Research Paper

TRK inhibitors block NFKB and induce NRF2 in TRK fusion-positive colon cancer

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Abstract

Tropomyosin receptor kinase (TRK) fusion is one of the oncogenic driver causes of colon cancer, and tropomyosin 3-neurotrophic receptor tyrosine kinase 1 (TPM3-NTRK1) fusion has been detected in the KM12SM cell line. In the present study, we investigated anticancer mechanisms in the KM12SM cell line using three different form of dovitinib (dovitinib (free base), dovitinib lactate (mono acid), and dovitinib dilactic acid (diacid)) and four TRK inhibitors (LOXO-101, entrectinib, regorafenib, and crizotinib). Exposure of TRK inhibitors at concentrations of 10 nM resulted in the apoptosis of KM12SM cells, whereas regorafenib had no effect. Treatment with all inhibitors except regorafenib also significantly increased the expression levels of the genes nuclear factor -erythroid 2-related factor 2 (NRF2) and glutamyl cysteine ligase catalytic subunit (GCLC) in KM12SM. These drugs significantly reduced expression of the phosphorylated proteins NFκB and COX-2 in the KM12SM cell line, and significantly attenuated KM12SM cell migration, according to a Transwell migration assay. Together, these results suggest that TRK inhibitors block products of carcinogenesis by negatively regulating the NFκB signaling pathway and positively regulating the antioxidant NRF2 signaling pathway.

Key words: TPM3-NTRK1; TRK inhibitors; NRF2; NFκB

Introduction

Colorectal cancer can be caused by genetic mutations. Despite improved colonoscopy techniques, only 38% of colorectal carcinomas are detected at an early stage [1]. Gene rearrangements, the most type of common molecular alteration, confer oncogenic properties to genes. Therefore, the treatment approach for colorectal cancer patients should carefully consider the molecular profile of the tumor [2].

The tropomyosin receptor kinase (TRK) family (TRK A-C) are major oncogenic drivers of various cancers, such as colorectal cancer, thyroid cancer, glioblastoma, cholangiocarcinoma, and lung cancer [3-5]. TRK mutations are mutually exclusive with other oncogenic drivers, which makes TRK-positive cancer a distinct disease entity, similar to other oncogene-addicted cancers such as c-MET and ALK [6]. Multi-kinase agents have activity against TRK A-C, but differ in their activity against other kinases. Many chemo preventive mechanisms have been shown to induce apoptosis and detoxification enzymes, including antioxidative enzymes, and inhibit inflammation and tissue invasion [7-10].

Nuclear factor erythroid 2-related factor 2 (NRF2) regulates the expression of antioxidative enzymes such as glutamyl cysteine ligase catalytic subunit (GCLC) [11], which plays multiple roles against oxidative stress. NRF2 also reduces the expression of inflammation factors such as cyclooxygenase-2 (COX-2) [12, 13], and previous studies have shown that the NFκB pathway is involved in inflammation and carcinogenesis. Therefore, the aim of this study was to determine whether a range of TRK inhibitors might exert...
anticancer effects via the NFκB/NRF2 pathway. Using apoptosis, gene, protein, and cell migration analyses and three different form of dovitinib (dovitinib (free base), dovitinib lactate (mono acid), and dovitinib dilactic acid (diacid)) and four TRK inhibitors (LOXO-101, entrectinib, regorafenib, and crizotinib) TRK inhibitors, we tested this hypothesis in the KM12SM cell line through tropomyosin 3-neurotrophic receptor tyrosine kinase 1 (TPM3-NTRK1) fusion.

Methods

Cell culture and reagents

Human colon cancer cell lines KM12SM, HT29, and HCT116 were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were cultured at 100% humidity and 5% CO₂ at 37 °C. The NTRK inhibitors LOXO-101, entrectinib, dovitinib, dovitinib lactate, dovitinib dilactic acid, regorafenib, and crizotinib were purchased from Selleck Chemicals (Houston, TX, USA). We used the annexin V-APC/propidium iodide (PI) apoptosis detection kit (Thermo Fisher Scientific, Rockford, IL, USA).

Transwell migration assay

A Transwell migration assay was performed using a 24-well Transwell chamber (8.0-μM-diameter pores) inserted into 24-well plates. We added 1 x 10⁴ cells in 300 μL serum-free RPMI 1640 medium to the upper chamber. NTRK inhibitors were diluted in RPMI 1640 medium to a final concentration of 10 nM and added to the lower chamber of each well. After 24 h of incubation, the medium and unmigrated cells in the upper chamber were removed, and the lower sides of the membranes were fixed with methanol and stained with hematoxylin and eosin. Images were taken using bright field microscopy (200× magnification).

Growth inhibition assays

We measured the 50% inhibitory concentrations (IC₅₀) of the NTRK inhibitors in KM12SM, HT29, and HCT116 cells using a specific MTS assay. We tested NTRK inhibitor concentrations of 10, 1, 0.1, 0.05, 0.0025, 0.00125, 0.001, 0.0001, 0.00001, and 0.000001 μM for 48 h. On the day of the proliferation assay, the medium was removed, and 200 μL fresh medium was added to each well of a 96-well plate, followed by 20 μL MTS solution. The plates were incubated at 37 °C for 2 h in a humidified environment with 5% CO₂. The absorbance was measured at 490 nm using a microplate reader (Synergy 2 Multi-Mode Microplate Reader; BioTek, Winooski, VT, USA). The IC₅₀ was calculated using nonlinear regression analysis with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA).

Apoptosis analysis

KM12SM, HT29, and HCT116 cells were seeded into 6-well plates at a density of 5 x 10⁴ cells/mL and then treated with 10 nM of each NTRK inhibitor. Cell death was determined using an annexin V-APC/PI apoptosis detection kit (Thermo Fisher Scientific, Waltham, MA, USA) with a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). The percentages of intact and apoptotic cells were calculated using CytExpert software (version 2.0; Beckman Coulter).

Quantitative reverse-transcription PCR (qRT-PCR) analysis

To quantify mRNA expression, total RNA from each sample was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using Power SYBR Green PCR Master Mix and a LightCycler 96 instrument (Roche Applied Science, Indianapolis, IN, USA). The transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were used for sample normalization. The primer sequences used were as follows: NTRK1 (TRKA; forward: 5’-AAACCAG TGGATCTGCCAAC-3’, reverse: 5’-ACGTAGCCGA AGAAACCTCA-3’), COX-2 (forward: 5’-TGAGCATCTACGGTTTGCTG-3’, reverse: 5’-AACTGCTCATCaACCCATTC-3’), NFR2 (forward: 5’-AACCCAGTGCA ATCTGCCAAC-3’, reverse: 5’-ACGTAGCCGAAGA AACCTCA-3’), GCLC (forward: 5’-GCTGGATGTGG ATGGATGTGG GACCAAGATG-3’, reverse: 5’-GCCGATTAACCTCC TCAATCA-3’), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5’-TTCAACCC ATGGAGAGGC-3’, reverse: 5’-GGCATGGACTGT GGTCATGA-3’).

Immunoblot analysis

Immunoblot analysis was conducted using standard procedures. Commercially available primary antibodies were directed against anti-TRK (1:1,000; sc7268; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-NFkB p65 (1:1,000; sc372; Santa Cruz Biotechnology), anti-COX-2 (1:1,000; sc19999; Santa Cruz Biotechnology), anti-phospho-ERK (1:500; #9101; Cell Signaling Technology), anti-ERK (1:1,000; sc514302; Santa Cruz Biotechnology) and anti-GAPDH (1:4,000; sc32233; Santa Cruz Biotechnology).

Statistical analyses

All data were statistically analyzed using GraphPad Prism 5 software (GraphPad Software).
values are presented as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA), with \( P < 0.05 \) considered significant.

Results

Effects of NTRK inhibitors on colon cancer cells with and without NTRK gene fusion

We observed dose-dependent inhibitory effects of NTRK inhibitors in KM12SM (TPM3-NTRK1 fusion), HCT116 (NTRK3, p.I837T), and HT29 cells (Figure 1). The cells were treated with different concentrations of NTRK inhibitors for 48 h, and the optimal dose was determined by evaluating cell viability using an MTS assay. NRTK inhibitors inhibited cell viability in KM12SM cells, whereas NRTK inhibitors seldom had an effect on HCT116 and HT29 cells.

Effects of NTRK inhibitors on cell apoptosis

To evaluate the effects of NTRK inhibitors on cell death in KM12SM, HCT116, and HT29 cells, we examined apoptosis by staining with annexin V-APC/PI, followed by flow cytometry (Figure 2), to assess early apoptotic and late apoptotic cell populations. Among the cell types, the death rate was best for KM12SM cells with LOXO-101, entrectinib, dovitinib, dovitinib lactate, dovitinib dilactic acid, cabozantinib, and crizotinib treatment. Apoptosis was seldom observed in HCT116 and HT29 cells (Figure 2). The addition of 10 nM regorafenib to KM12SM cells for 48 h had no significant effect on cell death.

Anticancer effects of NTRK inhibitors in KM12SM cells

NTRK inhibitors activated the NRF2 signaling pathway in KM12SM cells

We performed qRT-PCR to determine whether NTRK inhibitors activate the NRF2 signaling pathway for TPM3-NTRK1 fusion colon cancer in KM12SM cells (Figure 3). NTRK1 and COX-2 genes were downregulated when KM12SM cells were treated with 1 \( \mu M \) of all NTRK inhibitors except regorafenib, whereas NRF2 and GCLC genes were upregulated. KM12SM cells treated with regorafenib showed downregulation only of COX-2.

NTRK inhibitors suppressed NFκB signaling pathway activity in KM12SM cells

To further investigate the mechanisms of action of NTRK inhibitors in KM12SM cells, we analyzed phosphorylated NFκB (phospho-NFκB), COX-2, ERK, and phosphorylated ERK (phospho-ERK) proteins (Figure 4). Western blotting showed downregulation of phospho-NFκB, COX-2, ERK, and phospho-ERK in NTRK inhibitor-treated KM12SM cells.

NTRK inhibitors attenuated migration in KM12SM cells

To determine the effects of NTRK inhibitors on KM12SM cell migration, we performed a Transwell migration assay (Figure 5). KM12SM cell migration was significantly decreased by NTRK inhibitor treatment compared with non-treated control cells.

Discussion

The major finding of the present study was that six low-dose (10 nM) TRK inhibitors (LOXO-101, entrectinib, dovitinib, dovitinib lactate, dovitinib dilactic acid, and crizotinib) can induce apoptosis and attenuate cell migration in KM12SM cells harboring TPM3-NTRK1 fusion by regulating the NFκB and NRF2 signaling pathways. Low-dose (10 nM) regorafenib attenuated cell migration by regulating the NFκB signaling pathway in KM12SM cells, but did not induce apoptosis.
TPM3-NTRK1 was originally identified in cases of colorectal cancer [14, 15]. TRK gene fusion is a highly therapeutically actionable driver of tumor growth. Several tumors are associated with gain-of-function mutations of genes coding for both serine/threonine and tyrosine kinases [16]. TRK gene fusion is also characterized by ligand independent kinase activity and can occur in humans in a mutually exclusive manner with respect to other oncogenic drivers [3, 17]. Although numerous 5′ gene fusion partners have been identified, all studied variants appear to respond to targeted therapy [18, 19]. In this study, we aimed to identify effective TRK inhibitors and determine whether they have anticancer effects via activation of the NFκB and NRF2 pathways in KM12SM cells with TPM3–NTRK1 fusion.

We found that three different form of dovitinib and four TRK inhibitors from a TRK inhibitor screening library, i.e., dovitinib, dovitinib lactate, dovitinib dilactic acid, LOXO-101, entrectinib, regorafenib, and crizotinib, all reduced the viability of KM12SM cells in vitro; therefore, these compounds were selected for the present study. LOXO-101 had no effect on the HCT116 and HT29 cell lines, which lack TRK fusion, whereas a low dose (10 nM) of the other drugs affected all three cell lines, with and without TRK fusion (Figure 1). Apoptosis in KM12SM cells was highly induced (> 50%) by a low dose (10 nM) of each TRK inhibitor, except regorafenib, whereas these drugs had very little effect on HCT116 and HT29 cells (Figure 2). The balance between cell division and apoptosis depends on both oxidative stress and the mutation of important signaling molecules [11, 20]. NRF2 is an antioxidant/detoxification enzyme that...
low-dose drug delivery may have been associated with NRF2 signaling, which induces the expression of GCLC antioxidant genes; these drugs also suppressed COX-2, ERK, and phospho-ERK enzyme activity (Figures 3, 4 and 6). All of the TRK inhibitors attenuated NFκB signaling (Figure 4 and 6). Our Transwell migration assay revealed that all of the tested TRK inhibitors suppressed KM12SM cell migration (Figure 5).

Figure 4. Protein expression levels of TRK, p-NFkB p65, COX-2, ERK and p-ERK in KM12SM cell lines. Protein expression levels of TRK, p-NFkB p65, and COX2 were determined using Western blot analysis. ***p < 0.001.

Figure 5. Transwell migration assay. Representative microscopic images of cells that migrated through the Transwell during the migration assay (hematoxylin and eosin staining, 200× magnification).
In conclusion, the results of this study demonstrated that in KM12SM cells with TPM3-NTRK1 fusion, TRK inhibitors that induce NRF2 signaling were effective in colon cancer treatment at low concentrations, whereas those that did not induce NRF2 signaling were effective at high concentrations.

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Author Contributions

The authors’ responsibilities were as follows: Sung-Hwa Sohn: Methodology, Validation, Formal analysis, Investigation, Writing- original draft, Writing-review & editing. Hee Jung Sul and Bohyun Kim: Methodology, Validation, Formal analysis, Investigation. Bum Jun Kim and Hyeong Su Kim: Conceptualization, Writing - review & editing. Dae Young Zang: Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision, Project administration, Funding acquisition. All authors agreed to be accountable for the work.

Competing Interests

The authors have declared that no competing interest exists.

Figure 6. Schematic of proposed mechanisms involved in the anti-cancer effects in KM12SM cells of NTRK inhibitors.

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