〈Regular Article〉

Sorafenib-induced apoptosis in colonic neuroendocrine carcinoma cells

Masahiro YAMAMURA[1)], Akira YAMAUCHI[2)]
Shigenobu TONE[3)], Yoshiyuki YAMAGUCHI[1)]

1) Department of Clinical Oncology,
2) Department of Biochemistry, Kawasaki Medical School
3) Laboratory of Molecular Developmental Biology, Graduate School of Science and Engineering, Tokyo Denki University

ABSTRACT **Background:** Neuroendocrine carcinoma (NEC) is a rare disease, and therapy for this malignant tumor is controversial. Conventionally, platinum doublet chemotherapy has been used for advanced gastroentero-pancreatic (GEP) neuroendocrine carcinoma (GEP-NEC), but the efficacy of molecular-targeted drugs for GEP-NEC is unknown. In this study, we investigated the antitumor effect of molecular-targeted drugs on colorectal neuroendocrine carcinoma cells.

Materials and methods: A colonic neuroendocrine carcinoma cell line COLO320 was treated with molecular-targeted drugs, and cell growth suppression and apoptosis induction were evaluated.

Results: The cytostatic effects of molecular-targeted drugs against COLO320 were higher in the order of sorafenib, sunitinib, rapamycin, and imatinib. Flow cytometry analysis showed that sorafenib induced G1 cell cycle arrest and a high rate of apoptosis. Sunitinib showed condensation and fragmentation of nuclear chromatin, but also necrosis with cell swelling. In contrast, sorafenib strongly induced apoptosis via condensation and fragmentation of nuclear chromatin. Sorafenib-induced apoptosis was due to caspase-3 activation, and this apoptosis was inhibited with a caspase inhibitor.

Conclusion: Sorafenib induces apoptosis in COLO320 cells and is a potential therapeutic agent for colonic neuroendocrine carcinomas.

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Key words：Neuroendocrine carcinoma, COLO320, Angiogenesis inhibitor, Sunitinib, Sorafenib, Apoptosis

INTRODUCTION

Neuroendocrine neoplasms (NEN) are rare tumors that exhibit a various morphological and functional characteristics. In 2019, the World Health Organization classified NEN into well-differentiated neuroendocrine tumor (NET) histologically exhibiting a neuroendocrine pattern and poorly-differentiated neuroendocrine carcinoma

Corresponding author
Masahiro Yamamura
Department of Clinical Oncology, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan
Phone : 81 86 462 1111
Fax : 81 86 464 1134
E-mail: yamamura@med.kawasaki-m.ac.jp

(NEC). Well-differentiated NETs were classified as NET G1 with a Ki-67 index < 3%, NET G2 with 3% to 20%, and NET G3 with > 20%. A poorly differentiated neuroendocrine tumor with a Ki-67 index of > 20% was classified as NEC[1]. NEC is a poorly differentiated, high-grade malignant tumor, previously termed poorly differentiated neuroendocrine carcinoma, which include small-cell carcinoma and large-cell NEC. The incidence of GEP-NEN is thought to have increased and is now reported to be approximately 7 per 100,000 persons/year[2]. GEP-NECs are rare accounting for 5-10% of GEP-NEN[3].

GEP-NEN is treated by resection if it is resectable; however, but drug therapy is chosen in cases of unresectable tumors or metastatic recurrence. Chemotherapy for advanced GEP-NEN differs between NET and NEC, and treatment strategies differ for primary pancreatic and gastrointestinal tumors in NET. Pancreatic NET is treated with the somatostatin analog lanreotide, the targeted drugs everolimus and sunitinib, and the cytotoxic anticancer drug streptozocin. Gastrointestinal NET is treated with the somatostatin analog octreotide and lanreotide, the molecular-targeted drug everolimus, and the cytotoxic anticancer drug streptozocin. On the other hand, as the treatment for GEP-NEC, the cytotoxic anticancer agents etoposide plus cisplatin and irinotecan plus cisplatin are used[4, 5]. The phase Ⅲ clinical trial for digestive NEC showed a 5.6 months progression-free survival (PFS), and 12.5 months overall survival (OS) for etoposide plus cisplatin, and a 5.1 months PFS, and 10.9 months OS for irinotecan plus cisplatin[6]. No effective second-line therapy has been established for patients who are resistant to these treatments. Currently, there are no indications for molecular-targeted drugs for NEC, and their effects on NEC are unknown.

In this study, we examine the effects of octreotide, imatinib, rapamycin, sunitinib, and sorafenib on colon neuroendocrine carcinoma cells, and clarified the antitumor effects of molecular-targeted drugs on neuroendocrine carcinoma.

MATERIALS AND METHODS

Cell line and reagents

The human neuroendocrine carcinoma cell line COLO320, was obtained from the RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin at 37℃ in humidified air with 5% CO_2.

Reagents

Octreotide (Anaspec, San Jose, CA, USA), rapamycin (Sigma-Aldrich, St Louis, MO, USA), imatinib (Selleckchem, Houston, TX, USA), sunitinib (Cayman Chemical, Ann Arbor, MI, USA), and sorafenib (Selleckchem, Houston, TX, USA) were purchased. Octreotide was dissolved in sterile water, and the other drugs were dissolved in dimethyl sulfoxide. Stock solutions of octreotide and rapamycin were stored at -20℃, and those of Imatinib, Sunitinib and Sorafenib were stored at -80℃ .

Cell viability assay

Cell viability was determined using a premix WST-1 cell proliferation assay system kit (Takara, Tokyo, Japan). Cells (5,000 cells/well) were plated in 96-well plates in 100 μl media, cultured overnight and continuously exposed to a range of concentrations of each drug for 72 h.

Next, 10 μl of premix WST-1/well was added and, the plates were incubated for 4 h. In viable and metabolically active cells, the tetrazolium salt WST-1 was cleaved to form a formazan-class dye by mitochondrial succinate-tetrazolium reductase, and absorbance was measured using a microplate

absorbance reader at 450 nm. The half maximal inhibitory concentration (IC_{50}) was defined as the concentration resulting in a 50% reduction in growth compared with the control cell growth.

Cell cycle assay

COLO320 cells were plated on a 6-well plates at 2×10^5 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with 1 μM or 10 μM sorafenib. The cells were then harvested with 0.02% EDTA and 0.25% trypsin, washed with buffer using a Cycle TEST^{TM} PLUS DNA Reagent kit (Becton Dickinson, CA, USA), stained with Propidium iodide (PI), and histograms were obtained using a FACS-Calibur flow cytometer (Becton Dickinson). Data were collected and analyzed from 1×10^4 cells, using CELL Quest analysis software (Becton Dickinson).

Apoptosis assay

COLO320 cells were plated on 6-well plates at 2×10^5 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with 1 μM or 10 μM sorafenib. Using an in situ cell death detection Kit, TMR red (Roche Diagnostics, Tokyo, Japan), cells were fixed with paraformaldehyde and, permeabilized with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate), and then with the TUNEL reaction solution. Immunofluorescence image were obtained using a fluorescence microscope. For quantitative analysis of apoptosis, cells were collected and subjected to FACS analysis using an Annexin V-FLUOS staining kit (Roche Diagnostics). The number of apoptotic cells was calculated as the total number of annexinV-positive and PI (negative plus positive) cells.

Detection of caspase-3 activation

Caspase-3 activation was analysed using the NucView 488 Caspase-3 Assay Kit for live cells (Biotium, San Francisco, CA, USA), a caspase-3 substrate that fluoresces when cleaved. We investigated the effects of caspase-3 suppression using Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-FMK), a caspase inhibitor (Peptide Institute, Minoh, Osaka, Japan). COLO320 cells were plated on 24-well plates at 2×10^4 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with various concentrations of sunitinib and sorafenib. A caspase-3 inhibitor, Z-VAD was added at 100-fold dilution to 500 μl of medium 1 h, before the addition of the drugs. Immunofluorescence images were obtained using a fluorescent microscope.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviation. We analyzed the data using Student's *t*-test .

$p < 0.05$ was considered significant.

RESULTS

Growth inhibitory effects of molecular target drugs on colonic neuroendocrine carcinoma cells

We evaluated the growth inhibition effect of molecular-target drugs in the colonic neuroendocrine carcinoma cell lines COLO320 using the premix WST-1 cell proliferation assay system kit. Treatment with each drugs for 72 h resulted in a dose-dependent decrease in cell viability (Fig.1A). The IC_{50} values (μM) were 16.1 for rapamycin, 15.9 for imatinib, 8.2 for sunitinib, and 0.29 for sorafenib (Fig.1B). Octreotide did not inhibit the growth of COLO320 cells.

Sorafenib induced cell cycle arrest

The cell cycle distribution in each phase is shown in Fig. 2. When treated with each concentration of sorafenib, G0/G1 phase cells were 58.7 ± 0.26 in control and 63.2 ± 0.66 in 1 μM sorafenib ($p < 0.05$). SubG1 phase cells were significantly increased by 45.0 ± 0.2 with sorafenib 10 μM

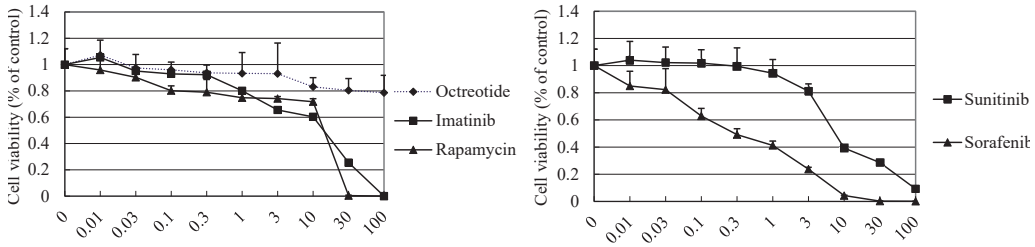

Fig.1. Growth-inhibitory effects of molecular-targeted drugs on colonic neuroendocrine carcinoma cells.
Human colonic neuroendocrine carcinoma cells COLO320 were plated on 96-well plates at 5×10^2 cells per well, cultured in a serum-containing medium for 24 h, and then incubated for 72 h with molecular-targeted drugs. Cell viability was performed using the premix WST-1 cell proliferation assay system kit (Takara, Tokyo, Japan). Values are the mean ± standard deviation of three independent experiments.

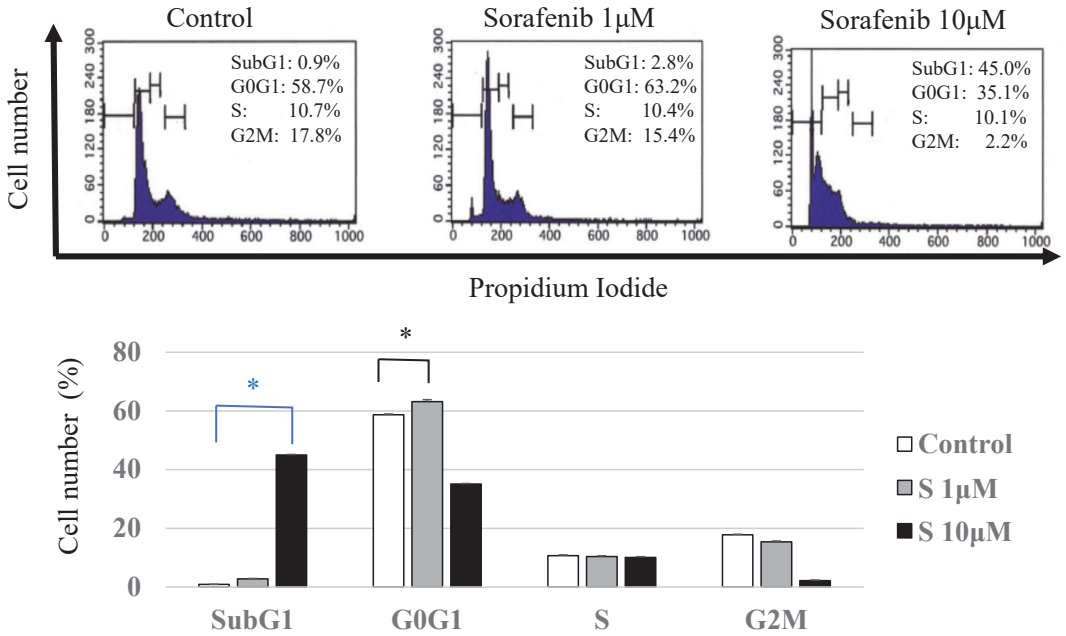

Fig.2. Cell cycle analysis in COLO320 cells with sorafenib treatment.
Human colonic neuroendocrine carcinoma cells COLO320 were plated on 24-well plates at 1×10^5 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with various concentrations of molecular-targeted drugs. Cell cycle distribution was analyzed by flow cytometry using propidium iodide staing. Values represent the mean ± standard deviation of three independent experiments. * $P < 0.05$ compared to the control group.

compared to 0.9 ± 0.06 in controls ($p < 0.05$). No change was observed in the S phase.

Sorafenib- and sunitinib-induced apoptosis

To evaluate sorafenib-induced apoptosis, the cells were treated with sorafenib for 48 h, stained with TMR red, and subjected to TUNEL assay. As shown in Fig. 3A, we confirmed apoptosis by sorafenib at 1 μM for 48h. Treatment with sorafenib 10 μM significantly increased the percentages of TUNEL-positive cells compared to untreated control cells. Sorafenib-induced apoptosis was evaluated using annexin V-FITC and PI staining by flow cytometry. Sorafenib treatment induced apoptosis in a dose-

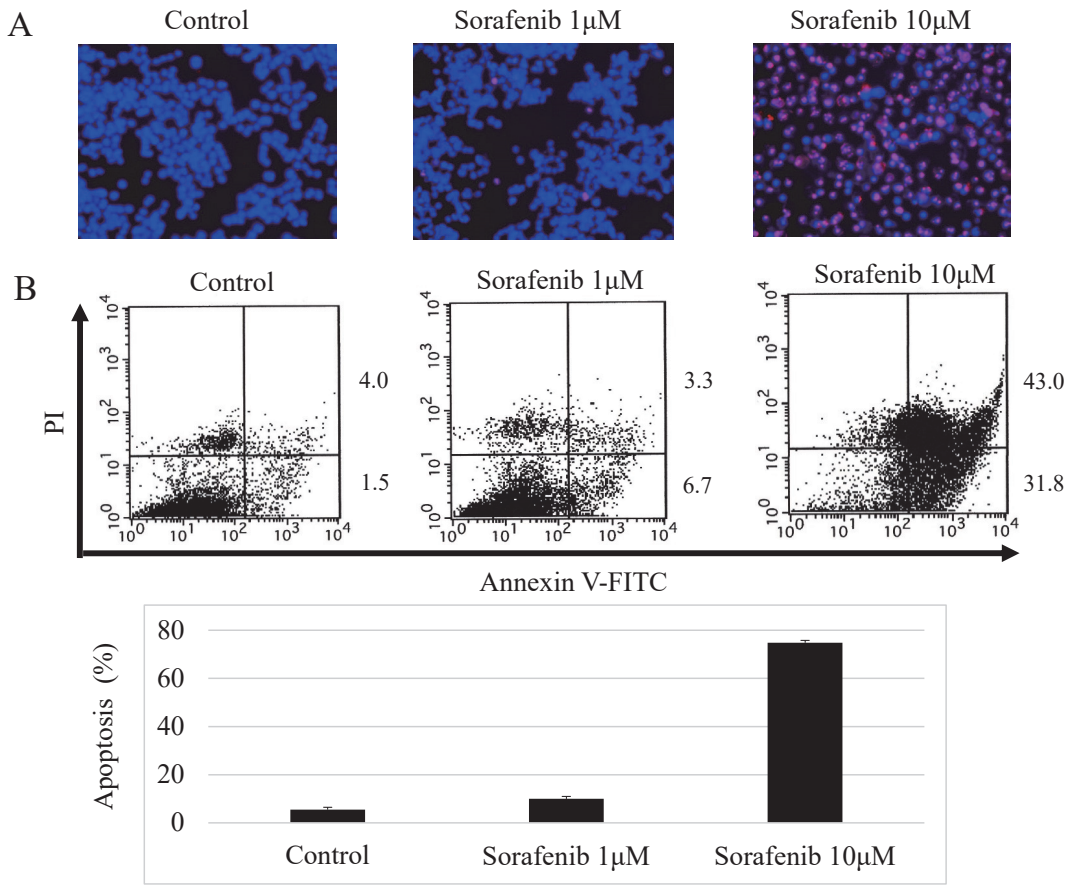

Fig.3. Evaluation of apoptosis in COLO320 cells with sorafenib treatment.
(A) Immunofluorescence image of COLO320 cells. Human colonic neuroendocrine carcinoma cells COLO320 were plated on 24-well plates at 1×10^5 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with 1 μM and 10 μM of sorafenib. (B) Flow-cytometry analysis of COLO320 cells. Apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide staining. Apoptotic cells were calculated by the number of annexinV-positive and PI (negative plus positive) cells.

dependent manner (Fig. 3B). Apoptosis is shown in the region on the right side of Fig. 3B. Apoptosis cells were 5.5 ± 0.5% in the control group, 10 ± 0.69% in sorafenib 1 μM, and 74.8 ± 0.66% in sorafenib 10 μM. In the 10 μM sorafenib-treated sample, many apoptotic cells were observed, similar to fluorescent staining.

Detection of morphological changes and caspase-3 activation by sunitinib and sorafenib

When COLO320 cells were treated with sunitinib and sorafenib, chromatin condensation and nuclear fragmentation by Hoechst33342 stain were observed in a dose-dependent manner, as well as necrosis, such as nuclear swelling and cell rupture (Fig. 4, left column). NucView488 staining showed that, both sunitinib and sorafenib activated caspase-3 in a dose-dependent manner (Fig.4, middle column). These COLO320 cellular changes were reduced in nuclear chromatin condensation and fragmentation by the administration of the caspase inhibitor Z-VAD-FMR prior to drug treatment (Fig.4, right column).

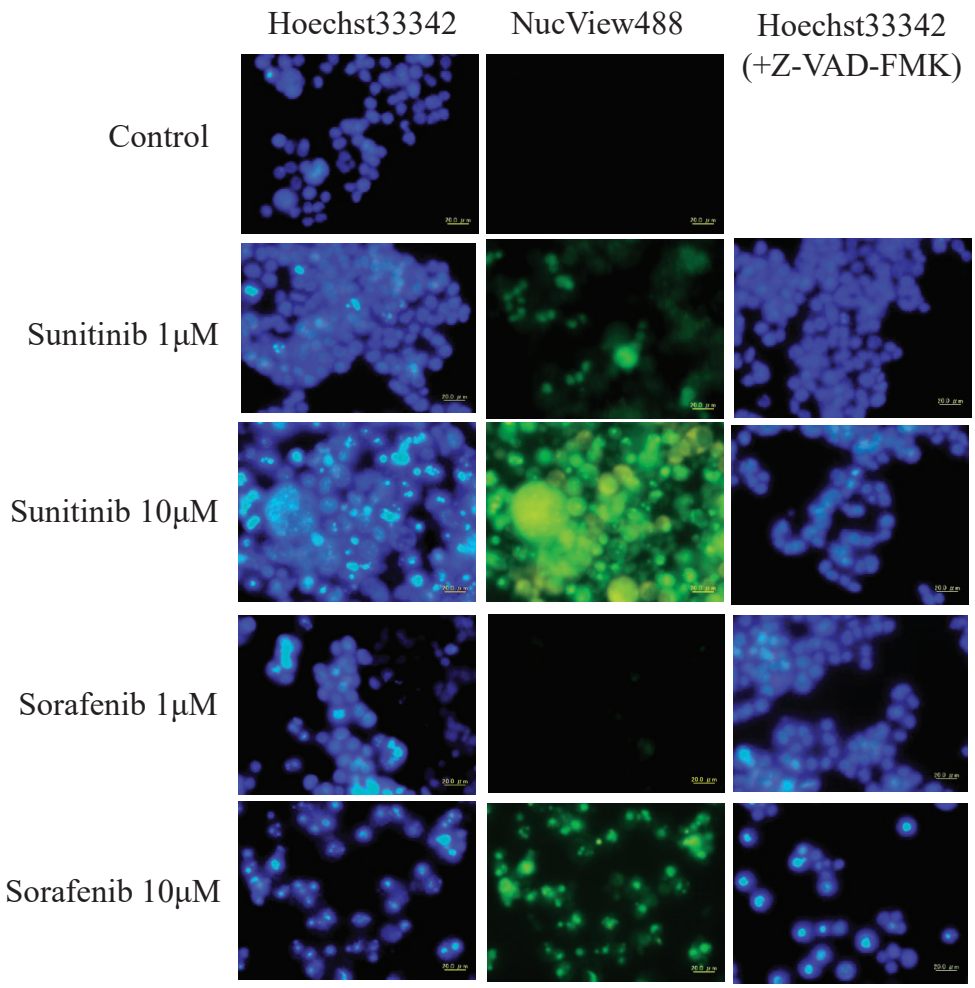

Fig.4. Immunofluorescence image of COLO320 cells.
Human colonic neuroendocrine carcinoma cells COLO320 were plated on 24-well plates at 2×10^4 cells per well, cultured in serum-containing medium for 24 h, and then incubated for 48 h with various concentration of molecular-targeted drugs. A caspase-3 inhibitor, Z-VAD-FMK was added 1 h before drug administration.
Immunofluorescence images were obtained using a fluorescence microscope.

DISCUSSION

In this study, we investigated the cytostatic effects of octreotide, a somatostatin analogue, and molecular-targeted drugs, rapamycin, imatinib, sunitinib, and sorafenib, against colonic neuroendocrine carcinoma cell line COLO320. Cell proliferation inhibition was observed in the following order: sorafenib, sunitinib, rapamycin, and imatinib. The IC_{50} value were estimated for sorafenib (0.29 μM), and sunitinib (8.2 μM) (Fig.1). Octreotide did not inhibit the growth of COLO320 cells.

Sorafenib inhibits receptor tyrosine kinases such as Raf, FLT-3, c-KIT, RET, VEGFR, and PDGFR[8]. Sorafrenib is currently used to treat hepatocellular carcinoma (HCC)[9], renal cell carcinoma (RCC)[10] and thyroid cancer[11]. The IC_{50} of sorafenib is reported to be 4.5-6.3 μM[9] for HCC cell lines and 0.17-6.87 μM for thyroid cancer[12]. The IC_{50} of sorafenib for COLO320 cells in this study was lower

than that for HCC cell lines. Sorafrenib inhibited tumor growth and angiogenesis in mice transplanted with HCC and RCC cells[9, 10], and inhibited tumor growth in mice transplanted with thyroid cancer cells[11]. Cell cycle analysis of sorafenib revealed G1 arrest at low concentrations and an increase in SubG1 at high concentrations (Fig.2). These data indicated that treatment with sorafenib resulted in cell cycle arrest at G0/G1 phase at 1 μM and increased apoptosis at 10 μM. Another in vitro analysis showed G1 arrest and induction of apoptosis in HCC cell lines[13]. In this study, NEC cells were treated with 0.1, 1, and 10 μM sorafenib to assess apoptosis, but sorafenib 0.1 μM treatment did not increase the number of apoptotic cells (data not shown). Sorafenib at 1 μM and 10 μM induced apoptosis in a dose-dependent manner (Fig.3). When COLO320 cells were treated with sunitinib or sorafenib, chromatin condensation and nuclear fragmentation were observed. Sunitinib also caused necrosis such as nuclear swelling and cell rupture. In NucView488 cells, both sunitinib and sorafenib activated caspase3 in a dose-dependent manner. Furthermore, the caspase inhibitor Z-VAD-FMK reduced the condensation and fragmentation of nuclear chromatin in COLO320 cells (Fig. 4). Therefore, we suggest that sorafenib-induced apoptosis occurs mainly via the caspase-3 pathway.

Poorly differentiated cancers generally progress rapidly and are less effective with molecular-targeted drugs. In this study, Sorafenib induced cytostatic activities and apoptosis in NEC cells. However, single-agent efficacy in NEC patients may be limited, as in other cancers. In the future, further basic research is required on the combined effects of sorafenib and cytotoxic anticancer drugs on NEC cells.

CONCLUSION

In this study, we showed that sorafenib induces apoptosis in colorectal neuroendocrine cancer cells via caspase-3 activation. Sorafenib is a novel therapeutic agent for colonic neuroendocrine carcinoma.

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DISCLOSURE

The authors declare that they have no conflicts of interest.

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