of 1.13%), but good Caco-2 permeability with a low efflux ratio ($AB=190 \times 10^{-6}$ nm/s, $ratio \leq 2.0$). We also characterized the drug-metabolizing liver enzymes of the system cytochrome P450 (CYP450) for their possible inhibition by BO-264 in human liver microsomes in order to evaluate the potential drug-drug interactions [150]. Accordingly, BO-264 was a moderate inhibitor of CYP2C9 ($IC_{50} = 2.63 \mu M$) and CYP3A (testosterone as substrate; $IC_{50} = 8.59 \mu M$) whereas it was a weak or not an inhibitor of CYP2CD6 ($IC_{50} = 18.46 \mu M$) and CYP3A ($IC_{50} =$ midazolam as substrate; $30.04 \mu M$), indicating that BO-264 has low activity on the tested P450 cytochromes. All these properties were summarized in **Table 4.2**.

Table 4.2. Summary of drug specific physicochemical properties

Molecular Weight (g/mol)	353.38			
Solubility at pH 7.4 (μM)^a	< 1			
LogD at pH 7.4^b	2.31			
Human PPB (%bound)^c	98.87			
T_{1/2} (min)^d	5 _(MLM)		12 _(HLM)	
Cl_{int} ($\mu\text{L}/\text{min}/\text{mg}$ protein)^e	329.2 _(MLM)		117.5 _(HLM)	
CYP (IC₅₀ μM)^f	<i>CYP2C9</i>	<i>CYP2D6</i>	<i>CYP3A4</i>	<i>CYP3A4</i>
	2.63 $\pm$ 0.19	18.46 $\pm$ 0.23	30.04 $\pm$ 4.24	8.59 $\pm$ 1.68
Caco-2 P_{app} (nm/s)^g	190.60 $\pm$ 61.82 _(A$\rightarrow$B)		241.03 $\pm$ 8.18 _(B$\rightarrow$A)	

^a Kinetic solubility in sodium phosphate buffer at pH 7.4 at 25°C after 2 h [151]. ^b LogD: distribution coefficient in octanol/sodium phosphate buffer (50 mM, pH 7.4) [152]. ^c PPB: plasma protein binding assessed by equilibrium dialysis in human at 37°C [151]. ^d T_{1/2}: half-life in mouse and human liver microsomes (MLM and HLM respectively) [153]. ^e Cl_{int}: intrinsic clearance in MLM and HLM [153]. ^f CYP: cytochrome P450 isozymes inhibition by BO-264 evaluated in HLM [154]. ^g P_{app}: apparent permeability coefficients in Caco-2 cell monolayer [155].

In order to evaluate pharmacokinetic profile including area under curve (AUC), maximum drug concentration in plasma, time to reach the maximum concentration, half-life, distribution volume, clearance and bioavailability of low dose BO-264, we monitored the *in vivo* change in plasma of female CD-1 mice. A single intravenous administration of BO-264 (5 mg/kg) to mice resulted in 894.7 ng*h/ml mean plasma exposure while it was 377.7 ng*h/ml after a single oral gavage administration (25 mg/kg) (**Table 4.3**). In addition, the terminal plasma half-life was decided as 0.881 h and 7.19 h for iv. and po. administrations, respectively. AUC_(0-inf), representing the total drug exposure across time

[156], was found to 896.7 h*ng/ml for iv. while it was 530.7 h*ng/ml for po. administrations. Bioavailability (%F), the parameter to reach the desirable plasma and organ to produce the pharmacological effect, was found as 11.8 (**Table 4.3**). It is mainly defined by solubility and permeability of the compounds after oral administration [157]; therefore, increasing these parameters through optimization steps might enhance pharmacokinetics and physicochemical properties.

Table 4.3. Pharmacokinetic parameters of BO-264 following intravenous and oral gavage administration of BO-264 dose formulations in female CD-1 mice

Treatment	Route	Dose (mg/kg)	T _{max} (h)	C ₀ (ng/mL)	C _{max} (ng/mL)	AUC _(0-last) (h*ng/mL)	AUC _(0-inf) (h*ng/mL)	CL (mL/hr/kg)	V _{ss} (mL/kg)	T _{1/2} (h)	% F ^a
BO-264	IV	5	0.083	3183	2296	894.7	896.7	5575.82	7089.4	0.881	11.8
	PO	25	0.25	NA	433.7	377.7	530.7	41107.31	488.61	7.19	

^a: Nominal doses and AUC_(0-inf) of IV and PO were used for oral bioavailability calculation; NA: Not applicable; V_{ss}: volume of distribution at steady state; oral bioavailability: %F. IV formulation: 0.05% HPMC, 2N HCl, pH 1-2. Oral (PO) formulation: 0.05% HPMC, 0.3 g Tween 80, 2N HCl, pH 1-2. (n=25)

5. DISCUSSION

5.1. TACC3 expression in cancer and its association with disease progression

TACC3, an oncogenic member of the TACC family of microtubule- and centrosome-binding proteins, emerges as an attractive anticancer target that is mainly associated with disrupted microtubule stability and deregulated mitosis [61] causing worse prognosis in a broad spectrum of cancers [81, 82, 86, 99]. In line with the literature, we showed that TACC3 is significantly upregulated in tumor tissues of TCGA patients from different cancers compared to their normal counterparts. Its oncogenic persistence independent of tissue type makes it an attractive general target since other TACC family members (TACC1 and TACC2) either showed a reverse expression pattern between tumor and normal, i.e. upregulated in normal tissues, or the expression levels were not significantly different between tumor and normal. In addition, we corroborated our findings with other online available clinical patient data where we were able to show high TACC3 expression significantly resulted in worse overall survival of breast, lung and gastric cancer patients. We also demonstrated that higher grade tumors had higher TACC3 expression, further showing the effect of TACC3 levels on cancer aggressiveness. Therefore, in this dissertation we propose that targeting TACC3 to suppress its oncogenic functions could be a promising strategy for cancer treatment.

5.2. Targeting TACC3 as a therapeutic strategy for cancer

It has been shown that the inhibition of TACC3 using conditional TACC3 knock-out mice leads to tumor regression of thymic lymphomas which is accompanied by massive

apoptosis *in vivo* [90]. KHS101, a small molecule TACC3 inhibitor, was first identified to promote neuronal differentiation in rats [109] and has been shown to reduce tumor growth of glioblastoma xenografts [110]. Moreover, SPL-B, another TACC3 inhibitor, was demonstrated to inhibit the centrosome microtubule nucleation in ovarian cancer cells and suppress tumor growth in ovarian cancer xenografts [84]. However, these compounds targeting TACC3 remained as useful chemical probes to study TACC3 functions due to their low oral systemic stability (KHS-101) and high working doses (both KHS-101 and SPL-B). Given the strong clinical association of TACC3 with tumor aggressiveness and a strong prognostic power in several different cancers, developing a TACC3 inhibitor with high efficacy and better drug-like properties, will be of high significance. Therefore, we developed BO-264 as a novel TACC3 inhibitor with high and superior potency on different cancer cells compared to KHS101 and SPL-B *in vitro*. We have also demonstrated that BO-264 is also superior to SPL-B *in vivo*, significantly suppressing tumor growth in breast cancer xenografts, supporting its potential to be used as a promising therapeutic strategy in future cancer treatment.

Microtubule targeting agents (MTAs) interfering with microtubule dynamics are widely used in different clinical settings and are one of the most effective of chemotherapeutics (44). Unfortunately, these drugs mostly lack cancer cell specificity, causing cell death also in non-tumorous cells which, in turn, leads to severe side effects. Furthermore, patients treated with MTAs frequently develop resistance which limits their long-term clinical use (45). Therefore, therapeutic strategies which specifically and efficiently target cancer cells and spare normal cells become more important as it is anticipated to reduce toxicity [31]. Since TACC3 is enrolled in stabilization of

kinetochore fibers of mitotic spindles together with ch-TOG and clathrin complex [71], TACC3-targeting agents have a potential to emerge as cancer-specific microtubule inhibitors that might function in a similar fashion with MTAs in terms of the anti-tumorigenic effects. However, they are expected to cause less side effects since normal tissues do not have strong TACC3 expression levels. In this line, BO-264 exhibited cancer-cell specific growth inhibition tested in different cancer cells while it spared normal breast epithelial cells, MCF-12A. This effect is also consistent with a previous study showing that TACC3 deletion causes a massive apoptotic regression of mouse thymic lymphoma while it does not induce any abnormalities in normal tissues [90]. Moreover, Ohoka and his friends showed proteasomal degradation of TACC3 using SNIPER (TACC3) greatly reduced cancer cell viability whereas it minimally affected that of normal human fibroblasts [158]. It is also noteworthy that BO-264 showed promising anti-tumor effect with no major organ toxicity and did not affect the body weight in both breast and colon cancer models. We also tested very high doses of BO-264 (100-500 mg/kg) for short time in mice and did not observe any sign of major toxicity compared to vehicle-treated group. Therefore, being selective in favor of normal cells makes BO-264 a promising molecule to be developed and emphasize its cancer-specific profile, further supporting the connection between BO-264 and TACC3.

We showed a significant TACC3 overexpression in tumor tissues of various cancers compared to their normal counterparts using the TCGA and other published data, as well as in breast cancer cell lines as compared with a normal breast cell line, MCF-12A. This is further evidence that BO-264 specifically and efficiently target cancer cells which has high TACC3 levels. In line with this, ectopic TACC3 expression in MCF-12A

cells, whose basal TACC3 level is very low, enhanced cell survival. More importantly, TACC3 overexpression in these cells rendered them sensitive to growth inhibition by BO-264, further validating the specificity of our inhibitor. Furthermore, we detected a trend towards higher TACC3 expression in more aggressive breast cancer subtypes, basal and HER2+, compared to luminal A subtype, and they were more sensitive to TACC3 inhibition with BO-264. This also further supports our findings that higher TACC3 levels are found in higher tumor grade patients. Overall, these results suggest that TACC3 expression might be a determinant (but not the sole determinant) of BO-264 response, which might further help in stratifying patients that will benefit most from TACC3 inhibition by BO-264.

We found out that BO-264 treatment leads to a strong and dose-dependent induction of multipolar spindle formation and reduction in centrosomal TACC3 levels during mitosis and interphase, which suggest that BO-264 strongly affects centrosomal functions of TACC3 [72]. Centrosome amplifications are frequently observed in cancers and are strongly associated with cancer aggressiveness [159], which in turn may lead to multipolar spindle formations, increase in aneuploidy and errors in chromosome segregation [160]. Cancer cells with supernumerary centrosomes displaying centrosome amplification and multipolar spindles tend to cluster their extra centrosomes to produce a bipolar spindle before anaphase onset to prevent aneuploidy [159, 161], which is a vital process for cancer cells. Therapeutic strategies that target centrosome clustering have been suggested to be highly effective and promising in terms of mediating specific cell killing in the highly aggressive, centrosome-amplified tumors [162]. Previously, TACC3 has been shown to be involved in centrosome clustering through its Aurora-A mediated

phosphorylation regulated by integrin-linked kinase (ILK) [163]. In line with the literature, our findings show a strong induction of multipolar spindle formations and reduction in centrosome localization of TACC3 upon BO-264 treatment in JIMT-1 cells, which harbors amplified centrosomes [164], further supporting the importance of TACC3 for centrosome integrity and suggesting that TACC3 might be a good candidate for cells displaying centrosome amplification.

5.3. Targeting cells with FGFR3-TACC3 fusion using BO-264

We showed that BO-264 effectively inhibits the growth of cancer cell lines harboring the highly oncogenic FGFR3–TACC3 fusion protein. FGFR3-TACC3 oncogenic fusion proteins are detected in numerous solid malignancies and was shown to drive tumorigenesis by stimulating constitutive activation of FGFR signaling [95]. Due to their uniqueness, they are also emerging as attractive therapeutic targets which will enable selective targeting of fusion-harboring cancers. Balversa (erdafitinib), an FGFR family kinase inhibitor, has been recently FDA-approved for the treatment of patients with metastatic urothelial carcinoma with FGFR alterations, including FGFR3-TACC3 fusion [165]. However, response rates to FGFR inhibition are still limited to around 40% (3% with a complete response and 37% with a partial response) [165], suggesting that targeting TACC3 alone or in combination with FGFR inhibition might provide a superior anti-cancer activity in tumors with FGFR3–TACC3 fusion. We demonstrated that BO-264 inhibits proliferation in two different FGFR3-TACC3 fusion-carrying models to different extend potentially depending on their TACC3 levels, suggesting that our inhibitor might represent a novel strategy to combat FGFR3–TACC3 fusion-carrying cancers. In line with

this, KHS101, another TACC3 inhibitor, has been shown to suppress the FGFR3-TACC3 fusion protein, and dual inhibition of FGFR with a phase III panFGFR inhibitor, BGJ398 (infigratinib), and TACC3 with KHS101 synergistically inhibited cell growth through inhibition of ERK and AKT phosphorylation [166]. Considering the superior effect of BO-264 to KHS101 and decrease in ERK phosphorylation by BO-264 at low doses, combination of FGFR inhibitors with BO-264 might provide better efficiency towards suppression of tumor growth in FGFR3-TACC3 fusion protein-harboring patients. Moreover, drug resistance is one of the major problems limiting the efficacy of FGFR inhibition [167, 168] as in the case of FGFR3-TACC3 fusion whose key activating FGFR3 tyrosine residues are constitutively phosphorylated [169]. Considering the patient response to most advanced/approved FGFR inhibitors which is reported to be 25.4% for BGJ398 [170] and aforementioned response for erdafitinib, there are still a large patient population who are not responsive to the FGFR inhibition due to intrinsic resistance to FGFR TKIs. Therefore, dual suppression of FGFR and other signaling pathways; here TACC3 downstream by using BO-264, to overcome possible resistance mechanisms could be a better strategy rather than using a single-agent FGFR inhibitor, which might in turn enhance the sensitivity of the therapy.

FGFR3 and TACC3 genes are fused together at different breakpoints, which are found to occur within exon 16 to 19 of FGFR3 containing active kinase domain and within exon 4 to 13 of TACC3 containing TACC domain, which is highly conserved in all family members and distributed over the last seven exons (out of 16) [171, 172]. Moreover, Stefano and colleagues identified 9 different fusion breakpoints even in the cases which had the same FGFR3-TACC3 transcript splice variants, showing the noticeable diversity

of fusion transcripts and breakpoints [102]. A detailed breakpoint/fusion analysis was reported with TCGA data where FGFR3 and TACC3 fuse at 38 different positions [114]. Previously, it has been reported as different TACC3 breakpoints led to altered cell transforming capacity. Overexpression of one of the most commonly identified FGFR3-TACC3 fusion breakpoint (exon 18 of FGFR3 to exon 8 of TACC3) resulted in 3-fold higher cell transformation ability than the one harboring fusion protein with exon 18 of FGFR3 to exon 11 of TACC3 [98]. Therefore, the position where FGFR3 or TACC3 inhibitors target is important for drug efficiency. Since, BO-264 was effective in FGFR3-TACC3 fusion-harboring cell lines (RT112 with FGFR3 exon 18 and TACC3 exon 11; RT4 with FGFR3 exon 18 and TACC3 exon 4) and was able to decrease ERK1/2 phosphorylation, which is a marker for activated FGFR signaling, we estimated that BO-264 suppress FGFR3-TACC3 fusion protein which retains TACC domain. However, to understand the exact mechanism of action of BO-264 on FGFR3-TACC3 fusion protein, more studies are needed showing which domain(s) of TACC3 is interacting with BO-264.

5.4. Optimizing Drug Metabolism and Pharmacokinetics of BO-264

It has been known that TACC3 inhibition leads to tumor regression *in vivo* [90]. Therefore, the efficacy of BO-264 was tested on tumor growth of cancer xenografts/syngeneic models. Showing a dose-dependent tumor growth suppression in JIMT-1 xenografts (**Figure 4.21** and **Figure 4.22**) and significant efficacy also in other models prompted us to further characterize drug-like properties of BO-264 through its pharmacokinetics and physiochemistry analysis.

Several studies demonstrated that oral drug absorption and permeability coefficient have positive correlations [173]. Its high permeability through intestinal epithelial cell barrier measured with Caco-2 cell lines and low cytochrome P450 isozymes (CYP) inhibition in human and mice liver microsomes for potential drug-drug interactions and metabolism give meaningful physical picture for the BO-264 use. Lipophilicity was another important physicochemical property which BO-264 was tested for. It plays significant role for development of a potential drug in terms of solubility, membrane penetration, distribution and clearance [174]. Drug metabolizing enzymes of the cytochrome P450 system promote the conversion of lipophilic compounds to more hydrosoluble metabolites, which can be then easily removed from the body [157]. BO-264 has a moderate logD value, a way to represent lipophilicity, which might explain the low solubility and high clearance rate. Earlier, only drug candidates with high solubility were preferred for further implications; however, renewed rational drug design understanding now allows the use of poorly water-soluble drugs with efficient lipophilicity [175]. Therefore, optimizing lipophilicity in terms of providing an efficient interaction with the target, we can achieve a better solubility and stability of the drug.

Bioavailability of BO-264 was found to be 11.8%, which can be classified as low [176]. Bioavailability is the fraction of an oral dose, composed of parent compound or active metabolite that reaches either site of action or systemic circulation [177]. The intake of drugs with food simultaneously or after food might have strong impacts on drug release, ADME properties and bioavailability due to altered gastrointestinal tract's physiology, which might in turn affect the efficacy and safety of the treatment [178]. Possibly due to reduced diffusivity of drug in the viscous postprandial upper gastrointestinal tract, the

bioavailability might decrease when treatment is received after food intake [179]. Considering our PK study where the mice were fed with regular *ad libitum* diet, there is high possibility that the bioavailability was measured low. Therefore, conducting the PK studies when the mice are fasted might lead to increased bioavailability of BO-264. Despite low bioavailability, we have high potency *in vitro* and high efficacy *in vivo*. The possible explanation would be due to high V_{ss} (steady state volume of distribution) indicating high drug distribution into tissues which then leads to high and fast impact on the phenotype [180]. As indicated in a study, drugs with large volume of distribution are preferable to obtain a long terminal half-life, and it is also valid for the drugs which have relatively high clearance [181]. Moreover, considering IC_{50} of BO-264 on JIMT-1 cells (0.19 μ M) and significant tumor growth inhibition of JIMT-1 xenografts at 25 mg/kg BO-264 oral dose, C_{max} (maximum plasma concentration) obtained from a single oral administration (25 mg/kg) of BO-264 to CD-1 nude mice was found as 433.7 ng/ml (1.23 μ M), which could be sufficient to sustain the target inhibition and achieve therapeutic efficacy [182]. However, to better characterize the drug efficacy and evaluate its relationship with pharmacokinetic parameters, a detailed pharmacokinetic analysis composed of several doses should be assessed.

Importantly, this study is only the first step for finding a novel TACC3 inhibitor. While maintaining the potency and *in vitro/in vivo* non-toxicity, inhibitor-lead optimizations offering improvements to physicochemical properties and pharmacokinetics will be the focus of further studies. Optimization of compounds could be done by directly manipulating the functional groups of the chemical compound, implementing pharmacophore-oriented molecular design or establishing the structure-

activity relationship (SAR) to ensure more rational optimization [183]. Bioisosteric replacement methods, which is swapping functional groups with similar biological properties, and scaffold hopping, which is the replacement of core framework with another scaffold structure, are also preferred for improving the potency, drug-like properties and synthetic accessibility of compounds [184]. Zask and his colleagues achieved dramatic improvements in mTOR-targeting selectivity only by changing morpholine residues in their hit compounds [185]. Similarly, addition of a morpholine substitution to the structure of the YU-101, a potent proteasome inhibitor, led to the improvement in ADME properties, especially water solubility, which eventually resulted in discovery of Carfilzomib, an approved drug for the treatment of multiple myeloma [183]. Therefore, BO-264 can be developed further by incorporating different morpholines which hopefully lead to improved ADME and PK profiles, which might then promote better potency and efficiency. Furthermore, other chemical modifications to a salt form or to a pro-drug could reduce first-pass metabolism and enhance the bioavailability [175]. Overall, due to its high potency and minimum toxicity, BO-264 may serve as an excellent chemical tool to dissect the role of TACC3 in various cancers and as a promising chemotype for optimization for future drug development.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

This dissertation has identified a novel TACC3 inhibitor which is amenable to be developed as a promising anti-cancer drug candidate for breast, colon and possibly other cancer types with high TACC3 levels. In this thesis, I have elucidated the oncogenic profile of TACC3 mainly in breast and colon cancers. I have identified a new TACC3-targeting chemotype which demonstrates superior effects compared to the available TACC3 inhibitors. Moreover, I have unveiled the underlying mechanism of action of BO-264 which is induction of spindle assembly checkpoint-dependent mitotic arrest, DNA damage and apoptosis.

BO-264 (3-(4-methoxyphenyl)-N-(2-morpholinopyrimidin-4-yl)isoxazol-5-amine) was identified as a novel TACC3 inhibitor through our in-house screening of several different small-molecule compounds obtained *via* rational drug design in cytotoxicity assays combined with several biochemical and cell-based assays. We showed that BO-264 causes formation of aberrant, mostly multipolar spindles *via* SAC activation and reduction in centrosomal TACC3 expression/location in aggressive breast cancer models. This further leads to mitotic arrest, DNA damage and apoptosis at very low doses compared to other available TACC3 inhibitors. These phenotypes are recapitulated the siRNA-mediated knockdown of TACC3, indicating on-target specificity of BO-264. This specificity is further supported by shRNA- and siRNA-mediated knockdowns of TACC3 where BO-264 was not able to further induce mitotic arrest, DNA damage, apoptosis and formation of aberrant spindles, in the absence of TACC3. Oral administration of BO-264 in aggressive breast and colon cancer xenograft as well as syngeneic models showed a

significant suppression of tumor growth and improved overall survival of tumor-bearing mice without any major organ toxicity, showing a strong preclinical support for targeting TACC3 in aggressive cancers. Collectively, this superior cytotoxicity and anti-tumorigenic activity of BO-264 provides a novel anti-cancer drug candidate for future development for the treatment of breast, colon and potentially other cancers with high TACC3 levels.

Direct interaction between BO-264 and TACC3 was validated with different biochemical assays including DARTS, CETSA and ITC. However, none of these assays describes the position of binding. Identification of druggable cavities on protein surface is an important step to determine the binding site. Since TACC3 is lacking a complete co-crystal 3D structure, structure based virtual screening *via* docking simulations will be difficult to assess. However, TACC domain of TACC3 was previously shown to play a crucial role for TACC3 binding to the transcriptional coregulator FOG-1 [186] and a transcriptional effector HIF-1 β (ARNT) PAS-B domain for transcriptional responses to hypoxia using secondary structure and coiled-coil predictions [80]. Therefore, detailed 3D analysis of TACC3 using NMR spectroscopy, X-ray crystallography and biochemical methods combined with *in silico* docking simulations will be helpful to understand the full structure and identify the drug-binding site. To find the critical regions for interaction, truncated versions of TACC3 could be created and the binding can be tested using previously mentioned assays. Overall, determining the TACC3-BO-264 binding site is of great significance to better understand and explore the oncogenic function of TACC3 in cancer cells.

TACC3 is one of the central players in centrosome regulation making an important complex with ch-TOG and clathrin to stabilize kinetochore fibers by inter-microtubule bridging [71]. In addition to these TACC3 partners, several other context-specific TACC3 interactome were identified. We showed that the downstream pathways such as mitotic arrest, DNA damage and apoptosis activated by genomic knockdown of TACC3 using siRNAs were also recapitulated with pharmacological inhibition of TACC3 with BO-264. However, we still do not know whether it is also targeting TACC3 interactome or the protein-protein binding between its partners. Co-immunoprecipitation experiments might help to elucidate how the physical interactions between TACC3 and its partners be affected by BO-264 [187]. As mentioned before, TACC3 is activated upon phosphorylation by Aurora A kinase which then recruits TACC3 to microtubules [72]. It will be interesting to test whether BO-264 affects TACC3 phosphorylation or other possible post-translational modifications (PTMs), which in turn negatively affects its activation or proper functioning. It is, therefore, of importance to test its detailed action mechanism whether BO-264 is targeting context dependent TACC3 interactome or its PTMs. Furthermore, TACC3 has been shown to regulate interphase-related processes, such as transcription or cell migration, including regulation of interphase microtubule dynamics that influence kinesin-1 based cargo trafficking and cell shape [78, 188, 189]. Therefore, the effect of BO-264 on interphase functions of TACC3 need to be tested in future.

Depletion of TACC3 in cancer cells has been associated with induction of mitotic arrest and DNA damage. We demonstrated a high potency of BO-264 at nanomolar levels in *in vitro* experiments, which was accompanied by SAC-dependent mitotic arrest, DNA

damage and apoptosis. BO-264 also induced aberrant spindle formations, mainly multipolar spindles. These phenotypes phenocopied the siRNA or shRNA-mediated knockdowns, further showing the on-target specificity of BO-264. However, BO-264 similar to any given compound may still have off-targets e.g. different kinases, ion channels, nuclear receptors, drug transporters etc. Notably, BO-264 has diaminopyrimidine (DAP) fragment in its structure which is commonly observed in kinase inhibitors [190]. Therefore, to gain a better insight into its specificity, the binding profile of BO-264 can be tested using kinome panels as well as CEREP panels, containing receptors, enzymes, transporters and ion channels. Any possible off-target effect, i.e., modulation of other targets, might give rise to adverse effects. Although, BO-264 was shown not to cause any cytotoxicity in normal breast epithelium cell lines at low doses and no major organ toxicity in tumor bearing mice, it is still very important to identify potential off-targets for future development of the compound.

Showing promising *in vitro* and *in vivo* results, drug-like properties of BO-264 was evaluated with respect to metabolic stability, solubility, permeability and drug-drug interactions. BO-264 demonstrates high Caco-2 permeability with a low drug efflux ratio ($AB=190 \times 10^{-6}$ nm/s, $ratio \leq 2.0$) and a medium lipophilicity with a $\log D_{7.4}$ of 2.3. It is also a moderate inhibitor of CYP2C9 and CYP3A (testosterone as substrate) whereas it is a weak or not an inhibitor of CYP2CD6 and CYP3A (midazolam as substrate), showing that BO-264 has low activity on drug-metabolizing liver enzymes. However, it has low solubility, high clearance rate and high plasma protein binding (unbound fraction of 1.13%), which then may result in low systemic exposure. In addition to ADME profiling of BO-264, its pharmacokinetics were also evaluated where it has low bioavailability

(11.8%) which needs to be further improved with future studies. Inhibitor-lead optimizations with structural modifications using structure-property relationships while maintaining the potency and *in vitro/in vivo* effects is important to improve the physicochemical and metabolism related properties. Modifications on the functional groups can be considered to improve drug-like properties and enhance pharmacokinetic profiles which might, in turn, increase efficacy and help to minimize the potential safety liabilities due to formation of toxic substances or metabolites [191-193]. During drug development, elimination of off-target effects and hence toxicities is an important issue to be addressed. Otherwise it can prevent effective dosing of possible efficacious drugs. Small molecule inhibitors have been previously shown to bind to several different targets (minimum 6-11), in addition to their real target [194]. Therefore, off-target binding studies might help to identify unrelated targets, and also help defining mechanism of action through related targets. When a promising compound is identified during these steps, it will progress into preclinical development including animal safety and efficacy studies and then clinical development, which will be ultimately in the market [191, 195]

To sum up, the strong cytotoxic and antitumorigenic effects presented in this dissertation for aggressive breast and colon cancer models without any apparent organ toxicity provides a strong preclinical support for developing this inhibitor further for future clinical tests to treat breast, colon, and potentially other cancers.

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