A Novel Eg5 Inhibitor (LY2523355) Causes Mitotic Arrest and Apoptosis in Cancer Cells and Shows Potent Antitumor Activity in Xenograft Tumor Models

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Abstract

Intervention of cancer cell mitosis by antitubulin drugs is among the most effective cancer chemotherapies. However, antitubulin drugs have dose-limiting side effects due to important functions of microtubules in resting normal cells and are often rendered ineffective by rapid emergence of resistance. Antimitotic agents with different mechanisms of action and improved safety profiles are needed as new treatment options. Mitosis-specific kinesin Eg5 represents an attractive anticancer target for discovering new antimitotic agents, because Eg5 is essential only in mitotic progression and has no roles in resting, non-dividing cells. Here, we show that a novel selective Eg5 inhibitor, LY2523355, has broad target-mediated anticancer activity in vitro and in vivo. LY2523355 arrests cancer cells at mitosis and causes rapid cell death that requires sustained spindle-assembly checkpoint (SAC) activation with a required threshold concentration. In vivo efficacy of LY2523355 is highly dose/schedule-dependent, achieving complete remission in a number of xenograft tumor models, including patient-derived xenograft (PDX) tumor models. We further establish that histone-H3 phosphorylation of tumor and proliferating skin cells is a promising pharmacodynamic biomarker for in vivo anticancer activity of LY2523355. Mol Cancer Ther; 14(11); 2463–72. ©2015 AACR.

Introduction

During G2–M progression, duplicated centrosomes are separated by the microtubule-based bipolar mitotic spindle through a complex process involving chromosomes, mitotic kinesins, and microtubule-associated proteins (1–3). The bipolar mitotic spindle orchestrates the segregation of replicated chromosomes accurately and equally into two daughter cells to maintain genome stability during cell division. Antimitotic drugs such as antitubulins, including taxanes and vincas, are among the most effective cancer therapies in current clinical use (4). However, these antitubulins also have severe side effects as they disrupt the normal microtubule functions in resting cells, including neurons, which may lead to neuropathic disorders (5–7). Furthermore, emerging resistance to these antimitotic agents renders them virtually ineffective (8).

Eg5, a member of BIMC family of kinesins, is a plus-end directed homotetrameric kinesin (1, 9, 10). It plays a vital role in chromosome segregation and bipolar mitotic spindle organization and elongation by sliding antiparallel microtubules away from each other (11–14). Its function in bipolar spindle formation was found to be evolutionarily conserved (14–16). Eg5 is tightly regulated by mitotic kinesins and cell-cycle checkpoint controls (14, 15, 17). Overexpression of Eg5 in transgenic mice caused mitotic defects leading to genome instability and tumorigenesis (18, 19). Unlike microtubules that are present throughout the cell cycle and have important biologic functions in many nondividing cells, Eg5 is expressed and active only during mitosis (20), thus making it an attractive target for discovering new antimitotic drugs with a different mode of action and an anticipated improved safety profile (5, 21). The discovery of small-molecule Eg5 inhibitors spurred strong interest in their development as anticancer agents (6, 22–24).

Inhibition of Eg5 with small molecules disrupts mitotic spindle formation inducing spindle assembly checkpoint (SAC)-mediated mitotic arrest (25) and apoptosis in cancer cells, including taxol-resistant cancer cells (22, 25–27). Recent studies have demonstrated that both activation of SAC and subsequent mitotic slippage are required for apoptosis induction by the Eg5 inhibitors (25, 28). However, how activation of SAC and mitotic slippage are linked to induction of apoptosis is still not well understood.
Eg5 inhibitors generally show excellent anticancer activities in a range of xenograft tumor models (21). However, clinical experiences with several Eg5 inhibitors that entered phase I and II studies have been rather disappointing, resulting in termination of their clinical studies (6). The lack of clinically meaningful activities for these Eg5 inhibitors may be a result of their poor pharmacokinetics or dose selection, which was not optimized on the basis of an understanding of pharmacokinetic and pharmacodynamic relationships. Moreover, early Eg5 inhibitors were developed following a dosing paradigm established for antitubulins. The fact that cell-type variation and the length of time in sustained mitotic arrest contribute to differences in induction of apoptosis by Eg5 inhibitors (25, 29–31) reinforce the need for careful dose and schedule selection.

We describe here the discovery and characterization of LY2523355, a selective allosteric inhibitor of human Eg5, derived through an extensive structure–activity relationship program from a precursor compound identified by a phenotypic-based screening for mitotic arrest of HCT116 cells (32). We demonstrate that inhibition of Eg5 in cancer cells by LY2523355 causes rapid apoptosis following SAC-dependent mitotic arrest. We further show that abrogation of SAC by inactivation of cyclin-dependent kinase 1 (CDK1) or knockdown of mitotic arrest deficient 2 (Mad2) causes rapid exit from mitosis and antagonizes induction of apoptosis by LY2523355. LY2523355 shows highly schedule-dependent, broad-spectrum anticancer activity against tumor models, including patient-derived xenograft (PDX) models. We establish that histone H3 phosphorylation of tumor proliferating skin cells is a promising pharmacodynamic marker for LY2523355 activity.

Table 1. Summary of the sensitive and insensitive tumors to LY2523355

| Type of tumor | Regressed | Suppressed | Number of insensitive tumors (%)
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<tr>
<td>In xenograft tumor models with established cancer cell lines</td>
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<tr>
<td>Colorectal</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
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<tr>
<td>Non-small cell lung</td>
<td>3/5</td>
<td>2/5</td>
<td>0/5</td>
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<tr>
<td>Ovarian</td>
<td>1/5</td>
<td>1/3</td>
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<td>Breast</td>
<td>1/2</td>
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<tr>
<td>Multiple myeloma</td>
<td>2/2</td>
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<td>Overall</td>
<td>9/14 (64)</td>
<td>3/14 (21)</td>
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<td>In PDX tumor models</td>
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<tr>
<td>Non-small cell lung</td>
<td>7/12</td>
<td>4/12</td>
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<tr>
<td>Small cell lung</td>
<td>3/4</td>
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<tr>
<td>Colorectal</td>
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<td>2/4</td>
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<td>Gastric</td>
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<td>Sarcoma</td>
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<td>Liver</td>
<td>0/1</td>
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<td>Overall</td>
<td>16/37 (43)</td>
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The cancer cell lines used are: Colo-205, HCT-116, EBC-1, Calu-6, NCI-H460, PC-14, PC-14/CDOP, A2780, SK-OV-3, RMG-1, KPL-4, M059K, KMS-11, NCI-H929.

Materials and Methods

Cancer cell lines, reagents, and treatments

Cancer cell lines (A2780, A549, ARH-77, Colo-205, Calu-6, DU145, HCT-116, HT-29, Hep 3B2.1-7, LN-18, LN-229, M059K, MDA-MB-231, NAMALWA, NCI-H460, NIH:OVCAR-3, RPMI 8226, SK-BR-3, SK-OV-3, SW620, U-87 MG, HeLa) used for in vitro anticancer and mechanism studies at Eli Lilly and Company were all obtained from ATCC between September 2005 to July 2007, unless otherwise stated. The cell lines were authenticated by short tandem repeat (STR) assay and compared against existing reference standards at RADIL (now IDEXX Bioresearch) and were maintained in media per ATCC instructions and as described previously (33). Sources of the cancer cell lines used for the in vivo xenograft tumor model studies at Kyowa Hakko Kirin as summarized in Table 1 were as follows: HCT-116 cells were obtained in 1998, NCI-H460 cells in 1999, NCI-H929 cells in 2001, and Colo-205, Calu-6, SK-OV-3 cells in 2002 from ATCC; EBC-1 cells were obtained from JCRB Cell Bank in 2002; KPL-4 and KMS-11 cells were obtained from Kawasaki Medical University in 1997 and 2001, respectively; PC-14 and PC-14/CDOP cells were obtained from National Cancer Center Hospital in 2001 and 2002, respectively; A2780 cells were obtained from Japanese Foundation for Cancer Research in 2002; RMG-1 cells were obtained from Shiga University in 1999 and MX-1 cells were obtained from Central Institute for Experimental Animals in 1998. These cancer cell lines at Kyowa Hakko Kirin were not authenticated and were maintained as previously described (32).

Mouse anti-BubR1 antibody was purchased from Becton Dickinson Transduction Labs. Mouse anti-α-tubulin, actin, and anti-Mad2 antibodies were from Sigma-Aldrich. Rabbit anti-pericentromeric antibody was from Abcam. PARP1, Mcl1, Bcl2, p38, and anti-cleaved PARP (Asp214) antibodies were from Cell Signaling Technologies. Rabbit anti-phospho-histone H3 (Ser10) and phospho-histone H2AX (Ser139) and cyclinB1 antibodies were purchased from Millipore. Mouse anti-GAPDH antibody was from Meridian Life Science Inc.. Mad2 ON-TARGETplus SMART pool siRNA and DharmaFECT 1 transfection reagent were purchased from Dharmacon. Propidium iodide was purchased from Meridian Life Science Inc.. DAPI and Hoechst 33258 were from Meridian Life Science Inc.. CellTiterGlo 3D cell viability assay kit was from Promega. Dye 2 was from Dharmacon.

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for 1 hour and scanned with Acumen for mitotic index (MI) measurement based on DNA condensation as percentage of cells with condensed DNA. For MI based on histone H3 measurement based on DNA condensation as percentage of 1 hour and scanned with Acumen for mitotic index (MI) measurement. 

Cells were washed three times with 0.2% Triton-X 100 in PBS and stained for 15 minutes in PBS containing 10 mg/mL propidium iodide (Molecular Probes, Invitrogen) and 100 mg/mL RNaseA (Molecular Probes, Invitrogen). The stained cells were scanned with an Acumen Explorer eX3 microplate cytometer (TPP LabTech). The results were expressed as percentage of cells positive for phospho-histone H3-Ser10 or phospho-histone H2AX.

Caspase-3/7 activity after 48-hour treatment was measured using Apo-ONE Homogeneous Caspase-3/7 Assay (cat. no. G7791, Promega) and cell viability after 72-hour treatment was measured using CellTiter-Glo Luminescent Cell Viability Assay (cat. no. G7573, Promega), following the manufacturer’s instruction.

Animal models and in vivo pharmacology

Primary human-tumor xenograft models were established and maintained in nude mice as previously described (34). Antitumor efficacy in subcutaneous xenograft tumor-bearing mice with 10 mice per treatment group from either established cancer cell lines or fragments of human tumor explants was evaluated as tumor volume by serial caliper measurements and was calculated as described previously (32, 34). The p388 syngeneic tumor model was developed for high-content imaging analysis using female BDF1 mice, weighing 20 to 23 g, which were purchased from Charles River and acclimated in-house for one week before their use in experiments. p388 murine lymphocytic leukemia cells, obtained from the NCI in 2006, were authenticated by STR assay as described above and maintained in RPMI1640 medium containing 10% FBS. For inoculation, the cells were washed with serum-free medium three times and 1.25 million cells were implanted by intraperitoneal injection into mice. On day 5 after implantation, mice were treated with LY2523355, via either intravenous bolus or intravenous infusion at appropriate doses and durations. Mice were euthanized and the ascitic (intrapertitoneal) fluid containing the p388 tumor cells was drawn and analyzed by acumen, flow cytometry, and TUNEL assays for phospho-histone H3, G2-M, and apoptosis. For pharmacokinetic study, we collected the blood samples via cardiac puncture and generated plasma with EDTA and determined LY2523355 exposure in the plasma.

All in vivo studies were reviewed by Ethical Committees of Eli Lilly, Kyowa Hakko, and Oncotest, respectively, and were performed in accordance with the guidelines stated in the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organization of Medical Sciences.

Immunohistochemistry

Xenograft tumors and mouse skin (ears) were fixed in 10% neutral buffered formalin for IHC studies. The sections were trimmed, routinely processed, and embedded in paraffin. Three micrometer sections were immunohistochemically labeled for histone H3-serine 10 phosphorylation using a rabbit polyclonal primary antibody (Upstate, Millipore) without antigen retrieval and with 3,3’-diaminobenzidine as chromagen. Sections were counterstained with hematoxylin.

Results

Threshold concentration effect on cell-cycle–dependent mitotic arrest by LY2523355

Biochemical studies established that LY2523355 is a potent and selective inhibitor of the human Eg5 (Fig. 1A and Supplementary Fig. S1). Cell-cycle analysis showed that LY2523355 caused a dose-dependent increase of cells with 4N DNA content (Supplementary Fig. S2A). Immunofluorescence microscopy further revealed that LY2523355 arrested proliferating normal and cancerous human cells in mitosis with monopolar spindles in vitro (Supplementary Fig. S2B and S2C). Interestingly, inactivation of Eg5 by LY2523355 also inhibited Eg5 localization to the mitotic spindle, suggesting spindle localization of Eg5 is an active process and requires its functional activity (Supplementary Fig. S2C).

LY2523355-treated HCT116 cells also showed a dose-dependent increase in MI based on phospho-histone H3 or DNA condensation (Fig. 1B). Furthermore, there was a time-dependent increase in MI in LY2523355-treated HCT116 cells reaching a maximum at 12 hours (Fig. 1C). Importantly, LY2523355 at 10 nmol/L was sufficient to cause maximal mitotic arrest at all the time points and concentrations higher than 10 nmol/L had no additional effects either on the timing or the magnitude of mitotic arrest. Mitotic block by LY2523355 was closely correlated with its antiproliferation activity in cell culture as demonstrated by complete inhibition of HeLa cell growth at approximately 10 nmol/L (Supplementary Fig. S2D). Indeed, LY2523355 showed potent, broad-spectrum anticancer activity in vitro at low concentrations (IC50 values ranging from 0.4 nmol/L to 14 nmol/L) when tested against a panel of 21 cancer cell lines, which was further confirmed in proliferation assays with the NCI-60 panel (Supplementary Tables S1 and S2).

LY2523355 kills cancer cells as a result of mitotic arrest

LY2523355 at 10 nmol/L or above causes maximal mitotic arrest with monopolar spindle (Fig. 1B and Supplementary Fig. S2B and S2C). A substantial sub-G1 fraction of cells was observed 48 hours after LY2523355 treatment (Supplementary Fig. S2A), indicating that cell death occurred. To establish the causal relationship between mitotic arrest and cell death, real-time measurement of HCT116 cell growth after LY2523355 treatment and subsequent withdrawal was performed. LY2523355 caused maximal mitotic arrest and cell death 12 to 24 hours after addition and cells treated for less than 12 hours were able to resume growth after withdrawal of LY2523355 (Fig. 1D). The data thus strongly suggest that cell killing by LY2523355 is linked to cell-cycle arrest at mitosis. Furthermore, it was observed that LY2523355-induced mitotic arrest and apoptosis were closely associated with each other in a time- and dose-dependent manner (Fig. 1C and Supplementary Fig. S2E).

A role for SAC activation in cancer cell killing by LY2523355

DNA damage and cell death in LY2523355-treated HeLa cells, as indicated by histone H2AX phosphorylation and cleaved PARP (Fig. 2A), occurred in mitotic cells but not in interphase cells.
adherent). This confirmed that cell killing by LY2523355 actually occurred during mitotic arrest. Mitotic arrest was indicated by phosphorylation of histone H3 and the spindle checkpoint kinase, budding uninhibited by benomyl Related-1 (BubR1). Immunofluorescence microscopy of mitotic cells further revealed that mitotic cells had condensed DNA, phosphorylated histone H3, and also phosphorylated histone H2AX (Supplementary Fig. S3A).

A time course study of multiple mitotic and apoptotic cellular biomarkers showed that mitotic arrest clearly preceded apoptosis (Fig. 2B). LY2523355 treatment caused a dramatic increase in phosphorylation of histone H3, B-cell lymphoma 2 (Bcl2), and BubR1, and accumulation of cyclin B from 4 to 16 hours (Fig. 2B). After peaking at 16 hours, phosphorylation of histone H3, Bcl2, and BubR1, and protein level of cyclin B began to decrease substantially and no cyclin B was detectable 48 hours after LY2523355 treatment. Interestingly, the level of BubR1 protein also markedly decreased 24 hours after LY2523355 treatment. In contrast, apoptosis, indicated by PARP1 cleavage and a decrease in the level of antiapoptotic proteins Bcl2 and Mcl1, occurred 16 hours after LY2523355 treatment and continued up to 48 hours (Fig. 2B). Mcl1 appeared to be most sensitive to LY2523355 treatment; its protein level decreased appreciably at 8 hours and completely disappeared 24 hours after treatment (Fig. 2B). The marked decrease in multiple mitotic markers may indicate a mitotic slippage after a sustained LY2523355-mediated mitotic arrest.

CDK1 kinase activity is required to maintain activation of SAC during mitosis (35). Therefore, to investigate a potential role of SAC in apoptotic cell death caused by LY2523355, we used a selective CDK1 inhibitor, RO3306, to abrogate SAC activation and force cells to exit mitosis rapidly. Upon addition of RO3306

Figure 1. LY2523355 kills cancer cells during mitotic arrest. A, the chemical structure of LY2523355. B, dose-dependent mitotic arrest of HCT116 cells by LY2523355. MI was determined by fluorescent microscopy based on DNA condensation 18 hours after LY2523355 addition. C, mitotic block by LY2523355 is time dependent with a threshold concentration for maximal activity. MI was determined using Acumen high content technology. D, cell-cycle arrest at G2–M and cell killing by LY2523355 were closely correlated from 12 to 24 hours after LY2523355 addition. HCT116 cells were treated with 25 nmol/L LY2523355 for various time intervals and then LY2523355 was removed from the growth medium. Cell-cycle profiles of the treated HCT116 cells were analyzed (top graph) and the ability of the treated cells to grow after LY2523355 removal was measured in real time using Incucyte (bottom graph).
Effect of the Eg5 Inhibitor LY2523355 in Cancer

To independently confirm the role of SAC in cell killing by LY2523355, we silenced SAC protein Mad2 by siRNA. As compared with the parental cells, cells with silenced Mad2 failed to be arrested at mitosis and consequently had no activated caspase activity, indicating that SAC has an essential role in mitotic arrest and subsequent cell killing by LY2523355 (Supplementary Fig. S3B–S3D).

Schedule-dependent antitumor activity in vivo by LY2523355

As shown in Fig. 3A, the in vivo antitumor activity of LY2523355 was highly schedule-dependent, although all treatment groups received the same total amount of the compound. Strong antitumor efficacy was observed when LY2523355 was administered with a time interval of 4 days between dosing cycles and the best antitumor activity was observed with daily dosing for 3 consecutive days. However, animals with 3-consecutive day dosing also had significant body weight loss and some mortality. In contrast, LY2523355, when administered once every 7 days, had no significant antitumor activity. It was noted that LY2523355 administered once every 4 days not only showed excellent antitumor activity but also had minimal toxicity (no significant body weight loss; Fig. 3A). Therefore, this dosing schedule was chosen to explore antitumor activity by LY2523355 against a variety of mouse xenograft tumor models.

We compared antitumor activity of LY2523355 with other anticancer agents, including paclitaxel, docetaxel, and vinorelbine, all given at their maximum tolerated dose (MTD), in a lung and an ovarian xenograft tumor model. As shown in Fig. 3B, LY2523355 showed better or at least comparable efficacy to other anticancer agents.

LY2523355 has broad-spectrum antitumor activity in vivo

As summarized in Table 1, LY2523355 was studied in 14 subcutaneous xenograft tumor models with established cancer cell lines representing major human tumor histology of colorectal, non–small cell lung, ovarian, breast, and multiple myeloma, including drug-resistant tumors. LY2523355 showed marked antitumor activity in 86% of the xenograft tumor models, with 9 of 14 tumor models having pronounced tumor regression after treatment. Only two tumor models showed minimal sensitivity to LY2523355.

We further tested LY2523355 in 37 subcutaneous PDX tumor mouse models that are shown to largely retain their original tissue histology and cellular morphology. PDX models are hypothesized to closely represent the response to anticancer agents in human subjects (34). We found that LY2523355 had a range of antitumor activities: some tumors were exquisitely sensitive to LY2523355 and achieved total remission (e.g., small cell lung cancer), whereas some tumors were refractory to LY2523355 treatment. The in vivo efficacy data in PDX models are summarized in Table 1. Together, the data show that LY2523355 had broad-spectrum antitumor activity.

In vivo antitumor activity of LY2523355 is associated with mitotic arrest and death of cancer cells

To directly link LY2523355 antitumor activity to inhibition of Eg5 in vivo, we carried out immunohistochemical (IHC) analyses in the Colo205 tumor model. The animals were dosed at 1.1, 3.3, 10, and 30 mg/kg on Days 0, 4, and 8. As expected, LY2523355 showed dose-dependent antitumor activity (Fig. 4A). Immunohistochemistry of tumors one day after each dosing showed that...
LY2523355 caused a dramatic increase in cancer cells immuno-positive for histone H3 phosphorylation and that had a cellular phenotype consistent with monopolar spindles, indicating mitotic arrest of cancer cells (Fig. 4B). Quantitative analysis of histone H3 phosphorylation showed a correlation with antitumor activity in a dose-dependent manner (Fig. 4C). It is worth noting that at efficacious doses, the increase in histone H3 phosphorylation was followed by cell death on Day 2 after dosing (Fig. 4B). Furthermore, increased cell death was observed after each successive dosing and by Day 8, very few abnormal tumor cells remained, providing a cellular mechanism for marked tumor shrinkage by LY2523355.

To further correlate cancer-cell mitotic arrest with antitumor activity in vivo, we examined the effect of LY2523355 in two resistant and two sensitive PDX tumor models (Supplementary Fig. S4A). Immunohistochemistry showed that each tumor had a distinct tissue structure and a low level of phospho-histone H3-positive cells (Supplementary Fig. S4B, a). The number of cells positive for phospho-histone H3 and cell death increased dramatically in the two sensitive tumor models, whereas in the two resistant tumor models there was only a slight increase, if any (Supplementary Fig. S4B, b and c, and 4C). Moreover, IHC analysis of skin tissues observed a marked increase in phospho-histone H3-positive proliferating skin cells in all treated animals (Supplementary Fig. S4D). Taken together, the data demonstrated that the antitumor activity of LY2523355 is linked to Eg5 inhibition in cancer cells and mitotic arrest of proliferating skin cells may serve as a promising surrogate pharmacodynamic biomarker for dose and schedule assessments.

**Therapeutic threshold concentration and efficacy by LY2523355**

To understand the relationship between therapeutic threshold concentration, duration for which the threshold must be maintained for cell-cycle-dependent mitotic arrest, and subsequent cell killing, we used the p388 ascites tumor model which, being a liquid tumor, is amenable to high-throughput quantitative cell-cycle analysis as compared with immunohistochemistry in solid tumors. Cell-cycle analysis showed a clear threshold dose between 5 to 10 mg/kg for maximal G2-M arrest of cancer cells (Fig. 5A). Indeed, the 5-mg/kg dose markedly extended survival of mice bearing p388 tumors and higher doses did not proportionately increase survival benefit (Fig. 5B).

To further demonstrate the relationship between threshold exposure and cell-cycle arrest, we performed a series of infusion studies with a p388 tumor model. Following a 72-hour infusion, we determined steady-state exposure in the plasma and quantified phospho-histone H3-positive cancer cells in the ascites fluid by high-content imaging and also in skin biopsies by immunohistochemistry. As shown in Table 2, Fig. 5C and Supplementary Fig. S5A and S5B, LY2523355 at the threshold exposure level of about 10 ng/mL, as established in vitro, achieved maximal mitotic block in both cancer cells and proliferating skin cells. TUNEL staining...
showed that many treated p388 tumor cells were also apoptotic (Supplementary Fig. S5A).

Taken together, both in vitro and in vivo data clearly indicate that efficacy of LY2523355 is highly dosing schedule-dependent and that histone H3 phosphorylation of proliferating skin cells is a promising surrogate pharmacodynamic biomarker for dose and schedule evaluation of LY2523355 to study its anticancer activity.

**Discussion**

This article describes in vitro and in vivo studies to characterize the anticancer activity of a novel selective Eg5 inhibitor LY2523355. LY2523355 showed broad-spectrum antiproliferative activity against cancer cell lines. In preclinical xenograft tumor models, LY2523355 also showed a broad-spectrum anticancer activity with complete remission in some. We further demonstrated that in vivo efficacy of LY2523355 is highly dependent on threshold therapeutic concentration and dosing schedule. We also showed that increase in histone-H3 phosphorylation of proliferating skin cells is quantitatively correlated with anticancer activity of LY2523355, thus offering a promising surrogate pharmacodynamic biomarker for the evaluation of LY2523355 anticancer activity.

Our extensive cellular studies showed that the anticancer activity by LY2523355 is mediated through cell cycle-dependent mitotic arrest and requires sustained activation of SAC. LY2523355 activates SAC as a result of monopolar spindle formation, leading to mitotic arrest. Spindle defects such as monopolar spindles activate SAC to delay exit from mitosis by preventing the proteolysis of cyclin B, which is mediated by anaphase-promoting complex (APC), until spindle defects are corrected (35). However, Cancer cells often escape from SAC-mediated mitotic arrest via a poorly defined process known as mitotic slippage. Mitotic slippage has been shown to be caused by gradual degradation of cyclin B in the presence of a functional SAC (36, 37). The time course study with HeLa cells showed that cells that were arrested at mitosis began to escape from the mitotic arrest 16 hours after LY2523355 treatment, as indicated by the decrease of multiple mitotic biochemical markers. Interestingly, we observed that, in addition to cyclin B degradation, mitotic slippage is also associated with BubR1 degradation. Perhaps degradation of BubR1, an essential regulatory component of SAC, is part of the mechanism responsible for mitotic slippage. Analysis of multiple mitotic and apoptotic markers show that induction of apoptosis by LY2523355 clearly followed mitotic slippage. Our data are consistent with the previous report that both activation of SAC and mitotic slippage are required for rapid cancer cell killing (25). This is further supported by the compound wash-out experiment that showed that killing of cancer cells by LY2523355 requires 12 hours or more and coincides with sustained mitotic arrest. Furthermore, phosphorylation of Bcl2 and rapid degradation of Mcl1 observed in cells mitotically arrested by LY2523355 may also contribute to induction of apoptosis. Indeed, recent studies that shows CDK1-mediated phosphorylation of Bcl-xL/
Bcl2 to be a functional link between mitotic arrest and apoptosis (38), and that suppression of Mcl1 sensitizes cancer cells to the treatment of anticancer agents, including an Eg5 inhibitor (ARRY-520), in human multiple myeloma (39–41).

Consistent with Eg5 biology and mode of action of LY2523355 as discussed above, we demonstrated that anticancer activity of LY2523355 is highly schedule-dependent and has an optimal threshold exposure level and duration to achieve significant anticancer activity. Furthermore, the in vivo anticancer activity in sensitive xenograft tumor models but not resistant tumor models is associated with first mitotic arrest and then apoptosis in cancer cells very similarly as observed in vitro, indicating that LY2523355 has the same mechanism of anticancer activity in vitro in cell-based studies.

The salient finding in this study on characterization of LY2523355 anticancer activity in vitro and in vivo is that duration of threshold exposure is extremely important to anticancer activity by selective Eg5 inhibitors. This finding has significant implications for a successful effort in the discovery and development efforts not only on selective inhibitors of Eg5, but also on selective

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**Figure 5.**

Efficacy and therapeutic threshold concentration of LY2523355 in p388 ascites tumor model. A, cell-cycle analysis by flow cytometry of p388 tumor cells from ascites fluid 18 hours after dosing by intravenous bolus. B, survival extension by LY2523355. Note that LY2523355 achieved maximal cell-cycle arrest and significant survival extension benefit at 5 mg/kg. C, changes in phospho-histone H3-positive epidermal cells in the ear skin of vehicle- and LY2523355-treated mice. The top panel shows no phospho-histone H3-positive keratinocytes in a 1 mm length of epidermis from the ear of a mouse that received vehicle. In the bottom panel, multiple phospho-histone H3-positive mitotic cells are present in the same length of epidermis in a mouse that received LY2523355 (bar, 100 μm).
inhibitors of Plk1 and aurora kinases as novel new antimitotic cancer drugs. Our data on LY2523355 convincingly showed that compounds targeting these mitotic regulators must have good pharmacokinetic properties that give rise to sustained duration of threshold exposure through careful dose/dosing schedule selection to achieve robust anticancer efficacy. Thus, dosing schedules need to be optimized for each compound in accordance with their potency and pharmacokinetic properties to differentially kill sensitive cancer cells while sparing or minimizing toxicity to proliferating normal cells. This also provides a good biologic explanation why clinical studies on early generation Eg5 inhibitors, such as SB-715992, that followed the development paradigm of antitubulin drugs showed little clinical activity (6).

Microtubules as target for antitubulin drugs are always present throughout the cell cycle of dividing cancer cells. In contrast, Eg5 is only expressed briefly during mitosis. Furthermore, cancer cell-cycle progression is asynchronous and mitosis is completed very rapidly. Consequently, mitotic cells in tumor tissues are always only a small fraction of the total cancer cell population at any given time. Therefore, Eg5 inhibitors must be available at or above the threshold concentration relative to their potency for an adequate duration to achieve sustained mitotic arrest of most dividing cancer cells as they asynchronously progress into mitosis. The length of this required duration of threshold exposure would also depend on the cell-cycle time of proliferating cancer cells in individual tumors. The importance of sustained duration of threshold exposure of drugs that target cell-cycle regulators now individual tumors. The importance of sustained duration of threshold exposure of drugs that target cell-cycle regulators now depends on the cell-cycle time of proliferating cancer cells in dividing cancer cells as they asynchronously progress into mitosis while keeping target-mediated toxicity manageable. Histone H3 phosphorylation (pHH3), which closely correlates with its antitumor activity, is a promising pharmacodynamic biomarker that has the potential to guide dose and schedule selection for optimal cancer treatment of Eg5 inhibitors with improved safety profiles and new anticancer mechanisms as compared with antitubulins.

**Disclosure of Potential Conflicts of Interest**

T. Yin is a scientist Eli Lilly and Company. G.P. Donoho has ownership interest (including patents) in Eli Lilly and Co. A. Aggarwal has ownership interest (including patents) in Eli Lilly. E.H. Westin has ownership interest (including patents) in Eli Lilly. No potential conflicts of interest were disclosed by the other authors.

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**References**


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