Characterization of LY3023414, a Novel PI3K/mTOR Dual Inhibitor Eliciting Transient Target Modulation to Impede Tumor Growth

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Abstract

The phosphoinositide-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is among the most frequently altered pathways in cancer cell growth and survival. LY3023414 is a complex fused imidazoquinolinone with high solubility across a wide pH range designed to inhibit class I PI3K isoforms and mTOR kinase. Here we describe the \textit{in vitro} and \textit{in vivo} activity of LY3023414. LY3023414 was highly soluble at pH 2-7. In biochemical testing against approximately 266 kinases, LY3023414 potently and selectively inhibited class I PI3K isoforms, mTORC1/2, and DNA-PK at low nanomolar concentrations. \textit{In vitro}, inhibition of PI3K/AKT/mTOR signaling by LY3023414 caused G1 cell-cycle arrest and resulted in broad antiproliferative activity in cancer cell panel screens. \textit{In vivo}, LY3023414 demonstrated high bioavailability and dose-dependent dephosphorylation of PI3K/AKT/mTOR pathway downstream substrates such as AKT, S6K, S6RP, and 4E-BP1 for 4 to 6 hours, reflecting the drug’s half-life of 2 hours. Of note, equivalent total daily doses of LY3023414 given either once daily or twice daily inhibited tumor growth to similar extents in multiple xenograft models, indicating that intermittent target inhibition is sufficient for antitumor activity. In combination with standard of care drugs, LY3023414 demonstrated additive antitumor activity. The novel, orally bioavailable PI3K/mTOR inhibitor LY3023414 is highly soluble and exhibits potent \textit{in vivo} efficacy via intermittent target inhibition. It is currently being evaluated in phase 1 and 2 trials for the treatment of human malignancies.


Introduction

The phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway controls many hallmarks of cancer including cell growth, survival, motility, and metabolism [1]. It is considered the most frequently altered pathway in human tumors [2-4] including tumors of the breast, colon, ovary, gastric system, prostate, mesothelium, endometrium, brain, and lung [5-7]. Alterations in this pathway have been linked with increased neovascular formation, metastasis, and chemotherapy resistance. In addition, blocking PI3K/AKT/mTOR pathway activation leads to apoptosis or cell-cycle arrest in several different models [8-12]. Consequently, this pathway has become a focus of anticancer drug development.

The PI3K/AKT/mTOR signaling pathway is dysregulated by a variety of mechanisms in human tumors [5, 13-16]. The most common activating alteration is loss of the tumor suppressor PTEN through gene deletion or inactivating mutation [17-19]. Other common dysregulatory mechanisms reported include amplification or activating mutations of PI3K class I isoforms (p110α, β, δ, γ), AKT, PDK1, RAS, tuberous sclerosis protein 1 and 2 (TSC1/2), and mTOR [16, 20, 21].

Data from preclinical studies suggest that PI3K pathway inhibitors could have single-agent activity in cancers exhibiting PIK3CA mutations [22], HER2-amplified breast cancers [23], and PTEN-deficient cancers [5, 24]. These data highlight elements to consider when designing PI3K/AKT/mTOR pathway inhibitors such as the role and inhibition of specific class I PI3K isoforms in different tissues, the optimal extent and duration of pathway inhibition needed for antitumor activity, and whether continuous or intermittent pathway inhibition is better for achieving antitumor efficacy.
Numerous inhibitors of the PI3K/AKT/mTOR pathway—ranging from allosteric inhibitors of mTORC1 (rapalogs) and AKT to ATP-competitive inhibitors of AKT, PI3K (pan- and isoform-specific), PI3K/mTOR, mTOR, S6K, and AKT/S6K—are being evaluated in clinical trials [9, 11, 25]. Though generally tolerable with manageable adverse events, none has shown more than modest single-agent antitumor activity except for the recently approved PI3Kδ inhibitor idelalisib (formerly CAL-101), which showed activity in lymphoid malignancies [26-28]. The reason for this modest single-agent activity with other inhibitors is only partially understood. Besides patient selection and compensatory pathways, it has been reported that the solubility of oral PI3K/AKT/mTOR inhibitors may be limited under different pH conditions in the upper gastrointestinal tract, which in turn may affect their absorption and pharmacokinetic (PK) properties and thereby contribute to the variable activity observed [29, 30].

In this report, we describe the discovery and initial \textit{in vitro} and \textit{in vivo} characterization of LY3023414, a novel, highly soluble, small-molecule, dual kinase inhibitor of class I PI3K isoforms, mTOR, and DNA-PK.
Materials and Methods

Solubility assays

LY3023414, or 8-[5-(1-hydroxy-1-methylethyl)pyridin-3-yl]-1-[(2S)-2-methoxypropyl]-3-methyl-1,3-dihydro-2H-imidazo[4,5-c]quinolin-2-one, was prepared as previously described in patent WO 2012/097039 A1 (see Fig. 1A). Its solubility in various solutions was measured by rotating each mixture for 24 hours at ambient temperature, measuring the pH of the filtrate, and analyzing the sample by high-pressure liquid chromatography (HPLC). The solutions used to measure solubility included 0.1 N or 0.01N HCl, 30 mM acetate buffer at pH 4.5, 50 mM phosphate buffer at pH 6.0 or pH 7.5, simulated gastric fluid (SGF), simulated intestinal fluid fed state (fed), and simulated intestinal fluid fasted state (fasted) [31, 32].

Kinase assays

The selectivity and inhibitory potential of LY3023414 were assessed against a panel of 192 kinases in PC-3 cell lysates using the KiNativ™ platform (ActivX Biosciences Inc., La Jolla, CA) and a panel of 102 kinases as purified enzymes from Cerep (Poitiers, France). Together, the 2 kinase panels covered approximately 266 unique kinases. These kinases were tested with three concentrations of LY3023414 to measure inhibition and calculate approximate IC\textsubscript{50} values.

The IC\textsubscript{50} of LY3023414 for PI3K\textalpha{} was measured using 5 nM recombinant human PI3K\textalpha{} [33], 0.01 mM ATP with a 1.76 mM Triton X 100/ 0.04 mM PIP2/ 0.2 mM PS mixed micelle as the lipid substrate in a scintillation proximity assay (SPA) with neomycin-linked beads (Amersham Biosciences, Buckinghamshire, UK). The IC\textsubscript{50} of LY3023414 for PI3K\textbeta{} was measured using a mixed micelle SPA format with 0.04 mM ATP with a 0.27 mM Triton X 100/ 0.05 mM PIP2/ 0.04 mM PA mixed micelle as the lipid substrate. The IC\textsubscript{50}s of PI3K\textdelta{} and PI3K\textgamma{} were...
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(Adapta® assay kit; Life Technologies, Grand Island, NY) and of DNA-PK (Z'LYTE® assay kit; Life Technologies) were measured at Life Technologies (Grand Island, NY). The IC\textsubscript{50} for mTOR was measured using the LanthraScreen\textsuperscript{TM} kinase assay (Life Technologies).

**Co-crystallization of LY3023414 with PI3K\(_\gamma\)**

PI3K\(_\gamma\) was expressed and purified as described [33] and used to form co-crystals with LY3023414. The crystal structure of LY3023414 bound to PI3K\(_\gamma\) was determined as previously described [34]. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under contract number DE-AC02-06CH11357. Use of the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at Sector 31 of the Advanced Photon Source was provided by Eli Lilly and Company, which operates the facility.

**Cell culture**

All standard cell lines except A2780, ACC-MESO-1, and ACC-MESO-4 were obtained from the American Type Culture Collection (ATCC). A2780 cells were obtained from the National Cancer Institute (NCI) Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository and cultured in RPMI-1640 media and 10% fetal bovine serum (FBS). ACC-MESO-1 and ACC-MESO-4 cells were provided by the RIKEN BioResource Center through the National BioResource Project of the MEXT, Japan and cultured in RPMI-1640 media and 10% FBS as described previously (35). Companion cell lines to Oncotest PDX models (BXF-1218L, BXF-1352L, PXF-698L, PXF-1118L and PXF-1752L) were obtained from Oncotest GmbH (Freiburg, Germany) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) and 10% FBS. BxPC-3, Hs766T, NCI-H1155, and PANC-1 cells were cultured in
DMEM and 10% FBS. MIA PaCa-2 cells were cultured in DMEM, 10% FBS, and 2.5% horse serum. A549 cells were cultured in Ham's F-12 Kaighn's modification and 10% FBS. BT-474 cells were cultured in Hybri-Care medium (ATCC) and 10% FBS. MDA-MB-361 and MDA-MB-436 cells were cultured in Leibovitz's L-15 medium in the absence of CO₂. A-204, HCT-116, HT-29, and SK-OV-3 cells were cultured in McCoy’s 5A medium and 10% FBS. HT-1080, MCF-7, SK-MES-1, and U87 MG cells were cultured in minimum essential media (MEM) supplemented with 1 mM sodium pyruvate and 1 mM MEM non-essential amino acids. 786-O, AsPC-1, MSTO-211H, NCI-H1299, NCI-H1395, NCI-H1650, NCI-H1703, NCI-H1734, NCI-H1975, NCI-H1993, NCI-H226, NCI-H446, NCI-H460, NCI-H520, SJSA-1, and THP-1 cells were cultured in RPMI-1640 media and 10% FBS.

All cell lines were obtained directly from the suppliers and were thawed, expanded, and frozen as master stocks. ATCC lines were authenticated using short tandem repeat (STR) analysis at ATCC and independently reconfirmed by third-party STR analysis (with the exception of MDA-MB-361, MDA-MB-436, and THP-1). All other lines (including Oncotest PDX-derived cell lines) underwent third-party STR analysis and were matched to existing STR reference genotypes.

**Proliferation assays**

The CellTiter-Glo® luminescent cell viability assay system (Promega, Madison, WI) was used to measure the antiproliferative effects of LY3023414 after 2 cell doublings on cells plated on plastic or incubated for 2 weeks in soft agar with a collection of standard cell lines and human patient-derived tumor xenografts passaged in nude mice (Oncotest GmbH) [36]. For the soft agar assay, RKO and SK-OV-3 cells were obtained from ATCC; MOLT-4 and L-363 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).
(Braunschweig, Germany); DLD-1, HCT-116, HCT-15, and NCI-H460 cells were obtained from the NCI. These standard cell lines were genotyped by STR and matched to existing STR reference genotypes. Oncotest PDX models (including model MX1 originally derived at NCI) were characterized using the Affymetrix genome-wide human SNP Array 6.0 as well as whole-exome sequencing. Genetic identity analysis confirmed that all PDX models were derived from independent patient samples. Combination studies were conducted in which LY3023414 was mixed with other therapeutic agents in fixed ratios of concentrations corresponding to the \( IC_{50} \) equivalents of each single agent. The data were analyzed according to the method described by Zhao et al. [37]. The combination index at 50% inhibition (CI\(_{50}\)) was calculated. Effects were classified as synergistic (CI\(_{50} < 0.5\)), additive (0.5 \( \leq \) CI\(_{50} \leq 2\)), or antagonistic (CI\(_{50} > 2\)). Statistical significance (95% confidence interval excluding the value 1) was also required to declare synergy or antagonism.

**Cell cycle analysis**

The effects of LY3023414 on cell cycle were measured after 1 cell doubling. In brief, cells were collected, washed, fixed with 70% ethanol at 4\(^\circ\)C for 30 minutes, washed with cold DPBS, and then treated with RNase and propidium iodide for 20 minutes at 37\(^\circ\)C in the dark. Cells were analyzed using a Beckman Coulter FC500 cytometer and VERITY ModFit 3.2 software (Verity Software House).

**Apoptosis assays**

The effects of LY3023414 on caspase-3 and -7 in cells were measured using the Caspase-Glo\textsuperscript{®} 3/7 assay (Promega, Madison WI). The fold increase in caspase-3/-7 activity (treated/[untreated cells at time zero]) was normalized to the cell number using the ratio of viable untreated cells at time zero to treated cells at the different time points.
**Phosphorylation assays**

The AlphaScreen SureFire ® assay (PerkinElmer, Waltham, MA) and commercial reagents (TGR BioSciences, Adelaide, Australia) were used to measure the effect of LY3023414 on the formation of endogenous phosphorylation sites at pT389 S6K, pT308 AKT, pS473 AKT, pT37/46 4EBP-1, and pS240/244 S6 ribosomal protein in U87 MG cells. The multiplex format of Meso Scale Discovery (Gaithersburg, MD) ELISA technology (MSD-ELISA) was used to measure phosphorylation in all other cell lines.

Human whole blood was collected from consented healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated, treated with LY3023414, collected, left unwashed or washed with Tris buffered saline, and then lysed with 90 μL of MSD lysis buffer. Lysates were stored at -80°C for later MSD-ELISA measurements of pT389, p70S6K, and pT37/46 4E-BP1.

**Pharmacokinetic studies**

The PK profile of LY3023414 was assessed in male CD-1 mice (Harlan Laboratories, Indianapolis IN), male Sprague-Dawley rats (Harlan Laboratories, Indianapolis IN), and male beagle dogs (Charles River Laboratories, Wilmington MA). Mouse PK was calculated from a composite curve based on data from 3 mice per time point. Rat PK was assessed using data from 3 rats per group, with and without pretreatment with 100 mg/kg intraperitoneal aminobenzotriazole (ABT), a nonspecific inhibitor of cytochrome P450 (CYP) enzymes. Dog PK was determined using data from 4 dogs in a crossover experimental design. Plasma concentrations of LY3023414 were measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS) using an AB Sciex API4000. Noncompartmental PK parameters were calculated using Watson version 7.4 (Thermo Scientific, Waltham, MA).

Plasma protein binding was measured in vitro in rat and human plasma by equilibrium
dialysis. In brief, aliquots (100 μL) of plasma spiked with 1 mM LY3023414 (n=3) were dialyzed against an equal volume of 100 mM sodium phosphate buffer (pH 7.4) on a Teflon equilibrium dialysis plate and cellulose membranes (12-14 kDa molecular weight cutoff) (HTDialysis, Gales Ferry, CT). Dialysis was conducted at 37ºC on an orbital shaker (175 rpm) for 4.5 hours. Compound concentrations in matrix and buffer were then measured by LC/MS/MS.

**In vivo mouse studies**

Xenograft tumors were implanted subcutaneously in athymic nude (Harlan Laboratories, Indianapolis, IN), CD-1 nude mice (Charles River Laboratories, Wilmington, MA), and NMRI athymic nude mice (Harlan Laboratories, Horst, Netherlands). B6.Cg-Tg(IghMyc)22Bri/J mice (Jackson Laboratory, Bar Harbor, ME) and C57BL/6NTac mice (Taconic, Cambridge City, IN) were used in the Eμ-myc transgenic orthotopic mutant PI3Kα E545K-driven leukemia model similar to the AKT1 E17K cancer model previously described [38]. LY3023414 was formulated in 1% HEC in distilled water containing 0.25% polysorbate 80 and 0.05% Dow-Corning Antifoam 1510-US and administered by oral gavage (final volume 0.2 mL) at the indicated doses and schedules. Efficacy and in vivo target inhibition studies were carried out after tumor volumes reached 150-200 mm³.

Target inhibition studies were conducted at various time points after administration of a single dose of LY3023414 to mice harboring tumors. Tumors were harvested, flash frozen, lysed in MSD buffer, and then analyzed using the MSD-ELISA multiplex method. Whole blood was collected at the same time points for plasma concentration determinations. Threshold effective dose 50 (TED50) was determined by fitting a 4-parameter logistic model to percent response vs. dose to estimate the dose that corresponds to 50% activity. The model equation was:
Where \( y \) = percent response, \( x \) = dose, \( a \) = upper asymptote, \( d \) = lower asymptote, \( c = TED_{50} \), and \( b \) = slope. Threshold Effective Concentration 50 (TEC_{50}) was determined by fitting a 4-parameter logistic model to percent response vs. plasma concentration to estimate the concentration that corresponds to 50\% activity. The model equation was:

\[
y = d + \frac{(a - d)x^b}{x^b + c^b (a - 50)/(50 - d)}
\]

where \( y \) = percent response, \( x \) = concentration, \( a \) = upper asymptote, \( d \) = lower asymptote, \( c = TEC_{50} \), and \( b \) = slope.

Tumor volume was estimated using the formula \( l \times w^2 \times (\pi/6) \), where \( l \) = the larger measured diameter and \( w \) = the smaller perpendicular diameter. Percent delta T/C was calculated using the formula 100 \times \left[\frac{(T-T_0)}{(C-C_0)}\right] \) and percent regression using the formula 100 \times \left[\frac{(T-T_0)}{T_0}\right], where \( T \) and \( C \) are mean tumor volumes in the treated and control group, respectively. \( T_0 \) and \( C_0 \) are the corresponding baseline mean tumor volumes. For some studies, percent delta T/C was converted to percent delta tumor growth inhibition (TGI) using the equation, 100 – percent delta T/C. Tumor volume data were transformed to the log_{10} scale and analyzed using 2-way repeated measures ANOVA by time and treatment using the MIXED procedure in SAS software (Version 9.1.3). Combination effects were tested using 2\times2 interaction tests. To classify each combination effect, the expected additive tumor volume response (EAR), as defined by Bliss Independence, was calculated as \( EAR = T_1 \times T_2/C \), where \( T_1 \) is the mean tumor volume of treatment group 1, \( T_2 \) is the mean tumor volume of treatment
group 2 and \( C \) is the mean tumor volume of the control group. Then 2-fold limits were
determined above and below the EAR volume. For smaller volumes, \( \pm 20\% \) limits on the percent
regression scale were also calculated, and the wider limit above or below the additive volume
was used. If the observed mean volume in the combination group was within these limits, the
combination was classified as additive, regardless of the interaction test \( P \) value. If the observed
mean combination volume was below the lower limit, the combination was classified as
synergistic. Mean combination volume above the upper limit was classified as less than additive
if the combination was still better than the best single agent; otherwise, it was classified as
antagonistic. In each case, the \( P \) value of the interaction test was also reported.

Mice inoculated with \( p110\alpha (E545K)/\text{myc} \) leukemia cells were assigned in groups of
5 per treatment and in a group of 10 to vehicle control and were treated on Day 5 through Day 11
after inoculation. Blood samples were collected on Day 12 to assess leukemia progression.
Fixed nucleated leukocytes were counted within a specific region of the
forward-scatter/side-scatter (FS/SS) plot in each sample (defined as a region showing little or no
leukemic cells in normal mice yet significant leukemic cells in leukemic control mice). These
resulting counts were normalized to the number of leukemic cells per unit volume of blood with
a fixed addition of Beckman Coulter Flow-Count fluorospheres per sample and expressed as
leukemic cells per unit volume. Tumor growth inhibition of the leukemia was calculated using
the formula \( 1 - [(\text{Count}_{\text{treated}} - \text{Count}_{\text{naive}})/(\text{Count}_{\text{vehicle}} - \text{Count}_{\text{naive}})] \).

All animal studies were performed in accordance with American Association for
Laboratory Animal Care institutional guidelines. All Lilly-internal \textit{in vivo} experimental protocols
were approved by the Eli Lilly and Company Animal Care and Use Committee.
Results

LY3023414 shows high solubility across a wide pH range

LY3023414 was highly soluble in various media whose conditions were intended to mimic the full physiological pH range of the gastrointestinal tract (pH 1.5-7) as well as the fasted and fed states (Table 1). The compound demonstrated the highest solubility near pH 2, which corresponds to the fasted stomach pH, and maintained favorable solubility and dissolution properties for absorption at pH 4.5 and pH 7.5, as observed in duodenal and intestinal fluids. When these solubility data were applied to a gastroduodenal passage model [39], the simulation predicted that LY3023414 would closely approach the theoretical maximum absorption in contrast to other PI3K/mTOR dual inhibitors (ie, NVP-BEZ-235 and PF04691502; see Fig. S1), while absorption would be only slightly reduced by coadministration of gastric pH-altering proton-pump inhibitors.

LY3023414 inhibits class I PI3K, mTOR, and DNA-PK

As shown by testing against a total of 266 unique kinases, LY3023414 inhibited members of the PI3K superfamily at low nanomolar concentrations. Table 2 summarizes the inhibitory potential of LY3023414 against PI3K family members, including class I PI3K isoforms.

The co-crystal structure of LY3023414 bound to the active site of PI3Kγ (Fig. 1B) confirmed the ATP-competitive mechanism of action observed in kinetic experiments with PI3Kα. Kinetic studies demonstrated ATP-competitive inhibition and an inhibitory constant (Ki) of 8.5 nM (Fig. S2).

There was at least an order-of-magnitude difference between LY3023414’s inhibition of PI3K superfamily enzymes and that of the remaining purified enzymes in the kinase panel tested, thereby underlining the selectivity of LY3023414 for its target kinases (Tables S1 and S2).
cell-based assays, LY3023414 inhibition of PI3K and mTOR was assessed in the PTEN-deficient U87 MG glioblastoma cell line. LY3023414 inhibited the phosphorylation of AKT at position T308 downstream of PI3K at an IC₅₀ of 106 nM (Table 3). Similarly, LY3023414 inhibited phosphorylation of AKT at position S473 (IC₅₀ = 94.2 nM) by mTORC2 as well as phosphorylation of mTORC1 kinase targets p70S6K (position T389; IC₅₀ = 10.6 nM) and 4E-BP1 (positions T37/46; IC₅₀ = 187 nM). The downstream phosphorylation of S6RP at positions pS240/244 (IC₅₀ = 19.1 nM) by p70S6K was inhibited as well, indicating target inhibition along the entire PI3K/AKT/mTOR pathway by LY3023414. Similar IC₅₀ concentrations for PI3K and mTOR phosphorylation targets were observed in other cell lines with activated PI3K/AKT/mTOR pathways (Table S3).

To explore target inhibition as a potential surrogate PD marker, phosphorylation of 4E-BP1 (pT37/46) in human PBMCs from healthy donors (n=3) was measured after ex vivo treatment with LY3023414 for 2 hours. The median IC₅₀ for p4E-BP1 was 156 ng/mL (0.36 μM) (Fig. S3), which was slightly higher than the IC₅₀ in cell culture.

**LY3023414 inhibits tumor cell proliferation**

The ability of LY3023414 to inhibit cancer cell proliferation was evaluated in 32 human cancer cell lines from different tumor types in culture after LY3023414 treatment for 2-3 cell doublings in dose-response studies (Fig. 2A). LY3023414 demonstrated potent single-agent activity and IC₅₀ values below 122 nM in half of the cell lines tested. Among the more sensitive cell lines were ones originating from breast cancer (4 of 4 tested) and mesothelioma (5 of 6 tested), while none of the lung or colon cancer cell lines tested appeared to be highly sensitive. There was no obvious correlation between LY3023414 sensitivity and any particular aberration known to activate the PI3K/AKT/mTOR pathway (ie, PIK3CA<sup>mt</sup>, PTEN<sup>del</sup>) (see Table S4). The
broad antiproliferative activity of LY3023414 was confirmed in clonogenic assays comprising 93 tumor models across a variety of histologies grown for 2 weeks in soft agar in the presence of LY3023414 (Fig. S4).

With respect to LY3023414’s observed antiproliferative mechanism of action, the accumulation of cells in the G1 phase was similar to that described after treatment with other PI3K/AKT/mTOR pathway inhibitors [8, 9, 40] and thereby indicative of a cytostatic response (Fig. 2B). Minimal to no induction of apoptosis was observed in cell lines treated with antiproliferative concentrations of LY3023414. Only at substantially higher LY3023414 concentrations (ie, 2.5 µM) was induction of apoptosis noted, as measured by an increase in activated caspase-3 and -7 (Table S5). There was no apparent concordance in the rank-order of sensitive cell lines in proliferation versus apoptosis assays.

When evaluated in combination with various standard-of-care anticancer therapeutics (ie, cisplatin, docetaxel, doxorubicin, erlotinib, gemcitabine, irinotecan, paclitaxel, pemetrexed, rapamycin, and tamoxifen) in a panel of 21 cell lines, LY3023414 showed additive or synergistic antiproliferative activity in most cases (Table S6). The most statistically significant (ie, lowest) combination indices were observed for combinations of LY3023414 with erlotinib in pancreatic cancer and rapamycin in lung and renal cancer cell lines. For instance, the combination of rapamycin and LY3023414 exhibited combination indices ranging from 0.04 to 0.26 in multiple cell lines.

**LY3023414 exhibits high bioavailability in animals**

Pharmacokinetic analyses of orally or intravenously administered LY3023414 were performed in male CD-1 mice, male Sprague-Dawley rats, and male beagle dogs (Table 4; Fig. S5). After intravenous dosing, plasma clearance (Cl) of LY3023414 was moderate, ranging
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from 10 mL/min/kg in dogs to 22 mL/min/kg in mice and rats. Oral bioavailability was high (>95%) in rats and dogs, suggesting nearly complete absorption at doses of 10 and 3 mg/kg, respectively. Volume of distribution (Vdss) was moderate, and half-life (t1/2) ranged from 1 to 3 hours. The results of additional PK studies in female animals were consistent with those observed in males (data not shown). To assess the importance of oxidative metabolism in LY3023414 clearance, rats were also pretreated with ABT to inhibit CYP enzymes. Clearance was decreased approximately 2-fold and t1/2 was increased, suggesting that CYP metabolism accounts for approximately half of total clearance in rats. However, oral bioavailability was not affected by ABT treatment. LY3023414 was moderately bound to plasma proteins, with an ex vivo mean unbound fraction of 40% in rat plasma and 17% in human plasma.

**LY3023414 shows time- and dose-dependent target inhibition in vivo**

The ability of LY3023414 to affect the phosphorylation status of PI3K/AKT/mTOR pathway kinases was evaluated in the U87 MG xenograft model after a single oral administration of LY3023414. LY3023414 inhibited in a time- and dose-dependent manner the phosphorylation of all direct and indirect target kinase substrates assessed (Fig. 3; Table S7). The direct PI3K/mTOR substrates pAKT, pS6K, and p4E-BP1 were maximally inhibited around the maximum concentration (Cmax) of LY3023414 at approximately 30 minutes after oral dosing, while pS6RP as substrate of S6K was maximally inhibited 2-6 hours after oral dosing. At these time points, the average inhibition of phosphorylation across all target substrates in response to the highest dose of LY3023414 administered (30 mg/kg) was approximately 90% of that at baseline. The median LY3023414 dose required for 50% inhibition of target phosphorylation substrates 30 minutes after LY3023414 administration was 2.7 mg/kg and ranged from 0.3 mg/kg (for the pT389 site of p70S6K) to 10.8 mg/kg (for the pS240/244 sites of S6RP)
Table 5). The dose required for 50% inhibition close to the time of maximal inhibition of pS240/244 S6RP (4 hours after LY3023414 administration) was 4.5 mg/kg (Fig. 3, Table 5). Target inhibition was dependent on LY3023414 plasma concentration. The duration of target inhibition greater than 50% was plasma concentration dependent and varied between 2 and 8 hours depending on the phosphorylation substrate measured and LY3023414 dose administered (Fig. 3A).

**LY3023414 shows single-agent antitumor activity in vivo**

The ability of LY3023414 to inhibit tumor growth was studied in several xenograft models exhibiting mutations or deletions that activate the PI3K/AKT/mTOR pathway [41]. Treatment with LY3023414 at 3, 6, or 10 mg/kg twice daily orally for 28 days resulted in dose-responsive inhibition of tumor growth in the PTEN-deleted U87 MG xenograft model (Fig. 4A). This treatment produced similar tumor growth inhibition in models exhibiting PTEN truncation (786-O), activating PI3Kα mutation (NCI-H1975), and transgenic Eμ-myc mutant PI3Kα-driven leukemia models (Fig. 4B). Of note, the total daily dose of LY3023414 appeared to result in equipotent antitumor activity: 12 mg/kg once daily and 6 mg/kg twice daily produced similar delta T/C values (42% and 38%, respectively) in U87 MG.

To investigate further the effect of different daily and intermittent dosing schedules, 4 different treatment schedules were evaluated in the U87 MG xenograft model (Fig. 4C). LY3023414 administered at doses of 15 mg/kg twice daily and 30 mg/kg once daily produced equivalent delta T/C values throughout the dosing period, suggesting that the total dose determined LY3023414 antitumor activity regardless of treatment schedule chosen. Likewise, an intermittent schedule of 20 mg/kg twice daily for 7 days on/7 days off for 2 cycles and a continuous schedule of 10 mg/kg twice daily for 28 days (a total dose of 560 mg/kg over 28 days
in both cases) resulted in similar tumor growth inhibition. Even though some tumor growth was evident during the 7-day dosing holidays on the intermittent dosing schedule, the delta T/C values for the intermittent and continuous dosing groups were statistically similar.

LY3023414 was studied for activity as a single agent in a collection of patient-derived tumor xenografts. As shown in Table S8, LY3023414 demonstrated single-agent activity across a wide range of tumor histologies (including bladder, colon, gastric, head and neck, lung, breast, ovarian, and renal cell cancers). On the basis of the sensitivity of mesothelioma cell lines towards LY3023414 observed in *in vitro* assays, LY3023414 was evaluated in 6 patient-derived mesothelioma xenograft models (PXF-537, PXF-541, PXF-680, PXF-698, PXF-1118, PXF-1752). In these models, LY3023414 showed moderate to high single-agent activity; in 5 of them, delta T/C values ranged from –33.3% to 41.2%, and LY3023414 demonstrated higher average potency than did single-agent pemetrexed (Fig. 5). Single-agent activity of LY3023414 was observed without any obvious correlation to activating PI3K/mTOR pathway aberrations in these *in vivo* models (Table S4).

**Selected LY3023414 combinations demonstrate additivity *in vivo***

To determine whether the synergistic antiproliferative effect of LY3023414 *in vitro* would also be seen *in vivo*, LY3023414 (3 mg/kg) and rapamycin (4 mg/kg) were assessed for antitumor activity as single agents and in combination in several xenograft models. In testing 3 xenograft models (U87 MG, NCI-H1975, 786-O), the combination with rapamycin showed some evidence of potentiation in U87 MG, but the effect size varied and while meeting the prespecified definition of additive response (see Materials and Methods section) it was not statistically different from additive in any model tested (Fig. 6A; Fig. S6).
In 6 patient-derived tumor xenografts of squamous non-small cell lung cancer (LXFE-211, LXFE-397, LXFE-409, LXFE-646, LXFE-1022, and LXFE-1422), treatment with LY3023414, a cisplatin/gemcitabine doublet, or a LY3023414/cisplatin/gemcitabine combination had effects ranging from moderate tumor growth inhibition to near-complete tumor regression. The activity of the combination was, on the whole, additive when compared with that of LY3023414 or cisplatin/gemcitabine (Fig. 6B; Table S9).
Discussion

The PI3K/AKT/mTOR pathway is one of the most frequently activated signaling pathways in human cancers, but its role in cancer is complex and remains incompletely understood. Here, we demonstrate that the novel PI3K/mTOR inhibitor LY3023414 is a potent, highly soluble, and selective inhibitor of class I PI3K isoforms, mTOR, and DNA-PK that exerts strong antitumor activity in vitro and in vivo.

The incomplete understanding of the relative contribution of the different PI3K isoforms to solid tumor growth and the existence of signaling feedback loops makes the a priori design of an optimal kinase inhibition profile challenging. In the present study, screening of LY3023414 against approximately 266 kinases in either enzyme- or cell lysate-based assays identified only PI3K superfamily members as highly sensitive kinases, thereby underlining the compound’s relative selectivity. LY3023414 inhibited all class I PI3K isoforms, PI3Kα being the most sensitive and PI3Kβ the least. LY3023414 also inhibited DNA-PK and mTOR, although mTOR IC₅₀ values differed between assay formats by approximately 10-fold. This difference in potency may reflect differences in the forms of mTOR and substrates used in each assay format. The KiNativ cell lysate assay uses endogenous full-length mTOR (presumably present in mTORC1 or mTORC2 complexes) and endogenous substrates; the LanthaScreen kinase assay uses a truncated enzyme and a labeled substrate. Such assay-dependent variability in potency of enzymatic assays has been well described previously [42]. Consequently, we consider cell-based assays more reliable at predicting the in vivo potency of kinase inhibitors. In the cell-based assays we used, LY3023414 decreased phosphorylation of direct or downstream substrates of PI3K and mTOR at similar nanomolar concentrations, as evidenced by its inhibition of phosphorylation of AKT at position T308 (the first protein phosphorylation event downstream of
PI3K; 106 nM) and of pS473 AKT, pT389 p70S6K, and pT37/46 4E-BP1 (downstream of mTOR complexes; 11-187 nM), respectively.

The leading mode of action for the antitumor activity for PI3K/mTOR inhibitors is challenging to define and rather pleiotropic. In line with previous reports on other PI3K/mTOR inhibitors [40], treatment of cells in culture with LY3023414 resulted in accumulation of cells in the G1 phase of the cell cycle. Activation of caspase-3 and -7, a measure of apoptosis induction, did not correlate with rank-ordered IC$_{50}$ values and was strong only at concentrations much higher than those inhibiting cellular proliferation. LY3023414 had broad antiproliferative activity in a wide variety of cell lines grown in monolayer as well as clonogenic colony formation assays using cells from patient-derived tumor xenografts. In vitro combination of LY3023414 with commonly used standard-of-care anticancer therapeutics resulted in additive effects for most of the combinations but synergistic effects for combinations with rapamycin, gemcitabine, erlotinib and irinotecan.

The ability of a given pharmacological agent to engage its biological target is driven by its PK profile, and the initial step in oral absorption of a drug is dissolution in the gastrointestinal tract. The measured solubility and dissolution properties of LY3023414 were favorable for absorption if compared with published data for other PI3K/mTOR inhibitors [2, 30, 43]. The compound was quite soluble near pH 2, which corresponds to the initial pH encountered by LY3023414 in the stomach, and remained soluble in simulated gastric and intestinal fluids. The favorable solubility of LY3023414 between pH 4.5 and pH 7.5 is expected to minimize precipitation that can contribute to variable and lower absorption in the intestinal tract [29, 30]. Modelling in a gastroduodenal passage model indicated that absorption would be only slightly reduced by coadministration of gastric pH-altering proton-pump inhibitors.
The PK parameters of LY3023414 in rats and dogs suggest that the drug is extensively absorbed at relevant doses and subsequently cleared in part via oxidative metabolism by CYP enzymes. The moderate volume of distribution and clearance resulted in a half-life of 1-3 hours for LY3023414.

The *in vivo* PK properties of LY3023414 demonstrate that it achieves exposures needed to inhibit the PI3K/AKT/mTOR pathway for relatively short periods of time. Dose- and time-dependent pathway inhibition was observed in mice exposed to LY3023414 in the U87 MG xenograft model. Maximum inhibition was observed 30 minutes after oral administration for pAKT, p70S6K, and p4E-BP1 and 3-6 hours after oral administration for pS6RP. Pathway activation of mTOR substrates pS473AKT and pT389 p70S6K was observed 12 hours after oral administration of LY3023414 at doses of 15 and 30 mg/kg and returned to pretreatment levels by 24 hours. Activation of pS235/236 S6RP was observed 24 hours after oral administration of LY3023414 at doses of 15 and 30 mg/kg. The relatively brief (6-hour) pathway inhibition translated into efficacy in the U87 MG xenograft model that was independent of dosing schedule. Notably, the inhibition of tumor growth in the U87 MG xenograft model was similar regardless of whether the same total daily dose was administered in a single dose or 2 separate doses. A survey of LY3023414 activity in various tumor xenograft models confirmed this schedule-independent activity, as the same total daily or weekly summed dose achieved equivalent efficacy.

Inhibitors of the PI3K/AKT/mTOR pathway have been developed and are being tested for their potential anticancer activity alone and in combination with other anticancer agents [44, 45]. Dose-dependent single-agent activity of LY3023414 was observed in xenograft models with different types of aberrant PI3K/mTOR pathway activation (eg, PIK3CAmt, PTEN loss).
However, across all tumor models and in line with previous studies [44, 45], no obvious predictive marker for sensitivity to LY3023414 in vivo or in vitro was identified. Of the 28 human patient-derived tumor xenograft models we have studied in vivo, squamous non-small cell lung cancer and mesothelioma models were the most sensitive to LY3023414 monotherapy. The favorable PK properties of LY3023414 and relatively short exposure provided discontinuous inhibition of the PI3K/AKT/mTOR pathway that was of sufficient duration and intensity to result in significant antitumor activity in a variety of tumor xenograft models. The synergistic in vitro effect of LY3023414 combined with rapamycin in various cell lines was not as strong in xenograft models of the same cell lines, potentially reflecting the higher total constant LY3023414 exposure in the in vitro short-term experiments. Combination of LY3023414 with a cisplatin/gemcitabine doublet also demonstrated additivity in vivo, when tested in squamous non-small cell lung cancer patient-derived tumor models.

In conclusion, LY3023414 is a novel, potent ATP-competitive inhibitor that demonstrates activity against PI3K, mTOR, and DNA-PK, thereby inducing cell-cycle effects and inhibiting cancer cell viability. Downstream target inhibition by LY3023414 occurs rapidly via an intermittent “on/off” mechanism that may enhance the drug’s clinical tolerability, which may in turn allow LY3023414 to overcome some of the toxicities associated with PI3K/mTOR inhibitors and potentially reduce the emergence of feedback mechanisms leading to resistance. The physicochemical and absorption properties of LY3023414 are favorable, as evidenced by the molecule’s high solubility across a wide pH range and high oral bioavailability. On the basis of these findings, LY3023414 is currently being evaluated in phase 1 and 2 trials (see clinicaltrials.gov) in patients with advanced cancer.
Acknowledgements

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References


41. COSMIC Catalogue of somatic mutations in cancer.


**Table 1.** Solubility of LY3023414 under various physiologic conditions

<table>
<thead>
<tr>
<th>Sample media&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average pH</th>
<th>Average solubility of LY3023414 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N HCl</td>
<td>1.32</td>
<td>≥10</td>
</tr>
<tr>
<td>0.01N HCl</td>
<td>3.88</td>
<td>5.29</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>4.52</td>
<td>1.34</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>5.97</td>
<td>0.478</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>7.48</td>
<td>0.377</td>
</tr>
<tr>
<td>SGF</td>
<td>3.99</td>
<td>5.01</td>
</tr>
<tr>
<td>Fed</td>
<td>5.00</td>
<td>1.98</td>
</tr>
<tr>
<td>Fast</td>
<td>6.50</td>
<td>0.643</td>
</tr>
</tbody>
</table>

<sup>a</sup> pH 4.5 medium was 30 mM acetate buffer at starting pH 4.5; pH 6.0 and pH 7.5 media were 50 mM phosphate buffer at starting pH 6.0 or pH 7.5, respectively; determined in triplicate.

Abbreviations: SGF = simulated gastric fluid; Fed = simulated intestinal fluid fed state; Fast = simulated intestinal fluid fasted state.
Table 2.  Biochemical enzyme inhibition of PI3K family members by LY3023414

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recombinant IC50 (nM) ± SE (n)</th>
<th>Native cell lysate IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K α</td>
<td>6.07 ± 3.38 (2)</td>
<td>83</td>
</tr>
<tr>
<td>PI3K β</td>
<td>77.6 ± 40.1 (2)</td>
<td>600</td>
</tr>
<tr>
<td>PI3K δ</td>
<td>38 (1)</td>
<td>ND</td>
</tr>
<tr>
<td>PI3K γ</td>
<td>23.8 (1)</td>
<td>ND</td>
</tr>
<tr>
<td>mTOR</td>
<td>165 ± 92.5 (5)</td>
<td>16</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>4.24 (1)</td>
<td>9.3, 3.6</td>
</tr>
</tbody>
</table>

Abbreviations:  ND = not done, SE = standard error of the mean.
Table 3.  *In vitro* cell-based target inhibition(s) in the U87 MG cell line

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC$_{50}$ (nM)</th>
<th>SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K inh pT308 AKT</td>
<td>106</td>
<td>64.9 (4)</td>
</tr>
<tr>
<td>mTORC1 inh pT389 p70S6K</td>
<td>10.6</td>
<td>2.96 (4)</td>
</tr>
<tr>
<td>mTORC2 inh pS473 AKT</td>
<td>94.2</td>
<td>4.21 (4)</td>
</tr>
<tr>
<td>p70S6K inh p240/244 S6RP</td>
<td>19.1</td>
<td>2.04 (3)</td>
</tr>
<tr>
<td>mTORC1 inh pT37/46 4E-BP1</td>
<td>187</td>
<td>60.5 (4)</td>
</tr>
</tbody>
</table>

Abbreviation: inh = inhibition.
Table 4. Mean (± SD) pharmacokinetic parameters in male CD-1 mice, male Sprague-Dawley rats, and male beagle dogs following oral and/or intravenous administration of LY3023414

<table>
<thead>
<tr>
<th>Parameter, units</th>
<th>Mouse (n = 3/time point)</th>
<th>Rat (n = 3/group)</th>
<th>Dog (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 mg/kg IV</td>
<td>3 mg/kg IV</td>
<td>10 mg/kg PO</td>
</tr>
<tr>
<td>AUC_{0-24hr}, ng∙h/mL</td>
<td>4570</td>
<td>2300±143</td>
<td>5700±918</td>
</tr>
<tr>
<td>AUC_{0-inf}, ng∙h/mL</td>
<td>4580</td>
<td>2300±137</td>
<td>5860±895</td>
</tr>
<tr>
<td>C_{max}, ng/mL</td>
<td>5410</td>
<td>1714±308</td>
<td>2924±477</td>
</tr>
<tr>
<td>T_{max}, h</td>
<td>ND</td>
<td>ND</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>t_{1/2}, h</td>
<td>2.8</td>
<td>1.4±0.2</td>
<td>4.6±1.1</td>
</tr>
<tr>
<td>Cl, mL/min/kg</td>
<td>22</td>
<td>22±1</td>
<td>8.7±1.2</td>
</tr>
<tr>
<td>Vdss, mL/kg</td>
<td>2560</td>
<td>2760±317</td>
<td>3100±460</td>
</tr>
<tr>
<td>Bioavailability, %</td>
<td>ND</td>
<td>ND</td>
<td>97±15%</td>
</tr>
</tbody>
</table>

Abbreviations: ABT = aminobenzotriazole; AUC_{0-24hr} = area under the curve from time 0 to 24 hours; AUC_{0-inf} = area under the curve from time 0 to infinity; Cl = clearance; C_{max} = maximum concentration; IV = intravenous; ND = not determined; PO = orally; T_{max} = time to maximum concentration; t_{1/2} = half-life; Vdss = volume of distribution at steady state.
Table 5. Threshold effective dose and threshold effective concentration for LY3023414 pathway inhibition in U87 MG xenograft tumors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>TED50 (mg/kg)</th>
<th>TEC50 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT308 AKT</td>
<td>3.1</td>
<td>787</td>
</tr>
<tr>
<td>pS473 AKT</td>
<td>2.2</td>
<td>604</td>
</tr>
<tr>
<td>pT389 p70S6K</td>
<td>0.3</td>
<td>57</td>
</tr>
<tr>
<td>pS240/244 S6RP</td>
<td>4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>pS235/236 S6RP</td>
<td>2.3</td>
<td>589</td>
</tr>
<tr>
<td>pT37/46 4E-BP1</td>
<td>5.9</td>
<td>1777</td>
</tr>
</tbody>
</table>

<sup>a</sup> This was value at 4 hours; value at 0.5 hours was 10.8 mg/kg.

Abbreviation: ND = not determined; TED50 = threshold effective dose; TEC50 = threshold effective concentration.
Figure Legends

Figure 1. A. Chemical structure of LY3023414. B. Co-crystal structure of LY3023414 and p110γ. The structure of the complex of LY3023414 with PI3Kγ reveals that the compound binds in a pocket bounded by side-chain residues of Met953 and Ile963 on one side and Trp812 and Ile831 on the other. It makes a hydrogen bond with the main-chain amide of Val882 and a CH···O interaction with the main-chain carbonyl of Glu880. It additionally interacts with the tyrosine hydroxyl of Tyr867 and the main-chain amide and side-chain of Asp964. Since all the residues whose side chains form the binding pocket are conserved between PI3Kγ and PI3Kα, the PI3Kγ isoform binding can be considered reflective of the PI3Kα isoform binding mode and affinity of LY3023414.

Figure 2. A. Antiproliferative activity of LY3023414 in a panel of cancer cell lines of various origin. B. LY3023414 (LY) induces cell cycle accumulation in G1 as measured by fluorescence-activated cell sorting (FACS) analysis.

Figure 3. LY3023414 inhibits PI3K pathway targets in U87 MG xenograft tumors. A. Plasma concentrations after single oral administration of LY3023414 in mice at 3 mg/kg (inverted dark blue triangles), 6 mg/kg (light blue triangles), or 10 mg/kg (yellow squares). The TEC50 values are represented by dotted lines. B. Percent inhibition of phospho-signal in tumors at various phosphorylation sites on pathway proteins after single oral administration of LY3023414.

Figure 4. In vivo efficacy of LY3023414 in multiple tumor models and dosing schedules. A. U87 MG xenografts were treated with 3, 6, or 10 mg/kg twice daily (BID) or 12 mg/kg once daily (QD) for 28 days with oral administration of LY3023414. B. Multiple LY3023414 dose levels were assessed in xenograft (U98 MG, NCI-H1975, 786-O) or syngeneic graft (Eu-myc
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transgenic leukemia) models across various genetic backgrounds as noted. Del = deletion; mut = mutant; NSCLC = non-small cell lung cancer; WT = wild type. C. U87 MG xenografts were treated with 10 mg/kg BID continuously for 28 days or with 20 mg/kg BID on alternate weeks for an equivalent average dose of 140 mg/kg/week. Two other groups were treated BID with 15 mg/kg or QD with 30 mg/kg for an equivalent 30 mg/kg/day for 28 days.

**Figure 5.** Effects of single-agent LY3023414 or pemetrexed (disodium heptahydrate) on the tumor growth of 6 patient-derived mesothelioma xenograft models.

**Figure 6.** Combination activity of LY3023414 and standard-of-care drugs. A. *In vivo* efficacy of LY3023414 and rapamycin alone and in combination in U87 MG xenograft tumor cell line model. B. Single-agent arms each of twice daily oral 15 mg/kg LY3023414, weekly subcutaneous 3.2 mg/kg cisplatin, and weekly intravenous 60 mg/kg gemcitabine (HCl); or the combination for 28 days of dosing in the squamous non-small cell lung cancer (sqNSCLC) LXFE-211 patient-derived tumor model.
Figure 1

A

Molecular formula: $C_{23}H_{26}N_4O_3$
Molecular weight: 406.48

B
Figure 2

A

Abs IC<sub>50</sub> (µM)

Cell Line

B

Cell Cycle Analysis

G2

S

G1
Figure 3

A

LY3023414 (ng/ml)

Time (hr)

3 mg/kg
6 mg/kg
10 mg/kg

T308
S473
T389

B

% inhibition

Time (hr)

pT308 AKT

pS473 AKT

pT389 S6K

pT37/46 4E-BP1

pS240/244 S6RP

pS235/236 S6RP

% inhibition

Time (hr)
Characterization of LY3023414, a Novel PI3K/mTOR Dual Inhibitor Eliciting Transient Target Modulation to Impede Tumor Growth

Michele C. Smith, Mary M. Mader, James A. Cook, et al.

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