Novel TOPK inhibitor HI-TOPK-032 effectively suppresses colon cancer growth

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ABSTRACT

The serine-threonine MAPKK family member TOPK/PBK (T-LAK-cell-originated protein kinase) is heavily involved in tumor development, cancer growth, apoptosis, and inflammation. Despite the identification of TOPK as a promising novel therapeutic target, no inhibitor of TOPK has yet been reported. In this study, we screened 36 drug candidates using an in vitro kinase assay and identified the novel TOPK inhibitor HI-TOPK-032. In vitro, HI-TOPK-032 strongly suppressed TOPK kinase activity, but had little effect on ERK1, JNK1 or p38 kinase activities. HI-TOPK-032 also inhibited anchorage-dependent and -independent colon cancer cell growth by reducing ERK-RSK phosphorylation as well as increasing colon cancer cell apoptosis through regulation of the abundance of p53, cleaved caspase 7, and cleaved PARP. In vivo, administration of HI-TOPK-032 suppressed tumor growth in a colon cancer xenograft model. Our findings therefore demonstrate that HI-TOPK-032 is a specific inhibitor of TOPK both in vitro and in vivo that may be further developed as a potential therapeutic against colorectal cancer.
Introduction

The mitogen-activated protein kinase kinase (MAPKK) signaling pathway is a major component of the RAS/RAF/MEK/ERK signaling axis. T-LAK-cell-originated protein kinase (TOPK) is a serine-threonine kinase that is a member of MAPKK family and is involved in many cellular functions, including tumor development, cell growth, apoptosis and inflammation (1-5). Previous studies showed that TOPK is highly expressed in many cancers such as lymphoma, leukemia, melanoma, colorectal, breast cancer, lung and cholangiocarcinoma (3, 6-9). TOPK interacts with hDlg through TOPK’s C-terminal PDZ-binding motif (6, 10). During mitosis, TOPK and the cdk1/cyclin B1 complex promote cytokinesis through phosphorylation of PRC1 (8, 11-13) and positive feedback between TOPK and ERK2 promotes colorectal cancer formation (3). TOPK expression corresponds with H-Ras-induced cell transformation, UVB-induced JNKs activation and DNA damage-induced p53 expression (2, 14). Recently, TOPK was identified as a downstream target of EWS-FLI1 chimeric fusion protein (15). TOPK interacts with p53 and promotes tumorigenesis by inhibiting p53 functions (2). It was also reported to increase cell migration by modulating a PI3K/PTEN/AKT-dependent signaling pathway (16).
Although these studies suggest that TOPK performs an oncogenic cellular function and its inhibition might be useful in cancer therapy, a TOPK inhibitor has not yet been identified. Our goal herein was to identify a novel TOPK inhibitor and to investigate its efficacy against colon cancer. Herein, we report that a novel compound, HI-TOPK-032 is a potent TOPK inhibitor that augments the efficacy of cancer treatment.

**Materials and Methods**

**Reagents**

HI-TOPK-001 to -036 were synthesized or purchased from InterBioScreen (Moscow, Russia) (Supplemental Table.1). Active MEK1, inactive ERK2 (MEK1 substrate), active ERK1, active JNK1, active p38, ATF2(p38 substrate), histone H2AX (TOPK substrate) human recombinant protein and MBP (Myelin Basic Protein; TOPK substrate) for kinase assays were purchased from Millipore (Temecula, CA). The inactive N-terminal RSK2 (ERK1 substrate) and C-terminal c-Jun (JNK1 substrate) human recombinant protein for kinase assays were purified from *E. Coli*. The active TOPK human recombinant protein for the kinase assay was purchased from SignalChem (Richmond, BC). Antibodies to detect total TOPK, phosphorylated TOPK (T9), total CDC2, phosphorylated CDC2 (T15), total ERK, phosphorylated ERK (T202/Y204), total RSK,
phosphorylated RSK (T356/S360) and caspase 7 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to detect p53, PARP and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DNA stat-60 for genomic DNA isolation was obtained from Tel Test (Friendswood, TX).

**Cell culture**

All cell lines were purchased from American Type Culture Collection (ATCC) and were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. HCEC (human colonic epithelial cells) were cultured in basal media (HyClone, Logan, UT) supplemented with EGF (25 ng/mL), insulin (10 μg/mL), gentamicin sulfate (50 μg/mL) (all from Invitrogen, Grand Island, NY), transferrin (2 μg/mL), hydrocortisone (1 μg/mL), sodium selenite (5 nM) (all from Sigma, St Louis, MO) and 2% cosmic calf serum (HyClone, Logan, UT). HCT116 and HT29 human colon cancer cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic. HCT15 and
DLD1 human colon cancer cells were cultured in RPMI1640 medium supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic.

JB6 mouse skin epidermal cells were cultured in MEM supplemented with 5% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic. TOPK-WT or KO MEFs (mouse embryonic fibroblasts) were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.

**Lentiviral infection.**

The lentiviral expression vectors, including $Gipz-shTOPK$ and packaging vectors, including $pMD2.0G$ and $psPAX$, were purchased from Addgene Inc. (Cambridge, MA).

To prepare TOPK viral particles, each viral vector and packaging vectors ($pMD2.0G$ and $psPAX$) were transfected into HEK293T cells using JetPEI following the manufacturer’s suggested protocols. The transfection medium was changed at 4 h after transfection and then cells were cultured for 36 h. The viral particles were harvested by filtration using a 0.45 mm syringe filter, then combined with 8 μg/ml of polybran (Millipore, Billerica, MA) and infected into 60% confluent HCT-116 cells overnight.

The cell culture medium was replaced with fresh complete growth medium for 24 h and
then cells were selected with puromycin (1.5 μg/ml) for 36 h. The selected cells were used for experiments.

**Molecular modeling of TOPK and HI-TOPK-032.**

A TOPK structure was modeled using comparative modeling. The sequence of TOPK was downloaded from NCBI (GI: 83305809) and BLAST was used to search for homologous proteins in the RCSB Protein Data Bank. Results indicated that the sequence identity between the sequences of TOPK and proteins with known structures is below 30% and the sequence similarity is about 45%. The protein structure from 2F4J (PDB entry) was selected as the template structure to model the TOPK structure. The alignment of sequences of TOPK and 2F4J was generated by BLAST and edited in Prime v3.0. The secondary structure of TOPK was predicted by SSpro. The TOPK structure was built with Prime v3.0 followed by refining and minimizing loops in the binding site. Glide v5.7 was used for docking of TOPK and HI-TOPK-032. HI-TOPK-032 was prepared using LigPrep v2.5 and then assigned AMSOL partial atom charge. Flexible docking was performed with extra precision (XP) mode as described (17). The number of poses per ligand was set to 10 in post-docking minimization and at most 5 poses would be output. The other parameters were kept as default.
**Anchorage-independent cell growth**

Cells (8 \times 10^3 per well) suspended in complete growth medium (McCoy’s 5A, RPMI1640 or BME supplemented with 10% FBS and 1% antibiotics) were added to 0.3% agar with different doses of each compound in a top layer over a base layer of 0.6% agar with different doses of each compound. The cultures were maintained at 37°C in a 5% CO₂ incubator for 3 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.4) program (Media Cybernetics).

**Luciferase assay for reporter activity**

Transient transfection was conducted using jetPEI (Qbiogene, Carlsbad CA), and assays for the activity of firefly luciferase and Renilla activity were performed according to the manufacturer’s manual (Promega, Madison, WI). Cells (1 \times 10^4 per well) were seeded the day before transfection into 12-well culture plates. Cells were co-transfected with reporter plasmid (250 ng) and internal control (CMV-Renilla, 50 ng) in 12-well plates and incubated for 24 h. Colon cancer cells were treated with HI-TOPK-032 for 48 h. Cells were harvested in Promega Lysis Buffer. The Luciferase and Renilla activities were measured using substrates in the reporter assay system (Promega). The
luciferase activity was normalized to Renilla activity.

Western blot analysis

Cell lysates were prepared with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 × Protease inhibitor tablet). Equal amounts of protein were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Proteins were separated by SDS/PAGE and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with appropriate primary antibodies overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at a 1:5,000 dilution and the signal was detected with a chemiluminescence reagent (Amersham Biosciences Corp).

Cell proliferation assay

Cells were seeded (1 × 10^3 cells per well) in 96-well plates and incubated for 24 h and then treated with different doses of each compound. After incubation for 1, 2 or 3 days, 20 μl of CellTiter96 Aqueous One Solution (Promega) were added and then cells were
incubated for 1 h at 37°C in a 5% CO₂ incubator. Absorbance was measured at 492 nm.

**In vitro kinase assay**

The kinase assay was performed in accordance with instructions provided by Upstate Biotechnology (Billerica, MA). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-32P]ATP with each compound in 40 μl of reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol. After incubation at room temperature for 30 min, the reaction was stopped by adding 10 μl protein loading buffer and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each experiment was repeated twice. The relative amounts of incorporated radioactivity were assessed by autoradiography.

**Xenograft mouse model**

Athymic mice [Cr:NIH(S), NIH Swiss nude, 6–9 wk old] were obtained from Charles River and maintained under “specific pathogen-free” conditions based on the guidelines established by the University of Minnesota Institutional Animal Care and Use Committee. Mice were divided into four groups: 1) untreated vehicle group (n = 10); 2) 1 mg HI-TOPK-032/kg of body weight (n = 10); 3) 10 mg HI-TOPK-032/kg of body...
weight (n = 10); and 4) no cells and 10 mg HI-TOPK-032/kg of body weight (n = 10).

HCT116 cells (1.5×10^6 cells/100 μl) were suspended in serum free McCoy’s 5A medium and inoculated subcutaneously into the right flank of each mouse. HI-TOPK-032 or vehicle was injected 3 times per week for 25 days. Tumor volume was calculated from measurements of 2 diameters of the individual tumor base using the following formula: tumor volume (mm^3) = (length × width × height × 0.52). Mice were monitored until tumors reached 1 cm^3 total volume, at which time mice were euthanized and tumors were extracted.

**Statistical analysis**

All quantitative results are expressed as mean values ± S.D. Statistically significant differences were obtained using the Student’s t test or by one-way ANOVA. A p < 0.05 was considered to be statistically significant.

**Results**

**Knocking down TOPK expression inhibits cell proliferation.** Using lentiviral infection, we established HCT116 colon cancer cells stably expressing shMock, low levels of TOPK (#2 shTOPK) or medium levels of TOPK (#3 shTOPK). The abundance
of TOPK in these cells was confirmed by Western blot analysis (Fig. 1A). The effect of knocking down TOPK on proliferation was then assessed by MTS assay at 1, 2 or 3 days. Results indicated that cell growth was significantly decreased in a manner dependent on the level of TOPK expression (Fig. 1B). Proliferation was also decreased in TOPK knockout MEFs (mouse embryonic fibroblasts) compared to wildtype MEFs (Fig. 1C). Additionally, the effect of knocking down TOPK expression on anchorage-independent colon cancer cell growth was assessed. These results showed that anchorage-independent cell growth was strongly inhibited by knocking down the expression of TOPK and the inhibition was dependent on the level of TOPK expression (Fig. 1D).

**HI-TOPK-032 directly suppresses TOPK kinase activity.** We screened 36 compounds (30 μM concentration) by *in vitro* TOPK kinase assay (Supplemental Fig. 1A) and by cell proliferation assay (4 or 20 μM concentration) (Supplemental Fig. 1B). Finally, we selected 6 compounds to test for inhibition of TOPK (10 μM concentration) (Supplemental Fig. 1C). Based on screening results, we identified HI-TOPK-032 (Fig. 2A) as a potent TOPK inhibitor. To determine the effect of HI-TOPK-032 on TOPK or MEK1 kinase activity, we performed an *in vitro* TOPK or MEK1 kinase assay with
increasing concentrations of HI-TOPK-032. HI-TOPK-032 effectively inhibited TOPK kinase activity. However, HI-TOPK-032 at the highest concentration (5 μM) also inhibited MEK1 activity by 40% (Fig. 2B). Thus, this result showed that HI-TOPK-032 is a more potent inhibitor against TOPK compared to MEK1. Additionally, we used *in vitro* kinase assays to investigate the effect of HI-TOPK-032 on other MAP kinase family members, such as ERK1, JNK1 and p38 (Fig. 2C). These results showed that HI-TOPK-032 suppressed TOPK kinase activity, but not ERK1, JNK1 or p38 activity.

**Computer modeling of the TOPK and HI-TOPK-032 complex.** We performed molecular docking of the HI-TOPK-032 and TOPK complex in order to determine the binding orientation of HI-TOPK-032. HI-TOPK-032 occupies the ATP-binding site of TOPK (Fig. 3A, B) and fits the binding site very well (Fig. 3C). The compound forms hydrogen bonds with GLY83 and ASP151 and has a hydrophobic interaction with LYS30 (Fig. 3D). These results suggest that HI-TOPK-032 binds to the TOPK active site.

**HI-TOPK-032 inhibits colon cancer cell growth and induces apoptosis.** To determine whether total and phosphorylated TOPK are highly expressed in normal
colon or colon cancer cells, we performed Western blot analysis. Results showed that HCT116 colon cancer cells highly expressed phosphorylated TOPK compared with other colon cancer cells (Supplemental Fig. 2A) and these cells were used in further studies. To examine the effect of HI-TOPK-032 on colon cancer cell proliferation, growth was measured using the MTS assay at 1, 2 or 3 days after treatment with HI-TOPK-032. Results indicated that colon cancer cell growth was significantly decreased by HI-TOPK-032 in a dose-dependent manner (Fig. 4A). Additionally, we determined the effect of HI-TOPK-032 on anchorage-independent cell growth. Colon cancer cells were seeded with HI-TOPK-032 in 0.3% agar and incubated for 3 weeks. Data showed that anchorage-independent cancer cell growth was strongly suppressed by HI-TOPK-032 in a dose-dependent manner (Fig. 4B). Next, to examine the effect of HI-TOPK-032 on apoptosis, HCT116 colon cancer cells were treated with HI-TOPK-032 and then incubated for 3 days. Results showed that DNA fragmentation induced by HI-TOPK-032 was substantially increased in HCT116 colon cancer cells compared with untreated control cells (Fig. 4C). Previous studies suggested that overexpressing TOPK can induce neoplastic cell transformation. Therefore, we determined the effect of HI-TOPK-032 on transformation of JB6 cells stably overexpressing TOPK. Results indicated that overexpressing TOPK-mediated cell transformation was significantly suppressed by HI-
TOPK-032 (Supplemental Fig. 3A). We next investigated the effect of HI-TOPK-032 on downstream targets of TOPK in HCT116 colon cancer cells. Cells were treated with HI-TOPK-032 for 24 h and then cell lysates were examined by Western blot. In previous studies, TOPK was shown to inhibit the p53 signaling pathway, involved the G2/M transition, and activated the ERK signaling pathway. Therefore, inhibition of TOPK kinase activity by a TOPK inhibitor should be able to induce abundance of the p53 protein and its downstream target proteins, G2/M phase marker proteins (i.e., CDC2 phosphorylation) and reduce ERKs phosphorylation. Our results showed that the expression of p53, cleaved caspase7, cleaved PARP and phosphorylated CDC2 were all strongly increased by HI-TOPK-032 and phosphorylated ERK and RSK were remarkably decreased by HI-TOPK-032 (Fig. 4D). However, the expression of total TOPK was not changed. We determined whether HI-TOPK-032 affected the reporter activity of the activator protein-1 (AP-1), nuclear factor-kappaB (NF-κB) or cyclooxygenase 2 (COX2) in HCT116 or HCT15 cells. Cells were treated with HI-TOPK-032 for 48 h and then reporter activity was measured. Results show that the reporter activity of these genes was strongly inhibited by HI-TOPK-032 (Supplemental Fig. 4A, B, C).
The inhibition of TOPK by HI-TOPK-032 is dependent on the abundance of TOPK. The effect of HI-TOPK-032 on growth of shMock, #3 shTOPK (medium expression of TOPK) or #2 shTOPK (low expression of TOPK) colon cancer cells was assessed by MTS assay at 72 h. Results indicated that cells expressing shTOPK were resistant to HI-TOPK-032’s inhibitory effect on growth compared to cells expressing shMock (Fig. 5A). Similar resistance to HI-TOPK-032 was observed in TOPK knockout MEFs (Fig. 5B, C). Additionally, we examined the effect of HI-TOPK-032 on anchorage-independent colon cancer cell growth. Results showed that the inhibitory effect of HI-TOPK-032 on anchorage-independent cell growth in shTOPK cells was much less than its effect on shMock growth (Fig. 5D). These findings showed that the anticancer activity induced by HI-TOPK-032 is dependent on TOPK protein expression.

HI-TOPK-032 inhibits colon cancer tumor growth in a xenograft mouse model. To determine the antitumor activity of HI-TOPK-032 in vivo, HCT116 colon cancer cells were injected into the flank of athymic nude mice. Mice were injected with vehicle or HI-TOPK-032 at 1 or 10 mg/kg 3 times a week over a period of 25 days. Treatment of mice with 1 or 10 mg/kg of HI-TOPK-032 significantly inhibited HCT116 tumor growth by over 60% relative to the vehicle-treated group (Supplemental Fig. 5A, Fig.
Additionally, mice seemed to tolerate treatment with HI-TOPK-032 without overt signs of toxicity or significant loss of body weight similar to the vehicle-treated group (Fig. 6B). To validate the results of the in vivo xenograft model, we investigated the effect of HI-TOPK-032 on downstream targets of TOPK by Western blot analysis of HCT116 colon tumor samples. The expression of p53 was strongly induced and phosphorylation of ERK and RSK, a direct downstream protein of ERK, was markedly inhibited in the HI-TOPK-032-treated group (Fig. 6C). This finding suggested that HI-TOPK-032 inhibits HCT116 colon tumor growth through inhibition of TOPK in vivo. We could suggest that inhibition of TOPK by HI-TOPK-032 regulates multiple effects such as tumor development, cancer growth, apoptosis and inflammation mediated through the AP1 or p53 signaling pathway (Fig. 6D).

**Discussion**

Many groups have reported that the serine/threonine kinase TOPK may contribute to oncogenic cellular functions including tumor development, cancer growth and anti-apoptosis effects. These reports suggested that TOPK is a potential target for development of anticancer agents. Despite these findings, a TOPK inhibitor has not yet been found, possibly because the TOPK crystal structure has not yet been reported. To
develop a reliable homology model for TOPK, we performed a sequence-based homology search. We found that the sequences of TOPK and MEKs are highly conserved. Based on this sequence homology, we tested the affect of U0126, a well-known inhibitor of MEK, on the \textit{in vitro} TOPK kinase activity. U0126 inhibited TOPK activity by 25\% at the highest concentration (20 μM; data not shown). Based on these preliminary data, we selected 36 compounds with a similar structure to a MEK inhibitor and identified HI-TOPK-032 as a potent TOPK inhibitor. In addition, we built a homology model based on the known structure of MEK and performed a docking simulation between HI-TOPK-032 and the modeled TOPK protein. HI-TOPK-032 was docked to the active site of TOPK (Fig. 3). We then compared the inhibitory effect of various HI-TOPK-032 analogues on TOPK kinase activity \textit{in vitro}. However, none of the analogues had any effect (data not shown). We synthesized various analogues of HI-TOPK-032 with certain portions deleted, but none of these compounds were effective inhibitors of TOPK kinase activity (data not shown). These findings showed that the complete structure of HI-TOPK-032 is important for effectively inhibiting TOPK kinase activity.

Previous studies showed that TOPK directly interacts with the DBD domain of tumor suppressor p53 \(^2\). TOPK’s downstream target, tumor suppressor activated
pathway-6 (TSAP6,) reportedly binds to myelin transcription factor 1 (MYT1), which induces phosphorylation of CDC2 (Tyr15) (18-19). We investigated whether p53 expression or phosphorylation of CDC2 (Tyr15) is affected by HI-TOPK-032. These results indicated that the abundance of total p53 and phosphorylation of CDC2 (Tyr15) in colon cancer cells were both strongly increased by HI-TOPK-032 treatment and the phosphorylation of ERK, a direct downstream target of TOPK, was dramatically inhibited by HI-TOPK-032 (Fig. 4D). Notably, total p53 expression was highly induced and phosphorylation of ERK-RSK was inhibited in HI-TOPK-032-treated tumor tissues (Fig. 6D). These results strongly indicate that the inhibitory effect of HI-TOPK-032 in cell-based assays corresponds closely with the in vivo animal results.

In conclusion, we report that HI-TOPK-032 is a novel and specific TOPK inhibitor both in vitro and in vivo. These findings should be useful for further development of drugs targeted against TOPK. Future studies will focus on the efficacy of HI-TOPK-032 and characterize its therapeutic potential against colorectal cancer and other human cancers.

Conflict of Interest

The authors state no conflict of interest.
Acknowledgements

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References


**Figure Legends**

**Figure 1.** Knock-down of TOPK expression suppresses anchorage-dependent and -
**independent colon cancer cell growth.** (A) Colon cancer cells stably expressing knockdown of TOPK were established. The expression of TOPK was determined by Western blotting. (B) Knocking down TOPK suppresses anchorage-dependent proliferation of colon cancer cells. (C) Murine embryonic fibroblasts (MEFs) deficient in TOPK protein expression exhibit decreased proliferation. Anchorage-dependent cell growth was determined at 1, 2 and 3 days using the MTS assay. (D) Knocking down TOPK suppresses anchorage-independent growth of colon cancer cells. HCT116 colon cancer cells stably expressing *shMock* or *shTOPK* were incubated in 0.3% agar for 3 weeks. Colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. Data are shown as means ± S.D. of values from triplicate samples and similar results were obtained from two independent experiments. The asterisk (*) indicates a significant difference between knock-down or knock-out cells and control cells or between cells expressing low levels of TOPK vs. cells expressing a medium level of TOPK as determined by t test (*p < 0.05*).

**Figure 2. HI-TOPK-032 suppresses TOPK kinase activity.** (A) Chemical structure of HI-TOPK-032. (B) The effect of HI-TOPK-032 on TOPK and MEK1 kinase activities. Kinase activity was assessed by an *in vitro* kinase assay using TOPK (active, 500 ng),
histone H2AX (TOPK substrate, 500 ng), MEK1 (active, 300 ng) and inactive ERK2 (MEK1 substrate, 300 ng) and $[\gamma^{32}\mathrm{P}]\mathrm{ATP}$. (C) HI-TOPK-032 has no effect on ERK1, JNK1 or p38 kinase activity. The effect of HI-TOPK-032 on ERK1, JNK1 and p38 activity was assessed by an in vitro kinase assay using ERK1 (active, 500 ng), inactive RSK2 (ERK1 substrate, 1 μg), JNK1 (active, 50 ng), c-Jun (JNK1 substrate, 1 μg) and p38 (active, 200 ng) and ATF2 (p38 substrate, 500 ng) with $[\gamma^{32}\mathrm{P}]\mathrm{ATP}$. All data are represented as means ± S.D. of values from 3 independent experiments. Band density was measured using the Image J (NIH) software program. The asterisk (*) indicates a significant difference between TOPK kinase activity and MEK1 kinase activity as determined by t test ($p < 0.05$).

Figure 3. Computer modeling results indicate that HI-TOPK-032 binds to the TOPK active site. (A) Docking model of HI-TOPK-032 and the TOPK protein structure. HI-TOPK-032 is shown in sphere representation and carbons are colored white. TOPK is shown as a cartoon model. (B) Binding site of TOPK with HI-TOPK-032. The ATP-binding site of TOPK is shown in surface representation. HI-TOPK-032 is shown in stick representation and carbons are colored green. (C) Surface representation of TOPK with HI-TOPK-032. HI-TOPK-032 is shown in stick.
representation and carbons are colored green. TOPK is shown in surface representation and carbons are colored white. (D) Interaction between TOPK and HI-TOPK-032. Carbons on TOPK are colored white. HI-TOPK-032 is shown in stick representation and carbons are colored green. Oxygen, nitrogen and sulfur are colored red, blue and yellow, respectively.

Figure 4. HI-TOPK-03 exerts anti-cancer activity against colon cancer cells. (A)

HI-TOPK-032 inhibits colon cancer cell growth in a dose-dependent manner. Cells were treated with HI-TOPK-032 at various doses for 1, 2 or 3 days and proliferation was measured by MTS assay. Data are shown as means ± S.D. (N=5) and the asterisk (*) indicates a significant (p < 0.05) difference compared to untreated control. (B) HI-TOPK-032 inhibits anchorage-independent cancer cell growth. Colon cancer cells were incubated in 0.3% agar for 3 weeks with HI-TOPK-032. Colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. Data are represented as means ± S.D. of values from triplicates and similar results were obtained from 2 independent experiments. The asterisk (*) indicates a significant (p < 0.05) decrease in colony formation induced by HI-TOPK-032 compared to untreated control. (C) Effect of HI-TOPK-032 on apoptosis in HCT116 colon cancer cells. Cells were
treated with HI-TOPK-032 for 72 h in medium containing 10% FBS and apoptosis was analyzed by DNA fragmentation assay. (D) Effect of HI-TOPK-032 on TOPK downstream proteins in colon cancer cells. Cells were treated with HI-TOPK-032 for 24 h in medium containing 10% FBS and analyzed by Western blot. Similar results were observed from 2 independent experiments. Numbers indicate density.

**Figure 5. The anticancer activity of HI-TOPK-032 is TOPK-dependent.** (A) The inhibition of cell growth induced by HI-TOPK-032 is less apparent in knockdown TOPK cells. The effect of HI-TOPK-032 was examined in *shMock* and knockdown cell lines with medium expression of TOPK (#3 *shTOPK*) or low expression of TOPK (#2 *shTOPK*). Cells were incubated for 72 h and growth was determined by MTS assay. (B) Effect of HI-TOPK-032 on growth of wildtype TOPK MEFs and (C) TOPK knockout MEFs. Cell growth at 1, 2 or 3 days was measured by MTS assay. Data are shown as means ± S.D. (N=5) and similar results were obtained from two independent experiments. The asterisk (*) indicates a significant (p < 0.05) difference between HI-TOPK-032 treated cells and untreated control cells. (C) Effect of HI-TOPK-032 on anchorage-independent growth of cells expressing *shMock*, #2 *shTOPK* or #3 *shTOPK*. Each cell type was incubated in 0.3% agar for 3 weeks at 37°C/5% CO₂. Colonies were
counted using a microscope and the Image-Pro PLUS (v.6) computer software program. Data are represented as means ± S.D. of values from triplicate values and similar results were obtained from 2 independent experiments. The asterisk (*) indicates a significant (p < 0.05) decrease in colony formation induced by HI-TOPK-032 compared to untreated control cells.

**Figure 6. HI-TOPK-032 prevents xenograft tumor growth.** (A) HI-TOPK-032 suppresses colon tumor growth. HCT116 colon cancer cells were injected subcutaneously into the dorsal right flank of mice. Mice were injected with HI-TOPK-032 or vehicle 3 times a week for 25 days. Mice were monitored until tumors reached 1 cm³ total volume, at which time mice were euthanized and tumors were extracted. Tumor volume was calculated from measurements of 2 diameters of the individual tumor based on the following formula: tumor volume (mm³) = (length × width × height × 0.52). Data are shown as means ± S.E. of values obtained from the experiment. The asterisk (*) indicates a significant difference between tumors from untreated and treated mice as determined by t test (p < 0.05). (B) HI-TOPK-032 has no effect on mouse body weight. Body weights from treated or untreated groups of mice were obtained once a week. (C) HI-TOPK-032 inhibits TOPK-target protein expression in
HCT116 colon tumor tissues. The tumor tissues from groups treated with vehicle, 1 mg or 10 mg/kg/BW HI-TOPK-032 were immunoblotted with antibodies to detect total TOPK, p-TOPK, p53, total ERK, p-ERK, total RSK, p-RSK and β-actin. β-Actin was used to verify equivalent loading of protein. (D) Representative signaling pathway of TOPK mediated multifunction by HI-TOPK-032.
Figure 1. A+B+C+D by DJKim

A

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C

- TOPK-WT MEF
- TOPK-KO MEF

B

- shMock
- #3 shTOPK
- #2 shTOPK

D

- shMock
- #3 shTOPK
- #2 shTOPK

Number of colonies (8000 cells/well)

- shMock
- #3 shTOPK
- #2 shTOPK
Figure 2. A+B+C by DJKim

N-(12-cyanoindolizino[2,3-b]quinoxalin-2-yl) thiophene-2-carboxamide or HI-TOPK-032

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<th></th>
<th>active ERK1</th>
<th>active JNK1</th>
<th>active p38</th>
<th>inactive RSK2</th>
<th>c-Jun</th>
<th>ATF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI-TOPK-032 (µM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ERK1    
RSK2    
c-JUN    
p38    
ATF2
Figure 3. A+B+C+D by DJKim
Figure 4. A+B+C+D by DJKim

A

HCT16

Cell proliferation (% of control)

HCT15

Cell proliferation (% of control)

0 1 2 3 days

B

HCT16

Number of cell colonies (000 cells/well)

HCT15

Number of cell colonies (000 cells/well)

HI-TOPK-032

- 2 5 (μM)

C

D

HI-TOPK-032

- 2 5 (μM)

HI-TOPK-032

- 2 5 (μM)

p-CDC2

p-ERK

T-CDC2

T-ERK

p53

p-RSK

Caspase7

p-RRK

PARP

p-TOPK

1kb

0.5kb
Novel TOPK inhibitor HI-TOPK-032 effectively suppresses colon cancer growth

Dong-Joon Kim, Yan Li, Kanamata Reddy, et al.

Cancer Res  Published OnlineFirst April 20, 2012.

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