Novel TOPK Inhibitor HI-TOPK-032 Effectively Suppresses Colon Cancer Growth

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Abstract

The serine-threonine mitogen-activated protein kinase kinase family member T-LAK cell–originated protein kinase (TOPK/PBK) 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 extracellular signal–regulated kinase 1 (ERK1), c-jun–NH2–kinase 1, 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 show 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. Cancer Res; 72(12); 3060–8. ©2012 AACR.

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 H-Ras through the C-terminal PDZ-binding motif of TOPK (6, 10). During mitosis, TOPK and the cdk1/cyclin B1 complex promote cytokinesis through phosphorylation of protein regulator of cytokinesis 1 (PRC1; refs. 8, 11–13), and positive feedback between TOPK and ERK2 promotes colon cancer cell formation (3). TOPK expression corresponds with H-Ras–induced cell transformation, UVB–induced c-jun–NH2–kinase (JNK) 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 (Supplementary Table S1). Active MEK1, inactive ERK2 (MEK1 substrate), active ERK1, active JNK, 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. The inactive N-terminal RSK2 (ERK1 substrate) and C-terminal c-Jun (JNK1 substrate) human recombinant protein for kinase assays were purified from Escherichia coli. The active TOPK human recombinant protein for the kinase assay was purchased from SignalChem. Antibodies to detect total TOPK, phosphorylated TOPK (T9), total CDC2, phosphorylated CDC2 (T15), total ERK, phosphorylated ERK (T202/Y204), total RSK, phosphorylated RSK (T358/S366), and caspase-7 were purchased from Cell Signaling Technology. Antibodies to detect p53, PARP, and β-actin were purchased from Santa Cruz Biotechnology. DNA STAT-60 for genomic DNA isolation was obtained from Tel-Test, Inc.
Cell culture

All cell lines were purchased from American Type Culture Collection 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. Human colonic epithelial cells (HCEC) were cultured in basal media (HyClone) supplemented with EGF (25 ng/mL), insulin (10 μg/mL), gentamicin sulfate (50 μg/mL; all from Invitrogen), transferrin (2 μg/mL), hydrocortisone (1 μg/mL), sodium selenite (5 nmol/L; all from Sigma), and 2% cosmic calf serum (HyClone). HCT-116 and HT29 human colon cancer cells were cultured in McCoy 5A medium supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic. HCT-15 and DLD1 human colon cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic. JB6 mouse skin epidermal cells were cultured in minimum essential medium (MEM) supplemented with 5% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic. TOPK and 2F4J was generated by BLAST and edited in Prime v3.0. The alignment of sequences of homologous proteins in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) was used to search for the activity of firefly luciferase and Renilla activity were carried out according to the manufacturer’s manual (Promega). Cells (1 x 10^5 per well) were seeded the day before transfection into 12-well culture plates. Cells were cotransfected with reporter plasmid (250 ng) and internal control (CMV-Renilla, 50 ng) in 12-well plates and incubated for 24 hours. Colon cancer cells were cultured with HI-TOPK-032 for 48 hours. 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 radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4; 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 x protease inhibitor tablet). Equal amounts of protein were determined using the bicinchoninic acid assay (Pierce Biotechnology, Inc.). 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 hour 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 x 10^3 cells per well) in 96-well plates and incubated for 24 hours 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) was added and then cells were incubated for 1 hour at 37°C in a 5% CO₂ incubator. Absorbance was measured at 492 nm.

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In vitro kinase assay

The kinase assay was carried out in accordance with instructions provided by Upstate Biotechnology Inc. 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 mol/L HEPES (pH 7.4), 10 mmol/L MgCl2, 10 mol/L MnCl2, and 1 mmol/L dithiothreitol. After incubation at room temperature for 30 minutes, the reaction was stopped by adding 10 μL protein loading buffer and the mixture was separated by 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- to 9-week-old] were obtained from Charles River Laboratories and maintained under "specific pathogen–free" conditions on the basis of the guidelines established by the University of Minnesota (Minneapolis, MN) Institutional Animal Care and Use Committee. Mice were divided into 4 groups: (i) untreated vehicle group (n = 10); (ii) 1 mg HI-TOPK-032/kg of body weight (n = 10); (iii) 10 mg HI-TOPK-032/kg of body weight (n = 10); and (iv) no cells and 10 mg HI-TOPK-032/kg of body weight (n = 10). HCT-116 cells (1.5 × 10⁶ cells/100 μL) were suspended in serum-free McCoy 5A medium and inoculated s.c. 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 (mm³) = (length × width × height × 0.52). Mice were monitored until tumors reached 1 cm³ total volume, at which time mice were euthanized and tumors were extracted.

Statistical analysis

All quantitative results are expressed as mean values ± SD. Statistically significant differences were obtained using the Student 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 HCT-116 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 KO MEFs compared with WT MEFs (Fig. 1C). In addition, 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 μmol/L concentration) by in vitro TOPK kinase assay (Supplementary Fig. S1A) and by cell proliferation assay (4 or 20 μmol/L concentration; Supplementary Fig. S1B). Finally, we selected 6 compounds to test for inhibition of TOPK (10 μmol/L concentration; Supplementary Fig. S1C). On the basis of 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 carried out 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 μmol/L) also inhibited MEK1 activity by 40% (Fig. 2B). Thus, this result showed that HI-TOPK-032 is a more potent inhibitor against TOPK compared with MEK1. In addition, 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 conducted molecular docking of the HI-TOPK-032 and TOPK complex to determine the binding orientation of HI-TOPK-032. HI-TOPK-032 occupies the ATP-binding site of TOPK (Fig. 3A and 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 conducted Western blot analysis. Results showed that HCT-116 colon cancer cells highly expressed phosphorylated TOPK compared with other colon cancer cells (Supplementary Fig. S2A) 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). In addition,
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, HCT-116 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 HCT-116 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 (Supplementary Fig. S3A). We next investigated the effect of HI-TOPK-032 on downstream targets of TOPK in HCT-116 colon cancer cells. Cells were treated with HI-TOPK-032 for 24 hours and then cell lysates were examined by Western blot analysis. 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 ERK phosphorylation. Our results showed that the expression of p53, cleaved caspase-7, 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), NF-kB, or COX2 in HCT-116 or HCT-15 cells. Cells were treated with HI-TOPK-032 for 48 hours and then reporter activity was measured. Results show that the reporter activity of these genes was strongly inhibited by HI-TOPK-032 (Supplementary Fig. S4A–S4C).

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
hours. Results indicated that cells expressing \( \text{shTOPK} \) were resistant to the inhibitory effect of HI-TOPK-032 on growth compared with cells expressing \( \text{shMock} \) (Fig. 5A). Similar resistance to HI-TOPK-032 was observed in TOPK KO MEFs (Fig. 5B and C). In addition, 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 colon cancer cell growth in \( \text{shTOPK} \) cells was much less than its effect on \( \text{shMock} \) 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 \textit{in vivo}, HCT-116 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 HCT-116 tumor growth by more than 60% relative to the vehicle-treated group (Supplementary Fig. S5A; Fig. 6A; \( P < 0.05 \)). In addition, 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 \textit{in vivo} xenograft model, we investigated the effect of HI-TOPK-032 on down-stream targets of TOPK by Western blot analysis of HCT-116 colon tumor samples. The expression of p53 was strongly induced, and phosphorylation of ERK and RSK, a direct down-stream protein of ERK, was markedly inhibited in the HI-TOPK-032-treated group (Fig. 6C). This finding suggested that HI-TOPK-032 inhibits HCT-116 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 AP-1 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 antiapoptotic 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 carried out a sequence-based homology search. We found that the sequences of TOPK and MEKs are highly conserved. On the basis of this sequence homology, we tested the effect of U0126, a well-known inhibitor of MEK, on the in vitro TOPK kinase activity. U0126 inhibited TOPK activity by 25% at the highest concentration (20 \( \mu \text{mol/L} \); data not shown). On the basis of these preliminary data, we selected 36 compounds with a similar structure to an 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 conducted 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 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 DNA-binding domain (DBD) of tumor suppressor p53 (2). The downstream target of TOPK, 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

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 hours and growth was determined by MTS assay. Effect of HI-TOPK-032 on growth of WT TOPK MEFs (B) and TOPK KO MEFs (C). Cell growth at 1, 2, or 3 days was measured by MTS assay. Data are shown as mean ± SD (\( N = 5 \)) and similar results were obtained from 2 independent experiments. * 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 in 5% CO\(_2\). Colonies were counted using a microscope and the Image-Pro Plus (v.6) computer software program. Data are represented as mean ± SD of values from triplicate values, and similar results were obtained from 2 independent experiments. * indicates a significant (\( P < 0.05 \)) decrease in colony formation induced by HI-TOPK-032 compared with untreated control cells.
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 vivo 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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