**Original Article**

**ICG001 inhibits colon cancer growth by suppressing eIF3D signaling**

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**Abstract:** Recently, several clinical trials have examined the efficacy of small molecule inhibitors of eukaryotic translation initiation factor 3 subunit D (eIF3D) for the treatment of colorectal cancer (CRC). Unfortunately, none of these compounds has proven more effective than existing chemotherapeutic drugs, resulting in a significant unmet need for effective CRC therapies. Here, we examined the anticancer effects of ICG001, a novel eIF3D inhibitor, on CRC cells using both *in vitro* cell growth and apoptosis assays, as well as *in vivo* HCT-116 xenograft mouse model. ICG001 induced the caspase-dependent apoptosis of CRC cells by downregulating the expression of Bcl-2 in combination with increased expression of Bad and Bax. Western blot analysis demonstrated that ICG001 inhibited the expression of eIF3D and its downstream targets, PI3K and AKT, *in vitro*. Finally, ICG001 inhibited HCT-116 xenograft growth significantly in nude mice in a dose-dependent manner. Taken together, these data suggest that ICG001 inhibited the growth of CRC cells *in vitro* and *in vivo* via the inhibition of eIF3D signaling. These results provide a basis for further clinical investigation of ICG001 as a targeted therapy for CRC. Our findings may open a new avenue for the development of novel eIF3D inhibitors in the treatment of CRC and other cancers.

**Keywords:** ICG001, colon cancer, eIF3D, PI3K/AKT signaling pathway

**Introduction**

The incidence rate of colorectal cancer (CRC), one of the most common forms of cancer worldwide, is increasing annually [1]. Despite significant advances in the treatment of CRC, the overall survival of advanced and metastatic disease has remained virtually unchanged over the past 20 years, with the 5-year survival rate ranging from as low as 15% to nearly 90% for late- and early-stage disease, respectively [2]. There is currently no effective therapy to control the recurrence and metastasis of CRC. Therefore, a better understanding of the molecular mechanisms underlying CRC pathology is necessary to identify new therapeutic targets that can improve the management and treatment of colon cancer.

A wide array of tumor suppressors and oncoproteins have been identified in CRC, though significant gaps remain regarding the role of specific gene alterations and their downstream functions in the initiation and progression of CRC [3, 4]. Eukaryotic translation initiation factor 3 (eIF3) is a multiprotein complex consisting of 10-13 subunits, with multiple functions in translation. Mismatches between eIF3 subunits is associated with cancer progression [5, 6]. Eukaryotic translation initiation factor 3 subunit D (eIF3D), a member of the eIF3 family, plays a key role in translation initiation [7]. Recently, several studies have identified links between eIF3D and the development and progression of various tumors. Aberrant activation of eIF3D signaling due to eIF3D overexpression and/or gene mutations has been shown to play a role in the development and progression of several cancers, including breast cancer [8], prostate cancer [9], non-small cell lung cancer [10], and melanoma [11]. Zhang et al. [12] demonstrated that the overexpression of eIF3D significantly enhanced the development of gallbladder cancer development as well as the occurrence and development of ovarian cancer. Given these strong associations, eIF3D has received significant attention as a potential therapeutic target for cancer intervention.
Here, we examined the anticancer effects of ICG001, a novel eIF3D inhibitor, on CRC cells using in vitro cell growth and apoptosis assays, and an in vivo HCT-116 xenograft mouse model. ICG001 inhibited proliferation and induced apoptosis in CRC cells in vitro. Furthermore, ICG001 inhibited the growth of HCT-116 xenografts in vivo, with no obvious signs of toxicity. Mechanistically, ICG001 was found to inhibit the eIF3D-mediated PI3K/AKT signaling pathway in CRC cells both in vitro and in vivo. Our findings suggest that ICG001 is a novel eIF3D inhibitor, which may be explored for the treatment of CRC and other tumors.

Material and methods

Cell culture

Human CRC cell lines (HCT-116 and SW480) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in RPMI 1640 supplemented with 10% FBS. All cell lines were cultured in a humidified atmosphere at 37°C in the 5% CO₂.

Cell proliferation assay

Cell proliferation assays were conducted as described previously [13]. Briefly, cells, grown in six-well plates, were treated with ICG001 (0-2 mM) for 6 days, followed by cell counting with a Z1 counter (Beckman Coulter, Fullerton, CA, USA). Cells treated with the vehicle (DMSO) alone served as a control.

MTT assays

For quantifying the cell survival, MTT assay were performed. Briefly, cells were seeded on 96-well plates, and then treated with various concentration of ICG001 (0-2 mM) for 24-72 h followed by the addition of 50 μl (5 mg/ml) MTT to each well and incubation for 3 h. The reaction was stopped by adding 150 μl dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 570 nm using a plate reader.

Apoptosis assay

In this study, we determined the cell apoptosis by flow cytometry. Briefly, cells were treated with various concentration of ICG001 (0-2 mM) for 72 h. And then cells were collected following staining by Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Sparks, MD, USA). The flow cytometry was used with a FACs Calibur flow cytometer (Becton Dickinson). Cells treated with DMSO alone were used as a control.

Western blot analysis

After treatment, cells were harvested and lysed, and protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). Equivalent amounts of proteins (whole cell lysates) were separated on 8-15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat dry milk (dissolved in PBS containing 0.05% Tween 20) for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C, followed by probing with appropriate secondary antibodies conjugated to horseradish peroxidase overnight at 4°C. Immunoreactive bands were visualised by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA, USA).
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**Figure 2.** ICG001 induces apoptosis in HCT-116 cells. HCT-116 cells were treated with ICG001 (0-2 mM) for 72 h, followed by Annexin V/PI staining and flow cytometry. Representative diagrams are shown. (B) Data from (A) were statistically analysed. Means ± SEM. (n = 3). *P < 0.05, **P < 0.01.

**HCT-116 xenograft model**

All animal experiments carried out were approved by the Animal Care and Use Committee of Liaocheng People’s Hospital. Female 4-week-old Balb/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. HCT-116 cells (2 × 10⁶) were injected subcutaneously into the hind flank of each mouse. When the tumours reached a volume of approximately 100 mm³, mice were randomised into four groups. ICG001 was dissolved in 5% DMSO in saline. Mice were orally treated with vehicle control, 12.5 mg/kg ICG001, 25 mg/kg ICG001, 50 mg/kg and ICG001 once daily. Tumour volume [(length × width²)/2] was determined with a digital caliper. Tumour growth and mice body weight were monitored every three days. At the end of experiments, animals were killed, and the tumours were collected, photographed, and analysed. And parts of tumours were evaluated by haematoxylin and eosin, and immunohistochemistry with indicated antibodies.

**Statistical analysis**

All results were expressed as mean ± SEM from at least three independent experiments. The statistical significance of mean difference between two groups was determined using two-tail Student’s t-test and P values of less than 0.05 were considered significant.

**Results**

*ICG001 inhibits cell proliferation and induced cell death in CRC cells*

First of all, to assess the anticancer activity of ICG001 in CRC cells *in vitro*, we tested whether ICG001 inhibits cell proliferation and induces cell death in HCT-116 and SW480 cells. As shown in Figure 1A, treatment with ICG001 for 6 days inhibited cell proliferation of HCT-116 and SW480 cells in a concentration-dependent manner. Furthermore, treatment with ICG001 (0.2 mM) for 24-72 h reduced the cell viability in both HCT-116 and SW480 cells in a concen-
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ICG001 inhibits colon cancer in a concentration- and time-dependent manner, as detected by MTT assay (Figure 1B). Collectively, the results indicate that ICG001 is a potent anti-cancer agent for CRC cells.

ICG001 induces apoptotic cell death in CRC cells

To determine whether ICG001 induces apoptotic cell death, we performed Annexin V-FITC/PI staining, a method that is frequently used to detect apoptosis. As shown in Figure 2, treatment with ICG001 for 72 h induced apoptosis in HCT-116 cells in a concentration-dependent manner. ICG001 at 0.1-2 mM increased the apoptotic cells by approximately 1.3-3.3-fold, compared to the control.

To understand how ICG001 induces apoptosis, we next examined whether ICG001 alters the expression of pro-apoptotic and anti-apoptotic proteins in the cells. As shown in Figure 3, treatment with ICG001 for 24 h markedly down-regulated the expression levels of anti-apoptotic proteins (Bcl-2) and meanwhile up-regulated the proapoptotic protein Bad and Bax levels in a concentration-dependent manner. Furthermore, we observed that ICG001 induced caspase-dependent apoptosis. This was consistent with the data that ICG001 increased the cleavage of caspase-3 and caspase-9. Therefore, our results suggest that ICG001 induces caspase-dependent apoptotic cell death by down-regulating the expression of anti-apoptotic protein Bcl-2 and up-regulating the expression of pro-apoptotic protein Bad and Bax in CRC cells.

ICG001 inhibits PI3K/AKT signaling in CRC cells

To further illuminate the molecular mechanisms by which ICG001 inhibits CRC cell growth and invasion, we explored the PI3K/AKT signal-
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Figure 4. ICG001 inhibits the expression of elf3D and its downstream PI3K/AKT signaling pathways in HCT-116 cells. Serum-starved HCT-116 cells were treated with ICG001 (0-2 mM) for 24 h, followed by stimulation with elf3D (50 ng/ml) for 1 h. The cell lysates were subject to western blotting with indicated antibodies. Means ± SEM. (n = 3). ##P < 0.01, *P < 0.05, **P < 0.01.

Figure 5. In vivo anti-tumour activity and mechanism of action of ICG001. A-C. Nude mice bearing HCT-116 tumour cells were treated with ICG001 at the indicated doses or vehicle control alone over 28 days. The bodyweight of mice and tumor sizes were measured every three days and at the end of the experiments, the mice were killed and the tumour tissues were dissected and weighed. The data are expressed as the Mean ± SEM. of groups (6 mice per group). The representative images of isolated tumours are also shown. *P < 0.05; **P < 0.01.

ICG001 (0-2 mM) for 24 h, followed by stimulation with elf3D (50 ng/ml). As indicated in Figure 4, our western blot analysis showed that ICG001 inhibited the phosphorylated PI3K and phosphorylated AKT in a concentration dependent manner. Interestingly, ICG001 showed the most potent inhibitory effect on p-PI3K and p-AKT meanwhile the elf3D expression was shown to be lowest at 1 mM. Thus, our data further support that ICG001 inhibited CRC cell growth and invasion via blockade of p-PI3K, p-AKT and elf3D activations.

ICG001 inhibits HCT-116 xenograft growth in nude mice

To evaluate the anti-tumour activity of ICG001 in vivo, HCT-116 xenograft model was used. In this model, HCT-116 cells were injected subcutaneously into the hind flank of each nude mouse, then the mice were randomised into four groups (6 mice per group) when the tumours grew to a size of about 100 mm³. Next, the animals were orally given three doses (12.5, 25, and 50 mg/kg) of ICG001 or vehicle (control) every day. At the end of the experiment, we found that ICG001 dose-dependently inhibited the tumour growth (volume) (Figure 5A) compared with the vehicle. Similarly, treatment with ICG001 also inhibited the tumour weight increase significantly, compared with the vehicle treatment (Figure 5B). Of note, no obvious toxicity was observed in all the treated groups (Figure 5C).
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![Figure 6](image_url)

**Figure 6.** Tumour tissues were evaluated by H&E, and immunohistochemistry with indicated antibodies. Representative images are shown (200 ×).

cal analysis was conducted using HCT-116 tumour. As shown in **Figure 6**, ICG001 showed anti-cancer active (HE staining) and a decreased tumour cell proliferation (Ki67-positive staining) and a significant increase in apoptosis (LC3B-positive staining), compared with the vehicle. Moreover, ICG001 remarkably reduced the microvessel density (CD31 staining).

**Discussion**

The study provides the first evidence demonstrating the potent anticancer activity of ICG001 against CRC *in vitro* and *in vivo*, by targeting eIF3D signaling. *In vitro* assays suggest that ICG001 inhibited cell proliferation and induced cell death in CRC cells. Furthermore, we were able to show that ICG001 induced apoptosis by flow cytometry. As shown in **Figure 2**, treatment with ICG001 for 72 h induced apoptosis in HCT-116 cells in a concentration-dependent manner, while our Western blot results suggest that ICG001 induces caspase-dependent apoptotic cell death by downregulating the expression of the anti-apoptotic protein Bcl-2 and upregulating the expression of the pro-apoptotic proteins Bad and Bax in CRC cells.

The PI3K/AKT signaling pathway has been extensively studied due to its critical role in cancer progression [14], including the regulation of metabolism, tumor growth, survival, and metastases [15]. Recently, therapeutic compounds that target this pathway have been developed and are currently being evaluated in clinical trials for several malignancies, including colon cancer [16]. Given recent evidence that the PI3K/AKT signaling pathway is overactivated in cancer cells [17, 18], several drugs that target PI3K and AKT are in development, a number of which have been validated in clinical trials. Subsequent addition of ionizing radiation can improve the anticancer effects of these drugs [19, 20].

The signaling events triggered by PI3K and AKT are complex, with different, partially overlapping functions that regulate cell survival and
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therapeutic resistance. Treatment with 1 mM ICG001 for 24 h inhibited the phosphorylation of PI3K and Akt significantly (Figure 4). Therefore, the more potent in vitro anticancer activity of ICG001 may be attributed to the fact that ICG001 exhibited stronger inhibition of eIF3D as opposed to its downstream effectors (PI3K and Akt). Our in vivo results show that at high doses (50 mg/kg) ICG001 could significantly inhibit HCT-116 xenograft growth when orally administered to mice (Figure 5), consistent with the effect seen in vitro. Further research will be necessary to evaluate the pharmacokinetic profile of ICG001 in animals; such data would be instructive to help develop derivatives of ICG001 with improved pharmacokinetic properties.

Taken together, our data show that ICG001 inhibits proliferation and induced apoptosis in CRC cells in vitro and in vivo. ICG001 exerts its anticancer action, at least in part, by inhibiting eIF3D-mediated PI3K/AKT signaling pathways. Our findings suggest that ICG001 is a novel inhibitor of eIF3D with great potential for the treatment of CRC and other tumors.

Disclosure of conflict of interest

None.

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