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Activity of the Kinesin Spindle Protein Inhibitor *Ispinesib* (SB-715992) in Models of Breast Cancer

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Translational Relevance

Microtubule targeted therapies form an integral part of treatment regimens for breast cancers, most often in combination with other therapies. Of the clinically limiting toxicities of microtubule-targeted therapies, neuropathy is uniquely unrelated to anti-proliferative drug action. Inhibitors of Kinesin Spindle Protein (KSP) have emerged as candidate non-neurotoxic anti-mitotic cancer therapies. One of these KSP inhibitors, *ispinesib*, has been evaluated in a Phase II clinical trial in women with locally advanced or metastatic breast cancer, producing several partial responses. In this report we evaluate the activity of *ispinesib* as a single agent in models of several breast cancer subgroups and examined tolerability and efficacy of *ispinesib* combined with various standards-of-care. This is the first study to explore activity of a KSP inhibitor in models of breast cancer, and to identify attractive KSP inhibitor drug combinations. Our results highlight clinical settings in which KSP inhibitors may be of clinical utility.

Abstract

Purpose: *Ispinesib* (SB-715992) is a potent inhibitor of kinesin spindle protein (KSP), a kinesin motor protein essential for the formation of a bipolar mitotic spindle and cell cycle progression through mitosis. Clinical studies of *ispinesib* have demonstrated a 9% response rate in patients with locally advanced or metastatic breast cancer, and a favorable safety profile without significant neurotoxicities, gastrointestinal toxicities or hair loss. To better understand the potential of *ispinesib* in the treatment of breast cancer we explored the activity of *ispinesib* alone and in combination several therapies approved for the treatment of breast cancer.

Experimental Design: We measured the *ispinesib* sensitivity and pharmacodynamic response of breast cancer cell lines representative of various subtypes *in vitro* and as xenografts *in vivo*, and tested the ability of *ispinesib* to enhance the anti-tumor activity of approved therapies.

Results: *In vitro*, *ispinesib* displayed broad anti-proliferative activity against a panel of 53 breast cell-lines. *In vivo*, *ispinesib* produced regressions in each of five breast cancer models, and tumor free survivors in three of these models. The effects of *ispinesib* treatment on pharmacodynamic markers of mitosis and apoptosis were examined *in vitro* and *in vivo*, revealing a greater increase in both mitotic and apoptotic markers in the MDA-MB-468 model than in the less sensitive BT-474 model. *In vivo*, *ispinesib* enhanced the anti-tumor activity of *trastuzumab*, *lapatinib*, *doxorubicin*, and *capecitabine*, and exhibited activity comparable to *paclitaxel* and *ixabepilone*.

Conclusions: These findings support further clinical exploration of KSP inhibitors for the treatment of breast cancer.

Introduction

Chemotherapy remains a cornerstone in the treatment of breast cancer. Microtubule-targeted anti-mitotic agents feature prominently in therapeutic regimens. Among these, the taxanes *paclitaxel* and *docetaxel* form an integral part of most adjuvant therapy regimens, and play a major role in the treatment of metastatic disease. Additional microtubule-targeted therapies include *vinorelbine*, *vinblastine* and the recently approved *ixabepilone*. These therapies are usually administered as part of a combination regimen with an anthracycline (*doxorubicin*), an antimetabolite (*capecitabine*), a platinum (*carboplatin*), or with HER2-targeted therapy such as *trastuzumab*.

Therapeutic regimens containing microtubule-targeted agents often produce clinically limiting toxicities including myelosuppression, neuropathy, alopecia and gastrointestinal toxicities (1, 2). Neuropathy is the only toxicity unrelated to anti-proliferative activity, and is likely due to the effects of these drugs on neuronal microtubules (3). One strategy to identify novel anti-mitotic cancer therapies with improved tolerability profiles is to target mitosis-specific enzymes, eliminating target-related neurotoxicities (4).

Kinesin Spindle Protein (KSP) is a motor protein with an exclusive and essential role in mitosis (5-7). It is required early in mitosis to separate the centrosomes of the emerging spindle poles, thus driving establishment of a bipolar mitotic spindle. Failure to establish a bipolar spindle results in an extended cell cycle delay in mitosis, after which cells may experience a variety of fates including abnormal exit from mitosis, resumption of the cell cycle and apoptosis (8-12). The essential role of KSP in cell cycle progression through

mitosis in normal and tumor cells alike suggests that anti-tumor activity of KSP inhibitors is most-likely attributable to post-mitotic response pathways that remain poorly understood.

Ispinesib (SB-715992), an allosteric small molecule inhibitor of KSP kinesin motor ATPase (13), was the first small molecule inhibitor of KSP that advanced to cancer clinical trials. Results from multiple phase I and II clinical studies of *ispinesib* in patients suffering from a variety of cancers confirm the absence of significant neurotoxicities, alopecia or gastrointestinal toxicities (14-22). The most common toxicity associated with *ispinesib* was neutropenia, with a reproducible nadir 6-8 days following administration and full recovery by day 14.

In a preliminary Phase II trial in women with locally advanced or metastatic breast cancer progressing despite an anthracycline and a taxane treatment, *ispinesib* was administered once every 21 days and produced a response rate of 9%, with reductions in tumor size of 46% to 69% and response durations of 6.9 to 19.1 weeks (20). A Phase I/II is ongoing in patients with locally advanced or metastatic breast cancer previously untreated with chemotherapy for advanced disease, administering *ispinesib* on a 2 week schedule (23).

We have evaluated the activity of *ispinesib* as a single agent in models of specific breast cancer subgroups (ER positive, Her2 positive, triple negative) and examined tolerability and efficacy of *ispinesib* combined with various standards-of-care for breast cancer.

This study represents the first in depth biological examination of KSP inhibition in breast cancer. Our findings support the ongoing clinical investigation of *ispinesib* as a treatment option in breast cancer patients.

Materials and Methods

Cell culture

Cell lines were obtained from ATCC and from collections developed by Drs. Steve Ethier and Adi Gazdar. KPL4 was kindly provided by Dr J. Kurebayashi (Kawasaki Medical School, Kurashiki, Okayama, Japan). Cell culture reagents were from Cellgro-Mediatech (Herndon, VA).

Cell growth inhibition and data analysis

Cells were plated in log phase of growth in 96-well plates and treated for 72h with a range of *ispinesib* concentrations from 3.3×10^{-5} to 8.5×10^{-11} M. Cell growth was measured using Cell Titer Glo assays (Promega, Madison, WI) and luminescence read-out was recorded using BIO-TEK FLx800. Data were analyzed according to the method described previously by the NCI/NIH DTP Human Tumor Cell Line Screen Process (<http://dtp.nci.nih.gov/branches/btb/ivclsp.html>) (24). The % growth curve is calculated as $[(T-T_0)/(C-T_0)] \times 100$, where T_0 is the cell count on day 0, C is the vehicle control (e.g. 0.3% DMSO without drug) cell count on day 3, T is the cell count at the test concentration. The GI_{50} value is the drug concentration that results in 50% growth after 72h of drug exposure.

Western blot analyses

Cells were treated with 150nM *ispinesib* (3 to 7-fold GI_{50} values) and lysed in RIPA buffer, (50mM Tris pH7.5, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1%

SDS, 1% complete protease inhibitor cocktail [Roche Biochemicals, Indianapolis, IN]). 5ug of protein were separated on 4-20% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred onto PVDF membranes using Invitrogen's iBlot system. Primary antibodies for Bax, Bid, xIAP, Bcl2, p-Bcl2 (Ser70), and Bcl-X_L (54H6) were from Cell Signaling (Danvers, MA). Other primary antibodies used were: PARP (BD Pharmingen, San Diego, CA), GAPDH-6C5 (Santa Cruz Biotechnology, Columbia, SC), cyclin-B and cyclin-E-HE12 (Upstate-Millipore, Billerica, MA). Secondary antibodies were infra-red 680/800CW Licor (Lincoln, NE) and signal detection and analysis were performed on a Licor-Odyssey imaging system.

DNA cell cycle analysis by flow cytometry

Cells were treated with 150nM *ispinesib*, fixed in 85% ice-cold ethanol, resuspended in PBS containing 10ug/ml propidium iodide DNA stain (Sigma Aldrich, St. Louis, MO) and 250ug/ml RNase A (Sigma Aldrich), and analyzed with a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell cycle analyses were performed with FLOWJO (Treestar Inc, Ashland, OR).

Xenograft studies

Protocols for xenograft studies were approved by the Cytokinetics Institutional Animal Care and Use Committee (IACUC). Female mice (7-8 weeks) obtained from Charles River (Hollister, CA) were implanted on their flank with 10⁷ cells in 100ul 1:1 PBS/matrigel (BD Biosciences, San Jose, CA). Nu/Nu mice were used for all tumor models, except BT474 and MDA-MB-468 which were established in Fox-Chase SCID

mice. BT474 tumors were generated by subcutaneously implanting 30mm³ tumor fragments from previously established xenografts. For MCF7 xenograft formation, mice were implanted subcutaneously at the base of the neck with 90 day release 0.36mg 17 β -estradiol pellets (Innovative Research of America, Sarasota, FL) 3 days prior to tumor cells implantation. Tumor volume ($(\text{length} \times \text{width}^2)/2$) and body weight were measured twice weekly. For efficacy studies, drug treatment started when tumor volume was $\sim 100\text{mm}^3$ and mice were sacrificed at 60 days post treatment or when tumor volume reached 1500mm^3 . Drug treated mice were categorized as a partial regression (PR) if 3 consecutive tumor measurements were less than half the starting tumor volume on day 0 of treatment, a complete regression (CR) if tumor volume was less than 12.5mm^3 for 3 consecutive measurements, and a tumor free survivor (TFS) if it had no measurable tumor or remained a CR at the end of the study. Tumor growth inhibition (TGI) is defined as the percentage difference in tumor volume between vehicle and drug treated groups, determined on the final day when all tumor volumes in the vehicle group are below 1000mm^3 .

Unpaired t-test statistical analyses were performed on all xenograft studies to determine the significance of differences in tumor volume ($p\text{-value} < 0.05$) between vehicle and drug treated mice. Unpaired t-tests were also used to determine if a significant improvement in efficacy occurred when *ispinesib* was combined with approved agents commonly used in breast cancer, relative to the single agent alone

Drugs

All drugs were dosed at their maximum tolerated dose (MTD) unless otherwise stated, and drug volumes were 200 μ l/25g mouse. *Ispinesib* was formulated in 10% ethanol, 10% cremophor, 80% D5W (dextrose 5%) and dosed intraperitoneally (*i.p.*) on a q4dx3 schedule (3 doses given every 4 days) at 10mg/kg in Nu/Nu mice or 8mg/kg in SCID mice, unless otherwise stated. *Trastuzumab* (Genentech, South San Francisco, CA) was dosed *i.p.* twice weekly for 4 weeks at 10mg/kg. *Doxorubicin* (LGM pharmaceuticals, Boca Raton, FL) was formulated in 0.9% saline and dosed q4dx3 at 3mg/kg in Nu/Nu mice or on days 1, 7, and 21 at 2.5mg/kg in Fox-Chase SCID mice. *Lapatinib* (GlaxoSmithKline, London, U.K.) was formulated in 0.5% hydroxypropylmethylcellulose and 0.1% Tween-80 in water and was dosed orally twice daily for 3 weeks at 40mg/kg. *Capecitabine* (Roche, Basel, Switzerland) was formulated in 40mM citrate buffer (pH 6) in 0.5% methylcellulose and was orally dosed daily at 450mg/kg for 14 days. *Paclitaxel* (Natural Pharmaceuticals, Arcadia, CA) and *ixabepilone* (Bristol Myers Squibb, Princeton, NJ) were formulated in 10% ethanol, 10% cremophor, 80% D5W and dosed intravenously (*i.v.*) q4dx3 at their respective MTDs of 30mg/kg and 5mg/kg. Vehicle treated control mice were injected *i.p.* q4dx3 with a formulation of 10% ethanol, 10% cremophor, 80% D5W.

Immunohistochemistry

Mice with a tumor volume of $\sim 250\text{mm}^3$ received a single dose of *ispinesib* (10mg/kg). Tumors were dissected, fixed in 10% buffered formalin, embedded in paraffin, and 5 μ m tissue sections were mounted on slides. Antigen retrieval was performed by boiling in 50mM citrate buffer pH 5.5, sections were incubated in 3% hydrogen peroxide for 5

minutes at RT, washed in PBS-0.1% Tween and blocked in 10% goat serum (Jackson Laboratories, Bar Harbor, ME). For phospho-histone-H3 (PH3) staining, we used a 1:250 dilution of primary antibody and a 1:500 dilution of AlexaFluor 488nm secondary antibody (Molecular Probes-Invitrogen). Images were taken with a Nikon-Eclipse TE-2000U microscope at 10x magnification and captured using Metamorph software to quantify PH3 expression by computing the area-ratio of PH3 positive cells per total cells. For Ki67/cleaved caspase-3 double staining, primary antibodies and a Biocare's Mach2 secondary-HRP-ALP cocktail were used according to manufacturer's guidelines (Biocare Medical LLC, Concord, CA). Non-fluorescent images were taken on an Olympus-BX41 microscope at 20x magnification.

Results

Sensitivity of human breast cancer cell lines to *ispinesib in vitro*. We investigated the possibility that specific breast cancer subtypes might exhibit particular sensitivity to *ispinesib* in a panel of 50 human breast tumor cell lines of representative of diverse primary tumor histotypes and genetic backgrounds, and in three normal mammary epithelial lines, MCF10A, MCF10F and MCF12A (Fig. 1A) (25). Cells were treated with increasing concentrations of *ispinesib* and ranked according to the concentration of drug required to reduce growth by 50% (GI_{50}) (Fig.1A). All lines exhibited sensitivities between 7.4nM and 600nM, with most falling within a ten-fold range, between 7.4nM and 80nM. Three lines, all of luminal subtype, exhibited sensitivities between 100 and 600nM. Across this relatively narrow range of sensitivity we were unable to discern any obvious correlation with subtype, receptor expression or mutational status.

We selected two cell lines, BT474, a HER2 positive luminal cell line (GI_{50} for *ispinesib* of 45nM) and MDA-MB-468, a basal A triple negative cell line, (GI_{50} of 19nM) and characterized the kinetics of cell cycle and apoptotic responses to *ispinesib in vitro* following exposure to 150nM *ispinesib*, greater than 3-fold the GI_{50} value for both cell lines (Fig.1B). In the absence of drug, the proportion of cells with in G_2 or M phases of the cell cycle in MDA-MB-468 was twice that of BT474. After exposure to 150nM *ispinesib*, this proportion increased transiently in both lines, consistent with KSP-induced mitotic arrest. Maximal accumulation of mitotic cells occurred after 16h of treatment in MDA-MB-468 cells and 48h in BT474 cells. At 48h, MDA-MB-468 displayed a much higher proportion of apoptotic cells (sub-G1 DNA content) (35%), than BT474 cells

(13.6%), (Supplemental 1). These findings are consistent with a more rapid and penetrant onset of cell death following mitotic arrest in MDA-MB-468 than in BT474.

We also evaluated the effects of *ispinesib* on the abundance of cell cycle and apoptosis-related proteins (Fig.1C). Expression of the pro-apoptotic proteins BAX and Bid was higher in MDA-MB-468 than in BT-474, while the anti-apoptotic protein Bcl-X_L was lower. Bcl2 levels were not different between the two lines, though phosphorylation on Serine 70 was greater in BT474. The significance of this modification is unclear but has been previously associated with potentiating and abrogating Bcl2 anti-apoptotic activity (26).

The onset of apoptosis was preceded by accumulation of cyclin B, a marker of mitosis (27). In MDA-MB-468 cells, cyclin B expression was maximal at 16h and remained elevated for at least 48h, consistent with an abundance of mitotic cells. In contrast, in BT-474 cells cyclin B levels were generally lower, maximal accumulation was observed at 6h and diminished thereafter. Cyclin E, which normally accumulates to maximal levels in late G1 phase of the cell cycle (28), increased slightly in BT474 after *ispinesib* treatment, but in MDA-MB-468 cells was almost undetectable. The abundance of cyclin A was minimally affected by drug exposure and we observed no changes in the abundance of cyclin D (data not shown).

Efficacy of *ispinesib* as a single agent in pre-clinical breast cancer models. To determine the extent of *ispinesib* anti-tumor activity in breast cancer models *in vivo*, we chose cell lines that exhibited different *in vitro* sensitivity to *ispinesib* and represent

different subtypes of human breast tumors. Their rank from most sensitive to less sensitive to *ispinesib in vitro* is: MDA-MB-468 > HCC1954 = MCF-7 > BT474.

MCF-7 is a well characterized ER-positive luminal breast cancer cell line. MDA-MB-468 is a model for basal triple-negative breast cancer. To represent HER2 over-expressing breast cancer, we chose BT474, HCC1954 and KPL4, a breast tumor line of metastatic origin (29). The transcriptomic, genomic and functional characteristics of these cell lines, except KPL-4, have been characterized previously (25)

Mice bearing tumor xenografts of the lines listed were treated *i.p.* with *ispinesib* at its MTD (SCID:8mg/kg, Nude:10mg/kg) on a q4dx3 schedule, the most effective schedule against a variety of tumor xenografts (30). *Ispinesib* was active in all models tested (Fig.2 and Table 1), producing partial or complete regressions in each. However, the respective tumors differed in sensitivity as judged by the extent of tumor shrinkage, the number of regressions and extent of tumor re-growth.

The triple-negative xenograft model MDA-MB-468, among the most sensitive lines *in vitro* (Fig.1A) exhibited the greatest *ispinesib* sensitivity *in vivo*. Upon *Ispinesib* treatment, MDA-MB-468 tumors regressed completely in all mice, each scoring as tumor-free survivor at the end of the study and 30 days beyond (data not shown).

In the ER positive model MCF7, *ispinesib* caused tumor regressions in 5 out of 9 mice [1 partial regression (PR) and 4 complete regressions (CR), 2 of which were tumor-free survivors (TFS) at study end] and a tumor growth inhibition (TGI) of 92%.

Of the HER2 positive models, KPL4 showed the best response to *ispinesib* treatment. All 10 treated mice exhibited regressions (4 PR, 6 CR and 4 TFS). In the HCC1954 model, *ispinesib* caused regressions in 4 of the 5 treated mice (3PR, 1CR). However, in both of these models, tumor re-growth began 35 days after treatment in the less responsive tumors. In the third HER2 positive model BT474, *ispinesib* caused a CR in 2 of 10 mice, a lower TGI (61%) than that observed in the other models, and tumors had re-grown in all mice by the end of the study (mean tumor volume of 875mm³).

MDA-MB-468 xenografts are hypersensitive to *ispinesib*. To investigate further the hypersensitivity of the MDA-MB-468 tumors to *ispinesib*, we compared the anti-tumor activity of *ispinesib* with that of *ixabepilone* or *paclitaxel*, two anti-mitotic therapies approved for the treatment of breast cancer. We administered each agent on a q4dx3 schedule to two cohorts of tumor-bearing animals, receiving either the MTD or a lower dose. *Ispinesib* anti-tumor activity was comparable to that of *paclitaxel* and *ixabepilone* in terms of tumor growth inhibition, and regressions (Fig. 3A and supplemental 2). One out of 9 mice treated with the higher dose of *ixabepilone* (5mg/kg) developed limb-paralysis and was sacrificed early. No such toxicity was observed with *paclitaxel* or *ispinesib*.

We compared primary and secondary pharmacodynamic (PD) responses to *ispinesib* in MDA-MB-468 and the less sensitive BT-474 tumors. For primary PD response (mitotic delay or arrest), we stained tumor sections with the mitotic antigen phospho-histone-H3 (PH3) (31) (Fig.3B). Quantification of the immunofluorescence signal (Fig.3C) showed that PH3 expression increased in both tumor lines by 6h post treatment. At 48h, PH3

levels declined sharply in BT474 tumors but continued increasing in MDA-MB-468 to levels representing more than twice those in BT474. At 72h, PH3 expression returned to near untreated levels in both lines. For secondary PD responses (reduced proliferation, and onset of apoptosis) we stained tumor sections for markers of proliferation (Ki67) and apoptosis (cleaved caspase-3) (Fig. 3D). 48h after *ispinesib* administration to mice with MDA-MB-468 tumors we observed a sharp reduction in Ki67 expression (brown), a simultaneous marked induction of cleaved caspase-3 (pink), and decreased cellularity consistent with cell death and tumor shrinkage. In BT474 however, we observed a more modest decrease in Ki67 expression no noticeable induction of cleaved caspase-3, and little change in tumor cellularity. These responses to *ispinesib* *in vivo* were similar to those observed *in vitro*, with cell cycle arrest in mitosis and cell death occurring more efficiently and rapidly in MDA-MB-468 than in BT474.

Activity of *ispinesib* in combination with standards-of-care in breast cancer. We sought to identify potentially beneficial combination regimens of *ispinesib* with agents commonly used in breast cancer: the HER2-targeted therapies, *trastuzumab* and *lapatinib*, *doxorubicin* (anthracycline), and *capecitabine* (antimetabolite). In all combination studies we dosed the approved agent at MTD and optimal dosing schedule, and adjusted the dose of *ispinesib* as necessary to achieve a tolerated combination regimen.

We combined *ispinesib* with *trastuzumab* in two different tumor models overexpressing HER2: the luminal model BT-474 (Fig. 4A), and the metastasis-derived model KPL-4 (Fig. 4B). In both models, the absence of *trastuzumab* toxicity allowed combination with

the single agent MTD of *ispinesib*. The combination proved superior to treatment with either single agent. In BT474, the combined agents caused a TGI of 99% compared to 61% and 88% with *ispinesib* and *trastuzumab* respectively (Table 2) and cured 7 out of 8 mice; *trastuzumab* alone cured 4 out of 10 mice, and *ispinesib* alone cured none. In KPL-4, all 10 mice receiving the combination experienced partial or complete regressions, 4 remained tumor-free at the end of the study, and TGI was 97%.

The benefit of combining *trastuzumab* with *ispinesib* suggested that similar effects might be observed with *lapatinib*, a small molecule HER2/HER1-targeting therapy (32). Although *lapatinib* proved less effective as a single agent than *trastuzumab* in the BT474 model, the addition of a tolerable dose of *ispinesib* to the MTD of *lapatinib* improved the TGI from 57% to 81%. The combination did not increase the number of regressions (Table 2).

We also studied the combination of *ispinesib* with the anthracycline *doxorubicin* in two different models, MCF7 and MDA-MB-468 (Fig.4C and table 2). We used different host mouse strains for MCF7 and MDA-MB-468 tumors (Nude and SCID, respectively), and we found the MTD for *doxorubicin* differ slightly between these strains. At the *doxorubicin* MTD, concomitant administration of *ispinesib* increased the TGI in both models compared with that obtained with single agents but no change in the number of regressions was detected (Table 2).

Lastly, we assessed the anti-tumor activity of *ispinesib* in combination with *capecitabine* in the KPL-4 model (Fig. 4D). We found the MTD for *capecitabine* given orally every

day for 2 weeks was 450mg/kg, comparable to previously reports (33). The maximum dose of *ispinesib* that we could co-administer with *capecitabine* without treatment-related toxicity was half its single agent MTD (5mg/kg). In these conditions, although the mean tumor volume was similar to that in mice treated with *capecitabine* alone we observed a clear increase in the number of tumor regressions, including one tumor-free survivor and an increase in TGI (Table 2).

Discussion

In this study, *ispinesib* has demonstrated significant anti-tumor activity in diverse pre-clinical models of breast cancer, supporting its potential for therapeutic intervention in breast cancer. *In vitro*, *ispinesib* inhibited proliferation of all 53 breast cell lines tested, including 3 non-malignant and 50 cancer cell lines derived from distinct breast tumor types. GI₅₀ values spanned a 100-fold range and fall between 10nM and 100nM for most cell lines. *Ispinesib* exhibited no apparent specificity for histopathological sub-type (luminal A, luminal B, basal) or receptor status (HER2, ER/PR). Interestingly, its profile of activity differed from that of other anti-mitotic agents such as *paclitaxel* that inhibited cell growth over a larger concentration range and were more potent against models of basal breast cancer (34). Identification of genomic and transcriptomic differences correlating with relative sensitivity to *ispinesib* may reveal the basis for differential sensitivity of these cell lines in culture and as xenografts, providing biomarkers predictive of disease response to *ispinesib*.

Ispinesib was also active *in vivo* in various breast cancer subtypes, inducing complete regressions or cures in ER positive (MCF7), HER2 positive (BT474, HCC1954, KPL4) and triple negative (MDA-MB-468) models, suggesting that it might be useful in the treatment of a broad range of breast cancers.

Xenografts of the triple-negative MDA-MB-468 cell line were exquisitely sensitive to *ispinesib*. *In vitro* this cell line scored among the most sensitive. *In vivo*, all mice were cured and remained tumor-free for at least 30 additional days. The basis for this strikingly

efficacious response is unclear but our data suggest that cell cycle abnormalities might present a favorable environment for *ispinesib* activity.

Prior to drug treatment *in vitro* and *in vivo*, MDA-MB-468 cells displayed a relatively high proportion of cells in mitosis (with a $4N$ DNA content or positive for the mitotic antigen, phospho-histone H3) compared to the less sensitive BT474 cells. BT474 cells appear to transiently arrest in mitosis and then escape from M-phase, re-entering interphase as suggested by accumulation of cyclin E. The loss of cyclin E expression and the increased and longer duration of cyclin B expression in MDA-MB-468 cells are consistent with *ispinesib* inducing a penetrant and sustained mitotic arrest in these cells. This suggests a deregulation of the G1/S transition and interestingly, deregulation of cyclin E expression is commonly observed in breast cancer (35). MDA-MB-468 cells also harbor mutations in the regulators of the G1 checkpoint Rb and p53. Additional experiments will be required to determine if these cell cycle alterations play a role in relatively greater sensitivity of MDA-MB-468 to *ispinesib*.

The elevated expression of the pro-apoptotic proteins BAX and Bid, and the reduced expression of anti-apoptotic proteins phospho-Bcl-2 and Bcl-X_L are consistent with increased induction of apoptosis following *ispinesib* treatment. Previous observations have linked elevated BAX expression to the induction of apoptosis by KSP inhibition (8, 9) and differences in apoptotic responses have been proposed to be predictive of sensitivity to anti-mitotic drugs such as KSP inhibitors (10). Importantly, our *in vitro* observations were confirmed by pharmacodynamic studies *in vivo*. In both MDA-MB-468 and BT474 tumors we observed *ispinesib*-induced increases in the mitotic antigen

phospho-histone H3. *Ispinesib* treatment was also associated with cleavage of caspase-3, a marker of apoptosis, decreased staining for Ki67, a marker of active proliferation, and decrease in tumor cellularity. These *ispinesib*-induced pharmacodynamic changes were greater in MDA-MB-468 tumors compared with BT474 tumors, consistent with the greater rate of regressions observed in MDA-MB-468 xenografts compared with BT474 xenografts.

Ispinesib compared favorably with approved anti-mitotic agents (*paclitaxel* and *ixabepilone*) in the MDA-MB-468 model of the basal subtype of breast cancer with all three agents producing complete and partial regressions. Consistent with neuropathy being a common side-effect of *ixabepilone* therapy (3, 36, 37), we observed severe limb-paralysis in some mice receiving *ixabepilone*. However, neurotoxicity is uncommon in patients receiving *ispinesib*, and this side-effect was not recorded in preclinical studies, most likely due to the absence of *ispinesib*'s target KSP from post-mitotic neurons (6).

Our findings also show that pre-clinically *ispinesib* is well-tolerated when combined with *doxorubicin*, *capecitabine*, *trastuzumab* and *lapatanib*, therapies commonly used in treatment of breast cancer. Administration of *ispinesib* at doses tolerated with the MTD of these agents enhanced their anti-tumor activity as demonstrated by higher TGI values and increased tumor regressions.

A particularly beneficial combination was that of *ispinesib* with *trastuzumab*. The HER-2 positive models we tested were somewhat less sensitive to single-agent *ispinesib* than either the ER-positive or triple-negative models. However, in two models of HER2

positive breast cancer, *ispinesib* combined with *trastuzumab* enhanced the activity of either single agent by increasing TGI or the number of regressions and cures. HER2 inhibition is known to potentiate the activity of anti-mitotic agents such as *paclitaxel* (38, 39) and this drug combination is currently the standard-of-care for patients with advanced/metastatic HER2 positive breast cancer (40, 41). The improved efficacy of combining KSP inhibition and HER2 inhibition in the preclinical setting, together with the favorable clinical toxicity profile of *ispinesib* (20, 23) suggests that a combination with *trastuzumab* may be of clinical benefit.

In patients with advanced or metastatic breast cancer, *capecitabine* is a standard-of-care (42). A Phase I study demonstrated that the commonly used dose of *capecitabine*, 2000mg/m², is well-tolerated with the full recommended Phase II dose of *ispinesib* administered every 21 days (18mg/m²) (43). In preclinical studies, toxicity prevented us from combining *capecitabine* and *ispinesib* at the respective single agent MTD. However, we found that doses of *ispinesib* below MTD potentiated the anti-tumor activity of *capecitabine* administered at its MTD. These findings suggest that *ispinesib* and *capecitabine* represent a potentially beneficial combination for the treatment of advanced breast cancer, a setting where *ispinesib* has already demonstrated activity.

We have shown that *in vitro* and *in vivo*, *ispinesib* displays a broad spectrum of activity against breast cancer models representative of various human breast tumor types. *In vivo*, the addition of *ispinesib* enhanced the anti-tumor activity of several therapies that are current standards-of-care for the treatment of breast cancer. This robust preclinical anti-tumor activity, coupled with evidence of clinical activity and favorable tolerability profile

in patients with breast cancer, support the continued investigation of *ispinesib* as a promising therapeutic agent in breast cancer.

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Figure Legends

Figure 1: Anti-tumor activity of *ispinesib* *in vitro* against models of breast cancer. *A*, Growth inhibition at 50% (GI_{50}) induced by *ispinesib* was determined for 53 breast cell-lines of luminal, basal A, basal B and non-cancerous origin. *B*, Differences in cell cycle profiles in MDA-MB-468, a cell line sensitive to *ispinesib*, compared to a less sensitive cell line, BT474, after treatment with 150nM *ispinesib* (3- to 7-fold GI_{50}). *C*, Expression of cell cycle markers (cyclin A, cyclin B, and cyclin E), apoptotic proteins (Bax, Bid, p-Bcl2, Bcl2, Bcl-X_L) was analyzed by western blotting in MDA-MB-468 and BT474 cells following treatment with 150nM *ispinesib*.

Figure 2: Anti-tumor activity of *ispinesib* *in vivo* in pre-clinical models of breast cancer. Vehicle control (black) and *ispinesib* (green) at its MTD (10mg/kg in Nude mice, 8mg/kg in SCID mice) were dosed *i.p.* q4dx3 in models of ER positive (MCF7), Her2 positive (KPL4, HCC1954, BT474) and triple negative (MDA-MB-468) breast cancer. All cell lines were grown in Nude mice except BT474 and MDA-MB-468 grown in SCID mice. Arrows indicate the days on which *ispinesib* was administered.

Figure 3: Mitotic arrest and apoptosis induced in the MDA-MB-468 *ispinesib*-hypersensitive xenograft model. *A*, Anti-tumor activity of *ispinesib* compared with *paclitaxel* and *ixabepilone* in MDA-MB-468 xenografts in SCID mice. Arrows indicate the days on which the respective drugs were administered. *B*, Mice with MDA-MB-468

and BT474 xenografts were treated with a single 10mg/kg *i.p.* dose of *ispinesib*, tumors sections were stained for the mitotic antigen phospho-histone H3 (PH3, green) and nuclear DAPI (blue). PH3 images were taken at 10x magnification. *C*, Quantification of *ispinesib*-induced PH3 staining in MDA-MB-468 and BT474 xenografts (calculated as the area of PH3 positive signal relative to the area of DNA positive signal). *D*, Ki67 (brown) was used as a marker of cellular proliferation and cleaved caspase-3 (pink) as a marker of apoptosis; images were taken at 20x magnification.

Figure 4: *Ispinesib* enhances the anti-tumor activity of therapies approved for the treatment of breast cancer. Combination of *ispinesib* with *trastuzumab* in *A*, BT474 and *B*, KPL4 xenografts. *Ispinesib* was dosed *i.p.* q4dx3 at its MTD (10mg/kg) in Nu/Nu mice (KPL4 xenografts) and 8mg/kg in Fox-Chase SCID mice (BT474 xenografts). *Trastuzumab* was dosed *i.p.* twice weekly for 4 weeks at 10mg/kg. *C*, Combination of *ispinesib* with the anthracycline *doxorubicin*. Nu/Nu mice with MCF7 xenografts were treated *i.p.* q4dx3 with *ispinesib* (6mg/kg) and *i.v.* q4dx3 with *doxorubicin* (2.5mg/kg). *D*, Combination of *ispinesib* with *capecitabine* in KPL4 xenografts in Nu/Nu mice. *Ispinesib* was dosed q4dx3 *i.p.* at 5mg/kg (0.5xMTD), *capecitabine* was dosed at 450mg/kg (MTD) *p.o.* qdx14. Arrows indicate the days on which *ispinesib* was administered.

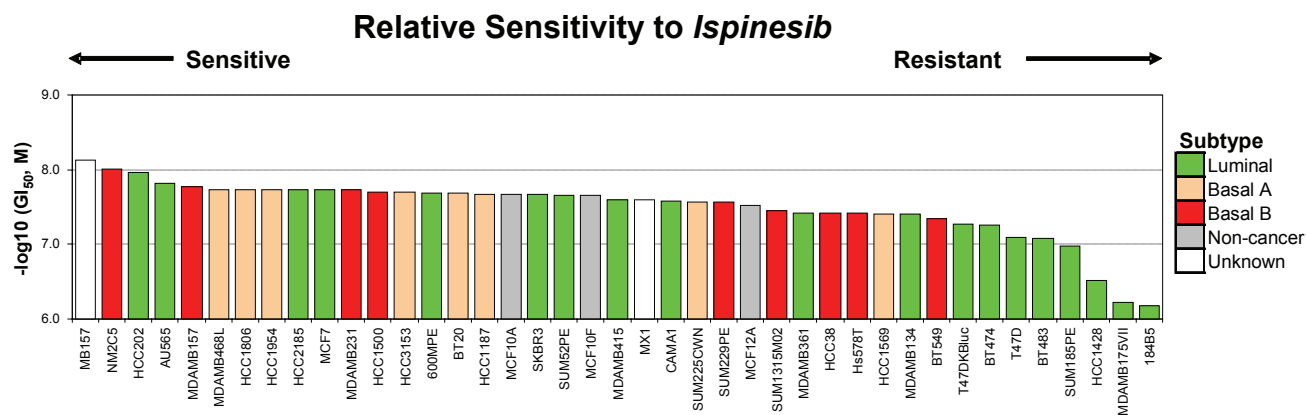
Supplemental 1: Percentage of BT474 and MDA-MB-468 cells in each phase of the cell cycle or undergoing apoptosis (sub-G₁ DNA content) at 0, 6, 16, and 48h following *ispinesib* treatment.

Supplemental 2: Summary of the anti-tumor activity (PR, CR, TGI) of *ispinesib*, *paclitaxel* and *ixabepilone* in MDA-MB-468 treated xenografts. p-values were determined on tumor measurements obtained just prior to tumors of vehicle-treated cohort reaching 1000mm³ endpoint.

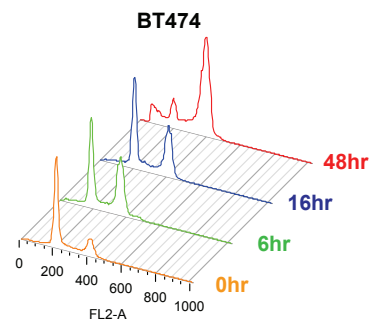
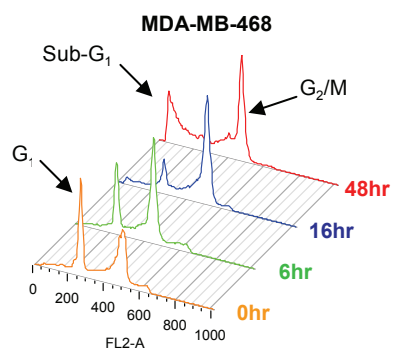
Supplemental 3: BT474 xenografts were treated with *ispinesib* in combination with *lapatinib*. *Ispinesib* was dosed at 3mg/kg (0.5xMTD in Harlan SCID mice) q4dx3 *i.p.* and *Lapatinib* was dosed at 40mg/kg (MTD) p.o. bid x11/6/17.

Figure 1 Purcell, JW et al

A



B



C

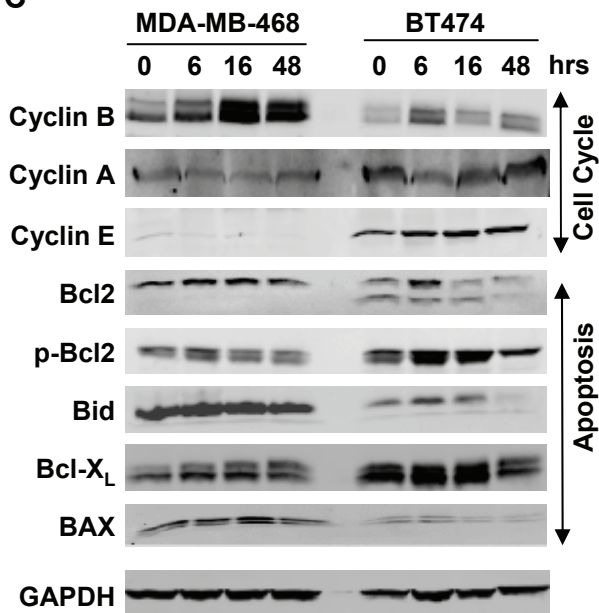
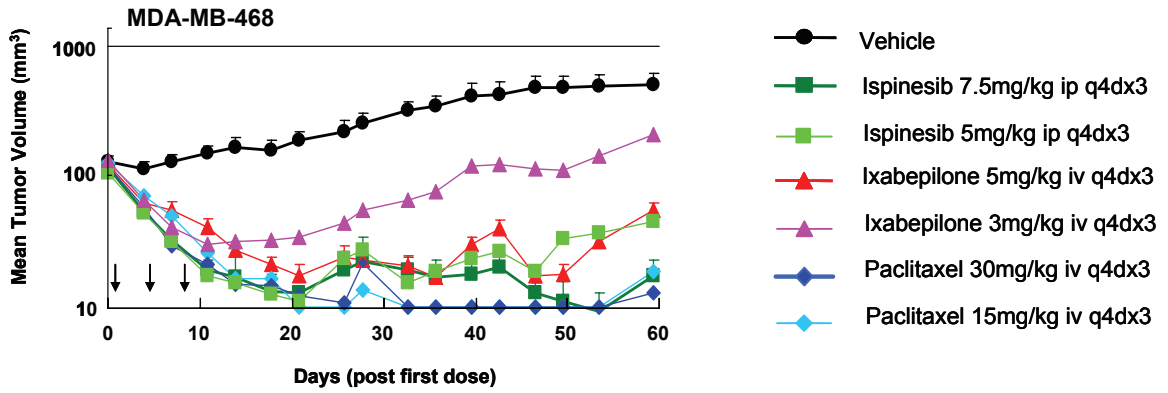
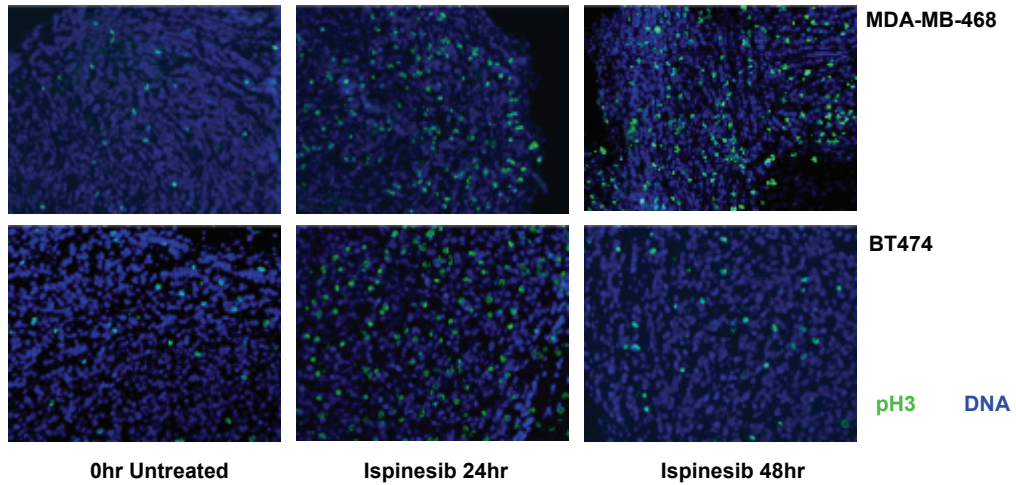


Figure 3 Purcell, JW et al

A



B



C

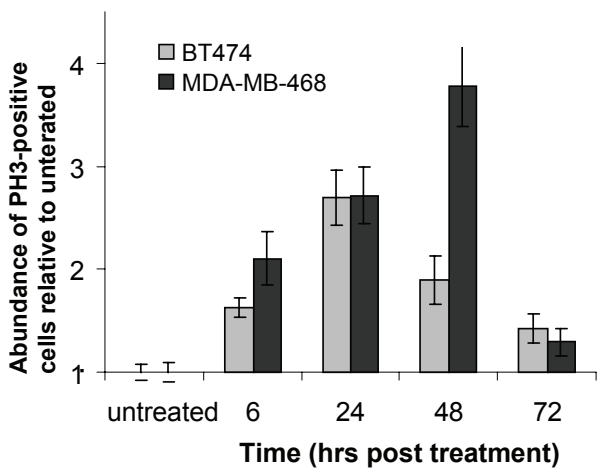
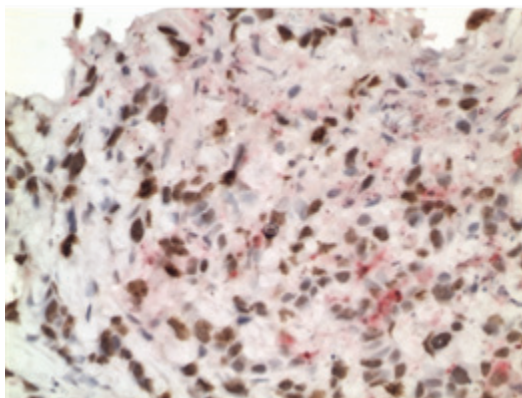
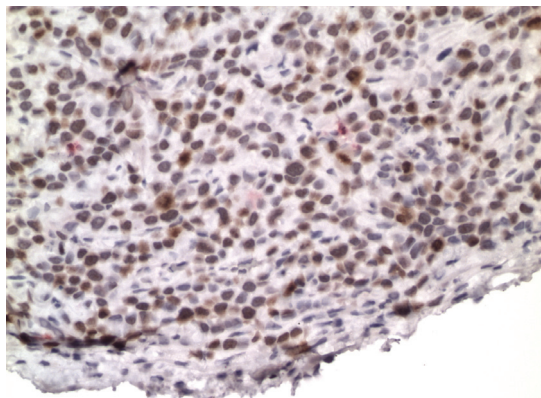
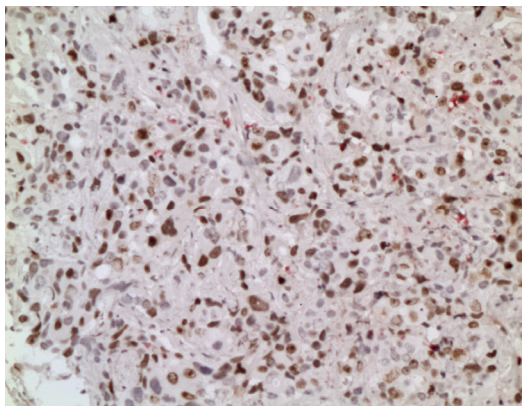
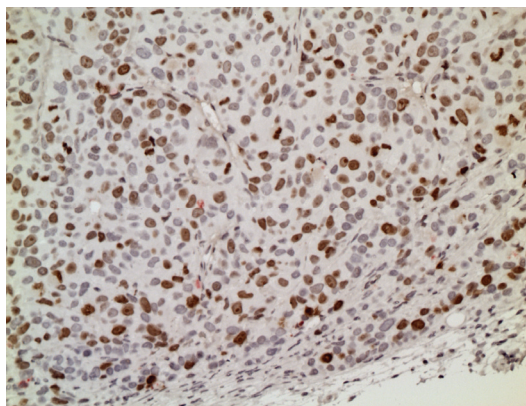


Figure 3 Purcell, JW et al

D



MDA-MB-468

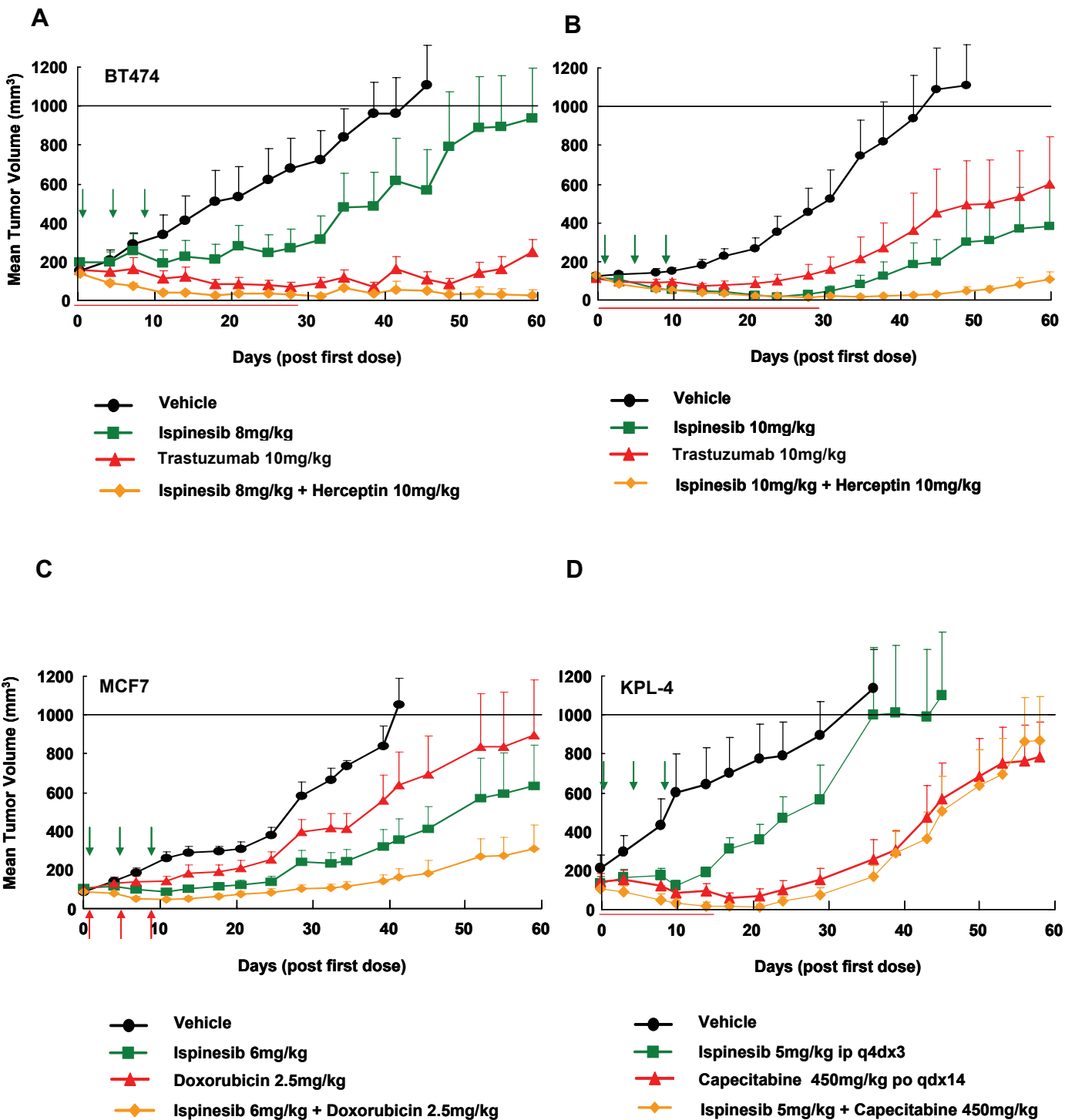


BT474

Untreated

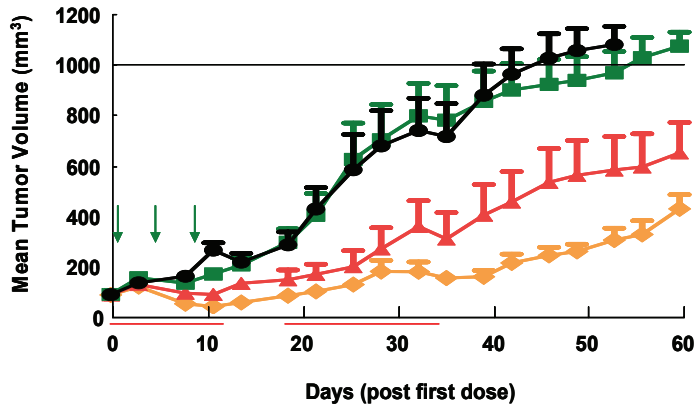
Ispinesib 48hr

Figure 4 Purcell, JW et al



		BT474	MDA-MB-468
0hr	sub-G₁	3.8	7.6
	G₁	65.3	35
	S	9.8	12.3
	G₂/M	21.1	45.1
6hr	sub-G₁	4.9	5.5
	G₁	43.3	27.4
	S	6.8	11.4
	G₂/M	45	55.7
16hr	sub-G₁	5.2	10.2
	G₁	45.8	15.2
	S	9.6	11.2
	G₂/M	39.4	63.4
48hr	sub-G₁	13.6	35.9
	G₁	14.5	7.68
	S	8.2	17.7
	G₂/M	63.7	38.7

	N	PR d60	CR d60	TGI d60 %	Weight Loss %	Tumor Volume Day 60	p-value (relative to control)
Ispinesib 7.5mg/kg	8	3	5	98	11	17	<0.001
Ispinesib 5mg/kg	8	5	3	97	5	47	<0.001
Ixabepilone 5mg/kg	8	6	2	97	8	59	0.005
Ixabepilone 3mg/kg	9	6	2	78	7	232	<0.001
Paclitaxel 30mg/kg	7	5	2	99	6	13	<0.001
Paclitaxel 15mg/kg	8	2	6	99	4	19	0.001



- Vehicle
- Ispinesib 3mg/kg
- ▲ Lapatinib 40mg/kg
- ◆ Ispinesib 3mg/kg + Lapatinib 40mg/kg

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