Characterization of Torin2, an ATP-Competitive Inhibitor of mTOR, ATM, and ATR

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Abstract

mTOR is a highly conserved serine/threonine protein kinase that serves as a central regulator of cell growth, survival, and autophagy. Deregulation of the PI3K/Akt/mTOR signaling pathway occurs commonly in cancer and numerous inhibitors targeting the ATP-binding site of these kinases are currently undergoing clinical evaluation. Here, we report the characterization of Torin2, a second-generation ATP-competitive inhibitor that is potent and selective for mTOR with a superior pharmacokinetic profile to previous inhibitors. Torin2 inhibited mTORC1-dependent T389 phosphorylation on S6K (RPS6KB1) with an EC50 of 250 pmol/L with approximately 800-fold selectivity for cellular mTOR versus phosphoinositide 3-kinase 3-kinase (PI3K). Torin2 also exhibited potent biochemical and cellular activity against phosphatidylinositol-3-kinase-like kinase (PIKK) family kinases including ATM (EC50, 28 nmol/L), ATR (EC50, 35 nmol/L), and DNA-PK (EC50, 118 nmol/L; PRKDC), the inhibition of which sensitized cells to Irradiation. Similar to the earlier generation compound Torin1 and in contrast to other reported mTOR inhibitors, Torin2 inhibited mTOR kinase and mTORC1 signaling activities in a sustained manner suggestive of a slow dissociation from the kinase. Cancer cell treatment with Torin2 for 24 hours resulted in a prolonged block in negative feedback and consequent T308 phosphorylation on Akt. These effects were associated with strong growth inhibition in vitro. Single-agent treatment with Torin2 in vivo did not yield significant efficacy against KRAS-driven lung tumors, but the combination of Torin2 with mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitor AZD6244 yielded a significant growth inhibition. Taken together, our findings establish Torin2 as a strong candidate for clinical evaluation in a broad number of oncologic settings where mTOR signaling has a pathogenic role. Cancer Res; 73(8); 1–13. ©2013 AACR.

Introduction

The mTOR is a highly conserved and widely expressed serine/threonine kinase that is a member of the phosphatidylinositol-3-kinase-like kinase (PIKK) family, which includes the serine/threonine kinases ATR, ATM, DNA-PK, and SMG-1 (1, 2). mTOR serves as a pivotal node in the PI3K/Akt/mTOR signaling pathway, which senses growth factor and nutrient signals and controls fundamental cellular processes such as cell growth, autophagy, translation, and metabolism (3, 4). Hyperactivation of this pathway through loss of negative regulators, such as PTEN, or mutational activation of receptor tyrosine kinases of phosphoinositide 3-kinase (PI3K) is a frequent occurrence in cancer (5). mTOR exists in at least 2 multiprotein complexes, which are named mTORC1 and mTORC2 (6, 7). mTORC1 contains mTOR, mLST8, and raptor as core components and regulates cell growth, protein synthesis, and autophagy through its downstream effectors, including S6K1, 4EBP1, and ATG13. mTORC2 consists of mTOR, mLST8, rictor, PR55ε, and SIN1 as core components and regulates cell survival and actin organization through effectors such as Akt, SGK1, and PKCα. Through its inclusion in these 2 protein complexes mTOR functions both upstream of Akt through...
mTORC2-dependent phosphorylation of S473 located in the hydrophobic-motif and downstream of Akt in the context of mTORC1 (4).

Rapamycin, a Streptomyces-derived natural product that is an allosteric inhibitor of mTORC1, has been critical to understanding the functions of mTOR (8). Rapamycin acts by forming a complex with the small 12 kDa protein FKBP-12, and binding to the FRB domain of mTOR, acutely inhibiting mTORC1 activity (9). Rapamycin does not acutely inhibit the activity of mTORC2, although prolonged treatment can disrupt its activity by destabilizing complex formation (10). Prolonged suppression of mTORC1 also results in disruption of a negative feedback loop and consequently results in hyperphosphorylation of Akt through activation of IRS1 and PI3K (11). Although rapamycin was long thought to completely disable mTORC1, a new class of ATP competitive mTOR inhibitors, such as Torin1 and PP242, have revealed that many mTORC1 functions, such as phosphorylation of the 4E-BP family of translational repressors, are resistant to rapamycin (12–14). Moreover, this new class of inhibitors also potently targets mTORC2. Together, these features have generated hope that the new generation of ATP-competitive mTOR inhibitors will exhibit broader clinical efficacy relative to the rapalogs.

Many members of this new class of ATP-competitive mTOR inhibitors have been developed using previously identified PI3K inhibitors as starting points. For example, LY294002, one of the original and most frequently used PI3K inhibitors, is structurally related to many dual PI3K/mTOR inhibitors, including SF1126, GSK1059615, BEZ235, XL765, PKI-587, PF-04691502, GSK2126458, and PKI-179, several of which have been advanced into clinical trials (15–22). mTOR inhibitors with little or no activity against PI3K, such as Torin1 and PP242, have served as important research tools to advance the basic understanding of the mTOR signaling pathway and AZD8055, WYE-125132, INK-128, and OSI-027 are currently undergoing clinical evaluation (23).

In the present study, we report detailed cellular and in vivo evaluation of Torin2, a compound recently developed to overcome the pharmacologic limitations of Torin1. Chemical proteomic profiling followed by cellular pathway profiling shows that Torin2, unlike Torin1, is also a potent inhibitor of ATR, ATM, and DNA-PK (29–31). Torin2 displays dramatic antiproliferative activity across a panel of cancer cell lines and elicited a combinatorial response with the mitogen-activated protein/extracellular signal-regulated kinase (MEK) kinase inhibitor AZD6244 against genetically engineered mutant KRAS-driven lung tumors.

Materials and Methods

Inhibitors

Torin1 and Torin2 were prepared as previously described (13, 29). AZD8055, PP242, and staurosporine were purchased from Haoyuan Chemexpress Co. Acridine orange was purchased from Invitrogen.

ATP competition assay

Human mTORC1 complex was obtained as reported (12). In vitro mTORC1 activity was assayed using the Lanthascreen time-resolved FRET assay (Invitrogen). Briefly, mTORC1 was incubated with serially diluted inhibitors (3-fold, 10 points) for 30 minutes in 5 μL of kinase buffer [25 mmol/L HEPES, pH 7.4, 8 mmol/L MgCl2, 6 mmol/L MnCl2, 4 mmol/L dithiothreitol (DTT)] in a 384-well low-volume white plate (Corning). The kinase reaction was initiated by the addition of an equal volume of the kinase buffer containing 0.6 μmol/L GFP-labeled 4E-BP1 and 20 μmol/L ATP. After incubation at room temperature for 90 minutes, the reaction was stopped by the addition of a 5 μL of solution containing 45 mmol/L EDTA and 4.5 mmol/L Tb-labeled antiphospho-4E-BP1 (T46) antibody. After 30 minutes, the FRET signal between Tb and GFP within the immune complex was read using an Envision plate reader (PerkinElmer). Each data point was duplicated and IC50 values were calculated using Prism4 software (GraphPad). For ATP competitiveness test, IC50 values were determined at a range of ATP concentrations in duplicate.

Immunoblot assays

ATR, ATM, and DNA-PK cellular activity: HCT-116 Cells were seeded in 6-well plates (0.5 × 105/well) and grown overnight. After 1 hour of pretreatment with appropriate compounds at 37°C, culture media was removed and saved. For ATR assay, the cells were treated with 50 μl of UV radiation energy using strata linker (10 gray Ionizing radiation for ATM and DNA-PK assay). The culture media were added back to the cells and incubated at 37°C. After 1 hour, cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer [40 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 10 mmol/L pyrophosphate, 10 mmol/L glyc erophosphate, 1% Triton X-100, and 1 tablet of EDTA-free protease inhibitors per 25 mL]. The soluble fractions of cell lysates were then isolated by centrifugation at 13,000 rpm for 10 minutes in a microcentrifuge. After the lysates from all the plates were collected, the concentration of the protein was normalized by Bradford assay. Fifty-microliter sample buffer was added to the normalized lysates and boiled for 5 minutes. Samples were subsequently analyzed by SDS-PAGE and immunoblotting. Results are shown in Fig. 1C–E.

Biochemical and cellular mTOR kinase assays

In vitro assay. mTORC1 was incubated with inhibitors [0.5 μmol/L, 1% dimethyl sulfoxide (DMSO)] in 5 μL of reaction buffer (25 mmol/L HEPES pH 7.4, 8 mmol/L MgCl2 and 6 mmol/L MnCl2) for 1 hour at room temperature. Then, drug–ATP competition was induced by the addition of 245 μL of the reaction buffer containing 500 μmol/L ATP, 4 mmol/L DTT, and 0.3 μmol/L GFP-4EBP1 (Invitrogen). The reaction mixture was dispensed (10 μL, triplicate) into a low volume white plate (Corning) and the kinase reaction was stopped at various times with 5 μL of stop solution (Invitrogen). The stop solution (5 μL) containing 4 nmol/L Tb-labeled p-4EBP1 (T46) antibody (Invitrogen) was added, then the FRET signal was read using Envision (PerkinElmer) after 30 minutes of incubation. Results are shown in Fig. 2A.

Cellular assay. HCT116 cells were treated with 100 nmol/L Torin2 or AZD8055 for 1 hour before they were thoroughly washed out by 3 × PBS and 1 × Dulbecco’s Modified Eagle’s Medium (DMEM) medium. Then cells were incubated...
in DMEM for indicated time before they are lysed and collected using M-PER (Pierce) according to the manufacturer’s instructions. Protein concentrations were measured and equal amount of proteins were loaded. Experiments were repeated 3 times and 1 set of results are shown in Fig. 2B.

**Ionizing irradiation assay.** Clonogenic cell survival in the human fibroblast AG01522 cell line was assessed by colony formation, using our standard protocols (32). Culture media was α-modified minimum essential medium (Sigma) with 20% FBS (HyClone), 100 μg/mL streptomycin, and 100 U/mL penicillin. A total of 100 nmol/L Torin2 was added 30 minutes before irradiation. The cells were irradiated using 250 kVp X rays (Siemens Stabilipan 2) and incubated for 24 hours, then reseeded into 60-mm Petri dishes. Colonies were stained with methylene blue after 12 days of incubation in a 37°C incubator supplied with 5% CO₂. Colonies containing at least 50 cells were scored under a bright field microscope. Plating efficiencies were calculated as colonies per number of cells plated and
surviving fractions as ratios of plating efficiencies for irradiated and unirradiated cells. All experiments consisted of 3 independent repeats Fig. 1F.

**Longer-term cellular signaling analysis**

HCT116 and HeLa cells were treated with 100 nmol/L of Torin2 or AZD8055 for indicated time before they are lysed and collected using M-PER (Pierce) according to the manufacturer’s instructions. Protein concentrations were measured and equal amount of proteins were loaded. Experiments were repeated 3 times and 1 set of results are shown in Fig. 2D and E.

**Apoptosis assays**

Different concentrations of AZD8055, Torin2, or staurosporine were added to 5 cancer cell lines for 16 hours before the cells were collected and analyzed by Western blot analysis using anti-PARP and anti-tubulin antibodies. Results are shown in Fig. 3B.

**Autophagy assay**

Hela cells were plated on coverslips, treated with different concentrations of Torin2 for 3 days before 1 μg/mL acridine orange was added for 15 minutes. Cells were washed in 3× PBS, fixed in PBS + 4% formaldehyde, and stained with 4’,6-diamidino-2-phenylindole (DAPI) before they are mounted in Prolong Gold (invitrogen). Pictures were taken in Nikon Imaging Center of Harvard Medical School (Boston, MA). Total fluorescence of acridine orange in each frame was quantified using MetaMorph and divided by the total number of cells within the frame. Numbers are then normalized to DMSO-treated cells to show the acridine orange fold changes. Experiments were repeated 3 times. Mean values are shown for each condition and error bars represent SDs. For Fig. 3D, Hela cells were treated with different concentrations of Torin2 for 3 days before fixed and stained with anti-LC3B antibody and DAPI. Pictures were taken in Nikon Imaging Center of Harvard Medical School. For Fig. 3E, different concentrations of AZD8055 or Torin2 was added to 5 cancer cell lines for 3 days.
A 3 d treatment

HCT116
AZD8055/Torin2 concentration

HeLa
AZD8055/Torin2 concentration

Calu 1
AZD8055/Torin2 concentration

H358
AZD8055/Torin2 concentration

H226
AZD8055/Torin2 concentration

B 16 h treatment

Control + Torin2 50 nmol/L

Control + Torin2 100 nmol/L

Control + Torin2 200 nmol/L

DMSO

Torin2–250 nmol/L

Torin2–1,000 nmol/L

G1

G2–M

S

Debris

2N  4N  2N  4N  2N  4N

PIKK Inhibitor Torin2 with Antitumor Activity

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before the cells are collected in M-PER as introduced earlier and analyzed by Western blot analysis using anti-LC3B and anti-tubulin antibodies. Results are shown in Fig. 3C–E.

Fluorescence-activated cell sorting analysis

Hela S3 cells were treated with Torin1, Torin2, or DMSO control for 48 hours. Cells were trypsinized, washed once in PBS, and fixed overnight at −20°C with 80% ethanol in PBS. Cells were washed 3 times with PBS. Finally, cells were resuspended in PBS containing 0.1% Triton X-100, 25 μg/mL propidium iodide (Molecular Probes), and 0.2 mg/mL RNase A (Sigma) and incubated for 45 minutes at 37°C. Samples were then analyzed and results are shown in Fig. 3F.

Tumor cell growth assay

All lung cancer cell lines (H2122, H358, H1792, A549, H441, H1355, H460, H226, H1299, and H292) were purchased from American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 media with 10% FBS at 37°C in a humified incubator with 5% CO2. To do combinational inhibition analysis, cells were plated into 96-well plates at 2,000 cells per well in 100-μL medium containing 4-fold serial dilution of AZD starting from 10 to 0.002 μmol/L and/or Torin2 starting from 0.2 to 0.04 μmol/L. After 3-day incubation, viable cells were counted using celliteter-glo (Promega). All reactions were carried out in triplicate. Results are shown in Fig. 4.

Mice cohort and drug treatment

Mice harboring a conditional activating mutation Lox-Stop-Lox-Kras (G12D) allele (35) were housed in a pathogen-free environment at the Harvard School of Public Health (Boston, MA) and were handled in strict accordance with Good Animal Practice as defined by the Office of Laboratory Animal Welfare, and all animal work was done with Dana-Farber Cancer Institute (Boston, MA) Institutional Animal Care and Use Committee approval. Mice were given Ad-Cre by nasal inhalation at 5 to 7 weeks of age to induce Kras G12D expression. After initial imaging, the mice bearing tumor were given Torin2 (40 mpg) or/and AZD6244 (25 mpg) garage daily. Torin2 was suspended in saline; AZD6244 was reconstituted in 0.5% methylcellulose (Fluka) and 0.4% polysorbate (Tween 80; Fluka).

MRI and PET/CT study

The mice were imaged by MRI biweekly to determine the reduction in tumor volume during the respective treatments, and 1:1,000 Whole Cell Stain (blue; Thermo Scientific) solution for 15 minutes. Cells were then washed 2 times with PBS and imaged in an imageWoRx high-throughput microscope (Applied Precision). The images were analyzed using ImageRail and the average GI50 of 6 experiments was calculated using DataPlex (33, 34). Results are shown in Table 1.

Combination study

Human non–small cell lung cancer cell lines H226, H358, and Calu-1 were grown in RPMI-1640/10% FBS/1% pen/strep/2 mmol/L L-glutamine at 37°C in a humified incubator with 5% CO2. To do combinational inhibition analysis, cells were plated into 96-well plates at 2,000 cells per well in 100-μL medium containing 4-fold serial dilution of AZD starting from 10 to 0.002 μmol/L and/or Torin2 starting from 0.2 to 0.04 μmol/L. After 3-day incubation, viable cells were counted using celliteter-glo (Promega). All reactions were carried out in triplicate. Results are shown in Fig. 4.

Table 1. Torin2 shows broad antiproliferative effects. GI50 was determined on the basis of a 72-hour growth assay.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>GI50, nmol/L</th>
<th>Tissue</th>
<th>Cell lines</th>
<th>GI50, nmol/L</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2122</td>
<td>13.2</td>
<td>Lung cancer</td>
<td>H1299</td>
<td>23.6</td>
<td>Lung cancer</td>
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<tr>
<td>H358</td>
<td>105.2</td>
<td>Lung cancer</td>
<td>H292</td>
<td>10.1</td>
<td>Lung cancer</td>
</tr>
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<td>19.8</td>
<td>Lung cancer</td>
<td>BT-20</td>
<td>219</td>
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<tr>
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<td>HS578T</td>
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<td>Breast cancer</td>
</tr>
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</tr>
<tr>
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<td>MCF7</td>
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<td>Breast cancer</td>
</tr>
<tr>
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<td>MDAMB231</td>
<td>108</td>
<td>Breast cancer</td>
</tr>
<tr>
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<td>74.6</td>
<td>Lung cancer</td>
<td>SKBR3</td>
<td>123</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>HCT-116</td>
<td>60.7</td>
<td>Colorectal cancer</td>
<td>Hela</td>
<td>29.3</td>
<td>Cervical cancer</td>
</tr>
</tbody>
</table>

Figure 3. Torin2 induces apoptosis and autophagy in vitro. A and B, cells were treated with the indicated concentrations of AZD8055, Torin2, or staurosporin overnight and analyzed by Western blot analysis using antibodies specific for the indicated proteins. C, Hela cells were treated with different concentrations of Torin2 for 3 days and then stained for acridine orange and DAPI. D, Hela cells were treated with indicated concentrations of Torin2 for 3 days stained with antibody specific for LC3B and DAPI. E, indicated cell lines were treated with increasing concentrations of AZD8055 or Torin2 for 3 days before the cells and analyzed by Western blot analysis using anti-LC3B and anti-tubulin antibodies. F, Torin2 effects on cell cycles.
as described previously (36). The tumor burden volume and quantification were reconstructed using 3D slicer software (http://www.slicer.org; ref. 37). The early effects of single-agent (AZD or Torin2) or combined dual-agent treatments on tumor glucose uses were studied in vivo using 2[18F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET). Each selected Kras tumor-bearing mouse underwent both baseline and post-Rx FDG-PET imaging, the latter of which was conducted after only 2 doses of respective single- or dual-agent treatments. The changes in tumor hypermetabolic activity, as quantified by the maximum standard uptake value (SUVmax) obtained from FDG-PET images, were compared for each treatment regimen. The operation of FDG-PET and quantification of SUVmax were described previously (38).

Immunohistochemical analyses

Hematoxylin and eosin (H&E) staining of tumor sections was conducted at the Department of Pathology at the Brigham and Women’s Hospital (Boston, MA). Antibodies of pAKT (S473), pS6K (T389), p4EBP1 (T37/46), pERK 1/2 (T202/204), and pCHK1(S345) were purchased from Cell Signaling. Immunohistochemistry was carried out on formal-fixed paraffin-embedded tumor sections using previously described methods (39).

Results

Torin2 is a potent and selective ATP-competitive mTOR inhibitor in vitro

Torin2 was discovered through a systematic medicinal chemistry effort to improve the pharmacologic and solubility properties of Torin1, a previously reported highly potent and
selective mTOR inhibitor (Fig. 1A; ref. 29). Biochemical kinase assays with increasing ATP concentrations show that Torin2 inhibits mTOR in an ATP-competitive fashion (Fig. 1B). To broadly survey the cellular targets of Torin2, we subjected the compound to profiling using the Kinativ chemical proteomics methodology (30). In this assay, the ability of Torin2 to protect kinases and other nucleotide-dependent enzymes from labeling with an electrophilic ATP–biotin compound in cellular lysates is measured using mass spectrometry. Kinativ profiling provides the most comprehensive coverage currently available for PIKK-family kinases, which bear the greatest homology to the ATP-binding site of mTOR. Comparison profiling of Torin2 and Torin1 revealed that Torin2 exhibits an apparent IC_{50} of less than 10 nmol/L against many PIKK family members including mTOR, ATR, ATM, and DNA-PK as well as PI3Kα, whereas Torin1 only strongly inhibits ATR, mTOR, and DNA-PK (Table 2 and full list shown in Supplementary Table S1; ref. 40).

To determine which of these targets are inhibited in a cellular context, we analyzed the phosphorylation status of downstream substrates following cellular treatment. As expected, T389 of S6k, a downstream target of mTORC1, was potently inhibited with an EC_{50} of 250 pmol/L and S473 of AKT, a downstream target of mTORC2, was potently inhibited with an EC_{50} of less than 10 nmol/L (Fig. 1C; ref. 13). Torin2 potently inhibits T308 of Akt, a direct substrate of PDK1 and an indirect substrate of PI3Ks, with an EC_{50} of less than 10 nmol/L. When the mTORC2 contribution to Akt phosphorylation is abrogated by introduction of a S473D mutant of Akt, the apparent EC_{50} against T308 is 200 nmol/L. This shows that Torin2 potently blocks the phosphorylation of Akt at both T308 and S473 sites and that the compound exhibits approximately 100-fold more potent functional inhibition of mTOR relative to PI3K activity (Fig. 1C and D and Supplementary Fig. S1). Torin2 inhibited the cellular activity of ATR with an EC_{50} of 35 nmol/L as assessed by phosphorylation status of S17 of Chk1 following exposure of the cells to UV-induced DNA damage (Fig. 1E). Torin2 inhibited the cellular activity of ATM with an EC_{50} of 28 nmol/L as assessed by phosphorylation status of S2056 of DNA-PK following exposure of cells to 10 gray ionizing radiation. In addition, torin2 shows a dose-dependent sensitization of the ionizing radiation treatment of the human fibroblast cell line AG01522, which is presumably due to the inhibition of the ATM or DNA-PK (Fig. 1F). The combined biochemical and cellular profiling results establish that Torin2 is a broadly active pan-PIKK family kinase inhibitor that most potently inhibits mTORC1 and mTORC2 at concentrations of less than 10 nmol/L but that also inhibits ATR, ATM, and DNA-PK at concentrations of between 20 and 100 nmol/L and that can inhibit PI3K at concentrations above 200 nmol/L. In contrast, Torin1 only exhibits moderate inhibition of DNA-PK (250 nmol/L) but is inactive against other PIKK-family kinases.

**Table 2.** Biochemical and cellular characterization of Torin2 in comparison with Torin1 IC_{50} was determined using ActivX with Kinativ technology

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Torin2/Torin1 IC_{50}, μmol/L</th>
<th>Torin2/Torin1 EC_{50}, μmol/L</th>
<th>Kinase</th>
<th>Torin2/Torin1 IC_{50}, μmol/L</th>
<th>Torin2/Torin1 EC_{50}, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
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<td>0.028/8.1</td>
<td>PIK3CA</td>
<td>&lt;0.01/0.26</td>
<td>N/D</td>
</tr>
<tr>
<td>ATR</td>
<td>&lt;0.01/0.1</td>
<td>0.035/8.1</td>
<td>PIK3CB</td>
<td>0.184.9</td>
<td>N/D</td>
</tr>
<tr>
<td>DNA PK</td>
<td>&lt;0.01/0.052</td>
<td>0.118/0.25</td>
<td>PIK3CD</td>
<td>0.018/1.6</td>
<td>N/D</td>
</tr>
<tr>
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<td>PIP5K2</td>
<td>0.99/8.10</td>
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<td>PIP5K3</td>
<td>2.0/8.10</td>
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<tr>
<td>PIK3C3</td>
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<td>N/D</td>
<td>SMG1</td>
<td>0.274.4</td>
<td>N/D</td>
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</tbody>
</table>

Note: EC_{50} was determined by Western blot analysis in Fig. 1C–E. Abbreviation: N/D, not determined.
**Torin2 limits negative feedback reactivation of Akt**

Prolonged treatment of cells with rapamycin is well known to result in hyperphosphorylation of Akt as a result of inhibition of the S6K/IRS-1/Akt negative feedback loop (11). To determine whether ATP-competitive mTOR inhibitors elicit a similar response, we examined the ability of Torin2 and AZD8055 to inhibit phosphorylation of Akt T308, AktS473, and S6K T389 over a 72-hour time course in HCT116 and HeLa cells (Fig. 2D and E and Supplementary Fig. S2). Treatment with Torin2 at 100 nmol/L was able to maintain strong suppression of both mTORC1 (S6K T389) and mTORC2 (AktS473) throughout the time course, however, by 72 hours the phosphorylation of Akt T308 was partially (HCT116) or completely recovered (HeLa). The data show that this feedback loop can reactivate Akt as assessed by T308 phosphorylation status despite potent inhibition of both mTORC1 and mTORC2. Treatment with 100 nmol/L AZD8055 also suppressed both mTORC1 and mTORC2 activity, however, the phosphorylation of T308 of Akt recovered more quickly.

**Torin2 inhibits proliferation of cancer cell, progression of the cell cycle, and induces apoptosis and autophagy**

Torin2 displays 2- to 3-digit nanomolar GI50s for inhibition of proliferation of a diverse panel of cancer lines including lung, breast, colorectal, and cervical (Table 1). In a 72-hour proliferation assay, Torin2 exhibits greater antiproliferative activity relative to AZD8055 in all 5 tested cancer cell lines (Fig. 3A). While the majority of tested cancer cell lines are potently inhibited, we did find 1 lung cancer cell line, Calu-1, which was relatively more resistant to both Torin2 and AZD8055. Both Torin2 and AZD8055 could induce apoptosis as measured by PARP cleavage at concentrations of 0.5 or 1 μmol/L in cells that exhibited strong inhibition of proliferation (HCT116, Hela) but much less in cells that were resistant (Calu 1 and H226), whereas only Torin2 induced strong apoptosis in H358 (Fig. 3B).

Both rapamycin and AZD8055 are potent inducers of autophagy (24), and so we also asked whether Torin2 would affect this process. The effect of Torin2 on autophagosome formation in Hela cells was first assessed by measuring the formation of punctate acidic vesicles in the cytoplasm using acridine orange as an indicator (Fig. 3C). A dose-dependent increase in punctate acidine staining was observed after 72 hours, consistent with induction of autophagy. A dose-dependent increase of LC3-1/II autophagosome markers was also observed in 5 different cell lines including HCT-116, Hela, Calu-1, H358, and H226 (Fig. 3D and E). Cell-cycle analysis using flow cytometry showed that Torin2 induced a dose-dependent decrease in G1 cells and an increase in S-phase, sub-G1, phase cells, and cell death (Fig. 3F and Supplementary Table S2).

**Torin2 and the MEK inhibitor AZD6244 synergistically suppress proliferation**

Our cellular profiling results suggest that cell lines carrying mutations in KRAS (such as H226, H358, and Calu-1) were somewhat less sensitive to Torin2 than other lines. Given that these lines show strong activation of the Ras/Raf/Mek/Erk pathway, we investigated the potential combinatorial effect of Torin2 with the potent allosteric MEK inhibitor AZD6244 (41). A 1:50 molar ratio of Torin2 to AZD6244 at concentrations that inhibited both mTORC1 and MEK activity resulted in the most differential growth inhibition relative to treatment with the single agents (Fig. 4 and Supplementary Table S3).

**Antitumor efficacy of combined Torin2/AZD6244 treatment in a KRAS-driven model of lung cancer**

The potent antiproliferative effect of the Torin2/AZD6244 combination in vitro suggested the possibility of similar efficacy in vivo. To determine whether Torin2 inhibits mTOR in vivo, we conducted a 2-day pharmacodynamics assay following a single daily dosing of 40 mg/kg in a genetically engineered KRAS mutant–driven lung cancer model (KRAS G12D model) and monitored pS6K(T389), pAkt(T308), and p4EBP1(T37/46) by immunohistochemistry. As expected, Torin2 strongly suppressed pS6K(T389) and p4EBP1(T37/46) and partly suppressed pAkt(T308). Treatment of mice with AZD6244 at 25 mg/kg resulted in a profound inhibition of pERK (41). Combined administration of Torin2 (40 mg/kg) and AZD6244 (25 mg/kg) showed strong inhibition of all pharmacodynamics markers. Having established the ability to inhibit the intended targets, we evaluated tumor size by MRI and pharmacodynamic markers after 4 weeks of treatment. Treatment with neither Torin2 (40 mg/kg, every day) nor AZD6244 (25 mg/kg, every day) alone resulted in a significant inhibition of tumor volume as determined by MRI. In contrast, the combination of 2 drugs showed significant reduction in tumor growth (P < 0.0001; Fig. 5A and B). Moreover, PET-CT showed a clear metabolic rate reduction in tumors treated with both compounds but not those treated with either compound alone (Supplementary Figs. S3 and S4). Examination of pharmacodynamic markers following 4-week treatment revealed some recovery of mTOR and MEK activity. In the Torin2 treatment group, pS6K(T389) and p4EBP1(T37/46) levels showed some recovery (Fig. 5C and D), whereas pERK1/2 levels was significantly increased. In the AZD6244 treatment group, the pERK1/2 levels had slightly recovered. In addition, the pCHK1(S345) of Torin2 group was slightly decreased indicating a inhibition of ATR kinase.

**Discussion**

We have described Torin2 as a potent orally bioavailable mTOR kinase inhibitor with significant selectivity over other protein kinases. In cells, Torin2 showed more than 800-fold selectivity against PI3K, approximately 1000-fold selectivity against ATR and ATM and 500-fold selectivity against DNA-PK. Like other mTOR active site inhibitors, Torin2 causes rapid dephosphorylation of rapamycin-sensitive mTORC1 substrates, such as S6K (T389), rapamycin-insensitive mTORC1 substrates, such as 4EBP1(T37/46), and mTORC2 substrates such as Akt(S473). Consistent with these effects, Torin2 is a potent inducer of phenotypes associated with mTOR inhibition, such as autophagy and, at higher concentrations, apoptosis.

Similarly to other mTOR inhibitors, including rapamycin, Torin2 also exhibited a bimodal effect on Akt activity. Acute
Figure 5. Single and combined effect of Torin2 and AZD6244 on tumor growth in vivo. A, MRI images of tumor size after treatment with vehicle, Torin2, AZD6244, or Torin2 + AZD6244 for the indicated times. B, measurements of tumor volume for animals treated as in A. C, sections of tumors from animals treated with the indicated compounds for 2 days, analyzed by immunohistochemistry for the indicated proteins. D, sections of tumors from animals treated with the indicated compounds for 4 weeks, analyzed by immunohistochemistry for the indicated proteins. E, sections from D were analyzed for levels of pChk1 by immunohistochemistry.
inhibition of Akt S473 phosphorylation reduces recruitment to the plasma membrane where PKD1 phosphorylates T308, a site in the activation loop that directly regulates Akt kinase activity. However, prolonged inhibition of mTORC1 de-represses a feedback loop that ultimately leads to PI3K hyperactivation (11). In this context, Akt is recruited to the plasma membrane despite dephosphorylation at S473, and Akt phosphorylation at T308 and kinase activity is reactivated to near normal levels. These effects are likely to be directly mediated through dual inhibition of mTORC1 and mTORC2 as the highly selective ATP-competitive mTOR inhibitor AZD8055 has been reported to exhibit the same bimodal effect on Akt activity (42). Nonetheless, the absence of phosphorylation at S473 is thought to limit levels of Akt activity below a threshold required for tumorigenesis (43). Thus, mTOR active site inhibitors may permit Akt activity within physiologic bounds while preventing the level of hyperactivation often observed with rapamycin and related rapalogs.

Torin2 retains the slow off-rate kinetics that we previously observed with Torin1, but not with other mTOR inhibitors. This long residence is potentially desirable because it results in sustained pharmacodynamics, which seem to compensate for the relatively short half-lives of Torin1 and Torin2 in vivo (13, 29). The recovery rate for Torin2 is, however, faster than for Torin1. Torin1 suppressed S6K T389 phosphorylation for up to 16 hours after removal of the drug, whereas Torin2 maintained suppression for only 4 hours (40). The difference was somewhat surprising given that Torin2 is a structural analog of Torin1, and molecular modeling indicates that both compounds use the same binding mode (13, 29). Moreover, both Torin1 and Torin2 show similar EC50s for mTOR substrates in cells, indicating that the difference in recovery times cannot be explained simply by different affinities for the binding pocket. One possible explanation is that Torin1 induces a conformational change in the kinase that is energetically more difficult to recover from.

There currently exists significant interest in the use of mTOR inhibitors as anticancer therapeutics, as evidenced by efforts from Wyeth, OSI, and Intellikine, etc. Like other mTOR inhibitors, Torin2 had broad efficacy against a panel of cancer cell lines although we were unable to associate any particular genetic alteration with resistance or sensitivity. Indeed, we identified only 1 cell line (Calu-1) that was markedly resistant. In general, cell lines harboring mutations in KRAS were more resistant to the effect of mTOR inhibition on proliferation, consistent with previous reports (44). However, cell lines with KRAS mutations exhibit a broad range of responses to mTOR inhibitors, and therefore a single lesion is unlikely to determine sensitivity. Analysis of a more comprehensive panel of tumor cell lines may better clarify the spectrum of alterations that determine sensitivity to the effects of mTOR inhibition.

We also asked whether Torin2 might synergize with other anticancer treatments. Unlike Torin1, Torin2 at slightly higher concentrations also targets the PIKK kinases ATM, ATR, and DNA-PK, which is similar with PI3K/mTOR dual inhibitor BEZ235 (45). Each of these kinases plays important roles in the response to DNA damage. BEZ235 has been shown to sensitize the chemotherapy treatment such as cisplatin due to the inhibition of ATR, and here we showed that torin2 can sensitize the radiation therapy in human fibroblast cells. In addition, we detected no synergy between Torin2 and these treatments (data not shown). One explanation may be that the cell-cycle arrest caused by mTOR inhibition protects cells from DNA damaging agents, which tend to act during S-phase. However, tumor cell lines that maintain proliferation when treated with Torin2 alone, such as Calu-1, may be more susceptible to combined treatment with DNA-damaging agents and deserve further investigation.

Previous work has indicated that molecules targeting the Ras/MAPK pathway might also synergize with mTOR inhibition (46). Various combinations of inhibitors have been reported to achieve better efficacy in a range of cancer models (46, 47) and some of these regimens are currently in clinical trials for treatment of solid tumors such as PF04691502(PI3K/mTOR)/PD-0325901(MEK) from Pfizer and GSK2126458(PI3K/mTOR)/GSK1120212(MEK) (48) from GSK. Here, we tested the efficacy of Torin2 either alone or in combination with the MEK inhibitor AZD6244 against a mouse model of lung cancer driven by expression of KRASG12D-mutant allele. Despite efficacy against cell lines harboring similar mutations in culture, neither Torin2 nor AZD6244 alone showed apparent antitumor activity in this model as single agents. Short-term treatment with either compound reduced target phosphorylation, such as pS6K(T389) for Torin2 and pErk1/2 for AZD6244 as determined by immunohistochemistry. However, a longer 4-week treatment resulted in partial reactivation of the targeted pathways. In tumors treated with Torin2, immunohistochemistry revealed increased levels of both pAkt(T308) and pS6K (T389) relative to short-term treatment. Likewise, tumors treated with AZD6244 exhibited partially recovered levels of pERK. Both observations of pathway reactivation are consistent with previous reports and further the paradoxical but increasingly common theme that prolonged inhibition of individual signaling pathways leads to compensatory activation through secondary mechanisms.

In contrast to their single-agent efficacy, 4-week treatment of tumors with a combination of Torin2 and AZD6244 significantly reduced tumor volume. Moreover, this regimen prevented the reactivation of each pathway as observed when each inhibitor was used alone. Why this occurs is unclear, as the currently understood mechanisms for mTOR reactivation do not involve the Ras/MAPK pathway, and vice versa. Thus, MEK and mTOR may play complementary and unappreciated roles in the reactivation of the other’s signaling pathways. Nonetheless, the maintenance of pathway suppression is likely an important contributor to the antitumor efficacy of dual-inhibitor regimen. The dual mTOR/PI3K inhibitor BEZ235 also synergized with AZD6244 in the same lung cancer model (46). As Torin2 does not significantly impair PI3K activity, our results indicate that the mTOR-specific activity of BEZ235 is key to its efficacy in this model.

We have described Torin2 as a potent pan-PIKK kinase inhibitor with significant activity and selectivity against mTOR, ATM, ATR, and DNA-PK and both in vitro and in vivo antitumor efficacy. As described previously for the dual mTOR/PI3K inhibitor BEZ235, Torin2 potently synergized with the MEK
inhibitor AZD6244 in a KRAS-driven model of lung cancer. The efficacy of Torin2 in this particular model is likely due mTOR inhibition. However, the capacity of Torin2 to target other PIKK kinases may prove useful in other contexts where mTOR inhibition alone is ineffective, potentially in combination DNA-damaging therapies.

Disclosure of Potential Conflicts of Interest
Pascale Janne is a consultant/advisory board member of Pfizer, Boehringer Ingelheim, Astra Zeneca, Roche, Genentech, and Sanofi Aventis and has expert testimony from Lab Corp. No potential conflicts of interest were disclosed by the other authors.

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Characterization of Torin2, an ATP-Competitive Inhibitor of mTOR, ATM, and ATR

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