Oral administration of FAK inhibitor TAE226 inhibits the progression of peritoneal dissemination of colorectal cancer

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Abstract

Peritoneal dissemination is one of the most terrible types of colorectal cancer progression. Focal adhesion kinase (FAK) plays a crucial role in the biological processes of cancer, such as cell attachment, migration, proliferation and survival, all of which are essential for the progression of peritoneal dissemination. Since we and other groups have reported that the inhibition of FAK activity exhibited a potent anticancer effect in several cancer models, we hypothesized that TAE226, a novel ATP-competitive tyrosine kinase inhibitor designed to target FAK, can prevent the occurrence and progression of peritoneal dissemination. *In vitro,* TAE226 greatly inhibited the proliferation and migration of HCT116 colon cancer cells, while their adhesion on the matrix surface was minimally inhibited when FAK activity and expression was suppressed by TAE226 and siRNA. *In vivo*, when HCT116 cells were intraperitoneally inoculated in mice, the cells could attach to the peritoneum and begin to grow within 24 h regardless of the pretreatment of cells with TAE226 or FAK-siRNA, suggesting that FAK is not essential, at least for the initial integrin-matrix contact. Interestingly, the treatment of mice before and after inoculation significantly suppressed cell attachment to the peritoneum. Furthermore, oral administration of TAE226 greatly reduced the size of disseminated tumors and prolonged survival in tumor-bearing mice. Taken together, a possible strategy for inhibiting peritoneal dissemination by targeting FAK with TAE226 appears to be applicable through anti-proliferative

and anti-invasion/anti-migration mechanisms.

Key words: focal adhesion kinase; TAE226; peritoneal dissemination; prolonged survival; anti-proliferation; colon cancer

1. Introduction

Peritoneal dissemination is one of the worst progression states of all gastrointestinal malignancies. Although improved surgical, chemotherapy and radiotherapy methods have been developed, peritoneal dissemination still takes place [1], especially in cases of colorectal cancer. In order to overcome this terrible abdominal malignancy, there is an urgent need to clarify the molecular mechanism of peritoneal dissemination and to identify agents that specifically prevent these mechanisms [2].

Peritoneal dissemination is a complex multistep process, but can be divided into three basic stages: the detachment of cancer cells from the primary tumor; attachment to the peritoneal surface in the abdominal cavity; growth, migration and invasion within the peritoneum [3]. Many adhesion molecules, matrix proteases, motility factors and angiogenic factors are involved in this process and so far no specific targeting therapy has been developed.

Focal adhesion kinase (FAK) is a 125-kDa non-receptor and non-membrane protein tyrosine kinase that is the canonical mediator of integrin signal pathways [4]. Growth factors or clustering of integrins facilitate the rapid phosphorylation of FAK at Tyr397 followed by subsequent full catalytic FAK activation [4, 5]. Activated FAK mediates several signaling pathways which are involved in cell adhesion, growth, proliferation, migration, survival and

angiogenesis, all of which are often dysfunctional in tumor cells [6, 7].

Researchers found that FAK mRNA was up-regulated in invasive and metastatic human tumor samples [8]. In fact, numerous studies have reported FAK overexpression in various tumor cells, including malignant tumors of the pancreas, ovary, cervix, bone, kidney, lung, prostate, brain, skin, thyroid, colon, head and neck, as well as in cases of acute myeloid leukemia [7]. Additionally, the overexpression of FAK correlates with increased tumor malignancy. Intense expression of FAK was detected by immunohistochemistry in colorectal tumors and colon cancer cell lines [9]. Therefore, we hypothesized that FAK may play a central role in cell attachment, migration, invasion, cell proliferation and angiogenesis during the process of peritoneal dissemination.

TAE226 is a novel ATP-competitive tyrosine kinase small-molecule inhibitor designed to target FAK and can effectively prevent FAK phosphorylation [10]. It has shown potent anticancer effects *in vitro* and *in vivo* in several types of malignancies [10-15].

Based on this background research, in the present study we examined the anti-tumor effect of TAE226 on colon cancer cells, especially focusing on whether TAE226 prevents the occurrence and progression of peritoneal dissemination using a mouse model in which HCT116 colon cancer cells were intraperitoneally inoculated to develop disseminated tumors in the peritoneum.

2. Materials and Methods

2.1. Cell lines and cultures

The colon carcinoma cell line HCT116 was cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gemini Biologicals, West Sacramento, CA, USA), 100 units/ml of penicillin G sodium, 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained in a monolayer culture at 37°C in humidified air with 5% CO_2. Three other colorectal cancer cell lines (RPMI4788, LoVo and DLD-1) were also used to assess their reproducibility in animal experiments.

2.2. Reagents

TAE226, a tyrosine kinase inhibitor of FAK, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland) through a materials transfer agreement with Okayama University. Stock solutions of the compound were reconstituted with dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and diluted with culture media before use. The final DMSO concentration in all cultures was 0.025%. For oral administration to mice, TAE226 was dissolved in 0.5% methylcellulose as a solvent to make a final concentration of 10 mg/ml.

2.3. Cell proliferation assay

Cell viability was assessed using the cell proliferation reagent WST-1 (Roche, Mannheim, Germany). After treatment with TAE226 for 48 h, WST-1 reagent was added to the cells and incubated for 1 to 4 h at 37°C. The absorbance (420-480 nm) of the samples against a background control was measured by microplate reader (Thermo Scientific, Rockford, IL, USA). Dose effect plots were created to calculate the IC_{50} of TAE226 using Calcusyn software (Biosoft).

2.4. Cell adhesion assay

Diluted rat tail collagen-I and human fibronectin (BD Biosciences, Franklin Lakes, NJ, USA) were coated to a 96-well plate, followed by blocking with 5% bovine serum albumin (BSA) for 1 h and rinsing with phosphate buffered saline (PBS) before use. Pretreated HCT116 cells with TAE226 (0-10 μM) were suspended in a serum-free RPMI-1640 medium supplemented with 0.5% BSA, and a cell suspension with either TAE226 or DMSO was added to the pre-coated plate and incubated for 1 h. After non-adherent cells were washed away with PBS, WST-1 reagent was added to the plate to evaluate the number of attached cells on the matrix.

2.5. Cell migration assay

A migration assay was performed using a cell culture insertion system (BD Falcon, San Jose, California, USA) [16]. Inserts with 8μm pore membranes were placed in a 24-well plate containing RPMI-1640 medium, in which 10% FBS stimulated cell migration as a chemotactic factor. HCT116 cells (30×10^4/insert) suspended in a serum-free medium with TAE226 were added to the top of the insert. After 12 h, the cells that did not migrate were removed by swabbing, and the cells that migrated to the lower surfaces of the inserts were fixed in methanol and stained using a Diff-Quick kit. The migrated cells were quantified under the microscope (20 ×magnification) by counting from five individual fields of each membrane.

2.6. FAK small interference RNA transfection

The FAK-siRNA (sense strand 5'-GAAUCAGUUACCUAACGGACA-3'; anti-sense strand 5'-UCCGUUAGGUAACUGAUUCCU-3') were synthesized by Sigma-Aldrich (St. Louis, MO, USA). Scrambled RNA duplexes (5'-GUACCGCACGUCAUUCGUAUC-3' and 5'-UACGAAUGACGUGCGGUACGU-3') bearing no sequence homology with any known human mRNA sequences were used as a control. Double stranded RNAs were transfected into cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The transfected cells were used for further experiments.

2.7. Western blot analysis

Cells treated with TAE226 or siRNA were lysed with a protein lysis buffer (M-PER, mammalian protein extraction reagent; Thermo Scientific, Rockford, IL, USA), and the protein concentration was determined using a BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels. The separated proteins were then transferred onto PVDF filter membranes (GE Healthcare, Buckinghamshire, UK) and incubated with primary antibodies at 4°C overnight, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 h. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was used for signal detection. The antibodies used for western blot analysis were the following: anti-human phosphorylated-FAK (Tyr397; BD Biosciences, Franklin Lakes, NJ, USA), anti-human total FAK (BD Biosciences); anti-actin (Sigma-Aldrich); Peroxidase-conjugated affinipure goat anti-mouse IgG and anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA).

2.8. Animal experiments

BALB/cAJc1-nu/nu 6-week-old male mice derived from C.B-17/Icr (Clea, Tokyo,

Japan) were used in this study. The mice were maintained under pathogen-free conditions and the mouse experiments were carried out in accordance with the standards and guidelines of the Department of Animal Resources at Okayama University and followed "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985). The anesthetized mice were subjected to animal models bearing disseminated tumors in the abdominal cavity by injecting $5x10^6$ cells for the following experiments: 1) transiently transduced cells with either FAK-siRNA or control-siRNA were injected, and the mice were sacrificed 48 h after injection to obtain the peritoneum, 2) cells pretreated with TAE226 for 2 h were injected, and the mice were sacrificed 48 h after injection to obtain the peritoneum, 3) the cells were injected into mice which received an oral administration of 100 mg/kg of TAE226 for 2 days before the cell injection and another 2-day administration thereafter, and then the mice were sacrificed to obtain the peritoneum, 4) the cells were injected and from the next day, TAE226 (60 mg/kg) or methylcellulose as a vehicle was orally administered once a day and continued for 14 days, and then the mice were sacrificed to obtain the peritoneum, 5) the cancer cells were injected and from the next day the mice were treated with a 5-day course of TAE226 (60 mg/kg) followed by 2-days off (no administration). The treatment continued up to 4 courses and then follow-up was done to track their survival periods.

2.9. Immunofluorescence Staining

Surgically resected tumor tissues from the mice were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen in liquid nitrogen. Fresh frozen sections of 6 μm were dried and fixed with acetone for 10 min. After washing with PBS, the sections were blocked with normal goat serum for 10 min followed by incubation with a primary antibody for phosphorylated-FAK (Tyr397; Abcam, Cambridge, MA, USA) for 60 min. After washing, the sections were subsequently reacted with goat anti-mouse IgG antibody labeled with FITC (ICN Pharmaceuticals, Costa Mesa, CA, USA) for 1 h. Propidium iodide was used for counterstaining. The slides were mounted with Perma-flour (Thermo Scientific) and examined under fluorescence microscopy. All procedures were done at room temperature.

2.10. Statistical analysis

Student's t-test was used to compare data between two groups. Data represent the mean ± SD. Overall survival was calculated using the Kaplan-Meier method and compared by the log-rank test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of TAE226 on FAK activity and proliferation of HCT116 colon cancer cells

Firstly, among four colorectal cancer cell lines (HCT116, RPMI4788, LoVo, and DLD-1), we found that HCT116 cells had the highest expression and activation of FAK (data not shown). When HCT116 cells were treated with TAE226 for 1 h, the inhibition of FAK phosphorylation at Tyr397 was noted at 1 μM and maximal inhibition was reached at 5 μM (Fig. 1A), without affecting the expression of FAK (Fig. 1A).

Next, a 48 h exposure to different concentrations of TAE226 induced a dose-dependent inhibition of cell proliferation in HCT116 cells (Fig. 1B). The 48 h treatment with TAE226 resulted in a 48.6% growth inhibition at a concentration of 0.1 μM and a 80.3% inhibition at 1 μM. The IC_{50} of TAE226 in HCT116 cells was 0.2185 μM（r=0.9890).

3.2. Effects of TAE226 on cell attachment and migration in vitro

FAK is intimately involved in the coordination of adhesion formation and turnover which regulates cell movement [17]. The migration of HCT116 cells was significantly inhibited by TAE226 in a dose-dependent manner (Fig. 1C), while the attachment of cells to the matrix was only minimally suppressed (Fig. 1D). Even the pretreatment of HCT116 cells with different

concentrations of TAE226 did not improve the inhibitory effect on cell attachment (data not shown). Although treatment with 5 μM of TAE226 significantly decreased the number of cells migrating through Boyden chambers with a 56.85%-reduction rate (Fig. 1C), TAE226 showed a minimal inhibitory effect on the attachment of HCT116 cells.

3.3. Silencing of FAK by siRNA had no effect on cell attachment in vitro and in vivo

Our data showed that the inhibition of FAK activity by TAE226 had a minimal effect on the attachment of HCT116 cells. Therefore, we wanted to explore whether the suppression of FAK expression by FAK-siRNA affected the HCT116 cell attachment. Western blot analysis showed that transfection with FAK-siRNA induced a significant decrease of FAK expression (Fig. 2A). However, the cell adhesion assay result indicated that the knockdown of FAK expression did not affect cell attachment to the matrix (Fig. 2B). Furthermore, when siRNA-transduced HCT116 cells were inoculated into the abdomen of mice, the silencing of FAK expression did not affect cancer cell attachment on the peritoneum and even began to grow equally to the control (Fig. 2C and D), indicating that the activity and expression of FAK is not so essential for physical cell attachment.

3.4. Effects of TAE226 on cell attachment in vivo

Since TAE226 demonstrated its potent inhibitory effects on cell proliferation and migration, our next question was whether the progression of peritoneal dissemination could be suppressed by TAE226. We first pretreated HCT116 cells with 10μM of TAE226 for 2 h, enough to suppress FAK activity, and subsequently inoculated those pretreated cells into abdomen of mice. Similar to the experiment using siRNA, the *in vitro* pretreatment of HCT116 cells with TAE226 did not show any apparent difference compared to the control treatment (DMSO) (Fig. 3A and B). On the other hand, when TAE226 was orally administered to the mice from 2 days before the inoculation and thereafter, the number of peritoneum-attached cells was significantly reduced (68%-reduction from control, p=0.004668; Fig. 3C and D). Since we could observe a similar reduction of developing peritoneal dissemination using other colorectal cancer cell lines (RPMI4788, LoVo and DLD-1, Supplementary Fig. S1), these reproducible data tempted us to consider that systemically saturating TAE226 in a mouse body through oral administration can increase the negative regulation of the progression of peritoneal dissemination.

3.5. In vivo effect of TAE226 on the development of peritoneal disseminated tumors

As we described above and [14, 15], TAE226 inhibited cancer growth and migration *in vitro* and *in vivo*, whereas it cannot fully suppress cell attachment or the occurrence of

peritoneal dissemination. Hence, we hypothesized that the best practice of TAE226 treatment is to reduce the progression of peritoneal dissemination, leading to prolonged survival, since disseminated tumor-bearing patients often have short survivals regardless of receiving multidisciplinary treatment. In order to prove this hypothesis, we extended the treatment periods with TAE226 and monitored the survival of treated mice with disseminated tumors. Fourteen-day treatment with TAE226 apparently reduced the size of disseminated tumors (Fig 4A and B) accompanied by a reduction of FAK activity (Fig. 4C), and the tumor area was significantly diminished (control vs TAE226 = 100.0 ± 20.80 vs 20.4 ± 9.80, p=0.000678; Fig 4D). Further extension of TAE226 treatment significantly prolonged the living periods of disseminated tumor-bearing mice (median survival time; control: 26.5 days, TAE226: 36.5 days, *p*=0.00082; Fig. 4E), indicating that targeting FAK by the oral administration of TAE226 can be a potential application for negatively controlling peritoneal carcinomatosis.

4. Discussion

FAK has been linked to cell adhesion, migration, proliferation, survival and angiogenesis, all of which are crucial for the progression of peritoneal dissemination. In this study, we found that TAE226 can significantly inhibit cell proliferation and migration, while the effect on inhibiting cell attachment appeared to be modest. Since several papers reported that focal adhesions can still form in FAK-deficient cells [18,19], it was reasonable to believe that the treatment with TAE226 or siRNA still allowed HCT116 cells to achieve initial cell attachment on the matrix. Hence, at the nascent focal adhesions, clustered integrins recruit FAK and FAK subsequently facilitates the maturation of focal adhesions [19]. Based upon this fact, the intrinsic value of the suppression the FAK activity by TAE226 is the inhibition of cell migration and subsequent proliferation, as we demonstrated above.

It is noteworthy that pretreatment of cancer cells by TAE226 did not affect their attachment to the peritoneum (Fig. 3A, B). However, the treatment of mice before and after inoculation reduced cell attachment to the peritoneum (Fig. 3C, D). This seems to be a contradiction. Of course it is possible that the TAE226 pretreated cells might recover their FAK activity during cell transplant to the peritoneum, however, as we mentioned above, we assume that FAK may not be essential for initial cell attachment, considering the result of the siRNA

experiments (Fig. 2). In addition, the pretreatment of mice with TAE226 might have provided a change of microenvironment which was unsuitable for cancer cells to attach, migrate and proliferate. Some papers described that the tumor microenvironment is a major part of the most important circumstances in cancer progression [20, 21]. Indeed, FAK is overexpressed not only in tumor tissues but in tumor-associated stromal cells, thus systemically saturating a mouse body with TAE226 could be a dual inhibition of FAK signaling in both cancer cells and tumor environment, which may be a unique and reasonable approach as a novel cancer treatment.

Controlling peritoneal dissemination is very difficult with existing therapeutic modalities, resulting in a poor outcome in general. One of the reasons is that generally disseminated tumors spread widely in the abdomen, which makes it difficult to target cancer cells without damaging normal abdominal tissues. On the other hand, stromal tissues adjacent to disseminated tumors are also involved in the progression of dissemination with the activation of some key molecules, including FAK. Thus, a systemic suppression of FAK activity by saturation with TAE226 enables a bidirectional negative regulation of dissemination as well as invasion and metastasis, possibly leading to better outcomes for tumor-bearing patients.

In conclusion, systemic saturation with TAE226 potently reduced the progression of peritoneal dissemination with prolonged survival, thus targeting FAK could be a potential alternative for advanced cancer treatment.

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Reference

[1] H. Mima, S, Yamamoto, M. Ito, et al., Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector, Mol. Cancer. Ther. 5(2006) 1021-1028.

[2] K. Hashimoto, K. Morishige, K. Sawada, et al., Alendronate inhibits intraperitoneal dissemination in in vivo ovarian cancer model, Cancer. Res. 65 (2005) 540-545.

[3] Y. Yonemura, Y. Endo, T. Obata, T. Sasaki, Recent advances in the treatment of peritoneal dissemination of gastrointestinal cancers by nucleoside antimetabolites, Cancer. Sci. 98 (2007) 11-8.

[4] J.T. Parsons, Focal adhesion kinase: the first ten years, J. Cell. Sci. 116 (2003) 1409-1416.

[5] M.D. Schaller, Biochemical signals and biological responseselicited by the focal adhesion Kinase, Biochim. Biophys. Acta. 1540 (2001) 1-21.

[6] V.M. Golubovskaya, Focal adhesion kinase as a cancer therapy target, Anticancer. Agents. Med. Chem. 10 (2010) 735-741.

[7] V.M. Golubovskaya, F.A. Kweh, W.G. Cance, Focal adhesionkinase and cancer, Histol. Histopathol. 24 (2009) 503-510.

[8] L.V. Owens, L. Xu, R.J. Craven, et al., Overexpression of the focal adhesion kinase

(p125FAK) in invasive human tumors, Cancer. Research. 55 (1995) 2752-2755.

[9] T. Murata , Y. Naomoto, T. Yamatsuji, et al., Localization of FAK is related with colorectal carcinogenesis, Int. J. Oncol. 32 (2008) 791-796.

[10] T.J. Liu, T. LaFortune, T. Honda, et al, Inhibition of both focal adhesion kinase and insulin-like growth factor-I receptor kinase suppresses glioma proliferation in vitro and in vivo, Mol. Cancer. Ther. 6 (2007) 1357-1367.

[11] J. Halder, Y.G. Lin, W.M. Merritt, et al., Therapeutic efficacy of a novel focal adhesion kinase inhibitor TAE226 in ovarian carcinoma, Cancer. Res. 67 (2007) 10976-10983.

[12] Q. Shi, A.B. Hjelmeland, S.T. Keir, et al., A novel low-molecular weight inhibitor of focal adhesion kinase, TAE226, inhibits glioma growth, Mol. Carcinog. 46 (2007) 488-496.

[13] V.M. Golubovskaya, C. Virnig, W.G. Cance, TAE226-induced apoptosis in breast cancer cells with overexpressed Src or EGFR, Mol. Carcinog. 47 (2008) 222-234.

[14] N. Watanabe, M. Takaoka, K. Sakurama, et al., Dual-tyrosine kinase inhibitor for focal adhesion kinase and insulin-like growth factor-I receptor exhibits anti-cancer effect in esophageal adeno carcinoma in vitro and in vivo,Clin. Cancer. Res. 14 (2008) 4631-4639.

[15] K. Sakurama, K. Noma, M. Takaoka, et al., Inhibition of focal adhesion kinase as a potential therapeutic strategy for imatinib-resistant gastrointestinal stromal tumor, Mol. Cancer. Ther. 8(2009) 127-134.

[16] T.B. Deramaudt, M. Takoka, R. Upadhyay, et al., N-cadherin and keratinocyte growth factor receptor mediate the functional interplay between Ki-RASG12V and p53V143A in promoting pancreatic cell migration, invasion, and tissue architecture disruption, Mol. Cell. Biol. 2006 26 (2006) 4185-4200.

[17] J.T. Parsons, A.R. Horwitz, M.A. Schwartz, Cell adhesion: integrating cytoskeletal dynamics and cellular tension, Nat. Rev. Mol. Cell. Biol.11 (2010) 633-643.

[18] D. Ilic, Y. Furuta, S. Kanazawa, et al., Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice, Nature. 377 (1995) 539-544.

[19] C. Lawson, S.T. Lim, S. Uryu, et al., FAK promotes recruitment of talin to nascent adhesions to control cell motility, J. Cell. Biol. 196(2012) 223-232.

[20] J.W. Franses, A.B. Baker, V.C. Chitalia, et al., Stromal endothelial cells directly influence cancer progression, Sci. Transl. Med. 3(2011) 66ra 5.

[21] A.C. Shieh, H.A. Rozansky, B. Hinz, M.A. Swartz, Tumor cell invasion is promoted by interstitial flow-induced matrix priming by strolmal fibroblast, Cancer. Res. 71 (2011) 790-800.

Figure legends

Fig.1. Effects of TAE226 on FAK activity, proliferation, migration and adhesion of colon cancer cells. A, The activation of FAK was suppressed by TAE226 treatment in 1 h in a dose-dependent manner. B, TAE226 treated HCT116 cells for 48 h with different concentrations. The cell viability of HCT116 cells treated with different concentrations of TAE226 was measured by WST-1 cell proliferation assay. C, A migration assay was performed using a cell culture insertion system. The migrated cells were stained and quantified. D, A cell adhesion assay was performed using a 96-well plate coated with type I collagen and fibronectin. After washing the non-adherent cells, attached cells were evaluated by measuring the absorbance of WST-1. Each column is given as a percentage of live, migrated or attached cells compared with the control (treatment with DMSO alone), which was set as 100%. Error bars represent the mean ± SD. *$p < 0.05$ vs. control.

Fig.2. Silencing of FAK expression by siRNA reveals the minimal inhibitory effect on cell attachment *in vitro* and *in vivo*. A, HCT116 cells were transfected with FAK-siRNA and control siRNA for 48 h and the cell lysates were prepared for western blot analysis. B, The ability of *in vitro* cell adhesion on collagen-I was compared between FAK-siRNA transduced HCT116 cells and control cells with scrambled siRNA. C, HCT116 cells transfected with siRNA were

inoculated in the abdomen and the peritoneal tissues were collected 48h after inoculation for the detection of peritoneal dissemination. Arrows in the H-E staining pictures indicate the cancer cells attached to the peritoneum. Scale bar: 200 μm. D, Peritoneum-attached cells were quantified in 10 randomly selected high power fields (40x) to draw histograms. Each column is given as a percentage of attached cells compared with the control, which was set as 100%. Error bars represent the mean ± SD.

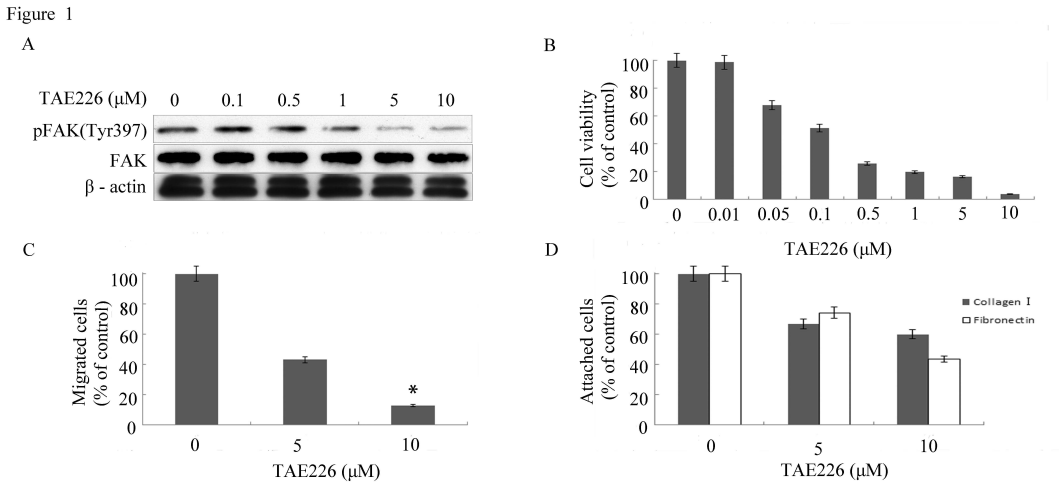
Fig.3. Systemic saturation of host animals with TAE226 exerts its anti-dissemination effect. A and B, HCT116 cells were pretreated with either 10μM of TAE226 or DMSO for 2 h before intraperitoneal inoculation. Peritoneum tissues were collected 48 h after inoculation, in order to detect the disseminated cancer cells by H-E staining (A) and to compare the number of peritoneum-attached cells between TAE226-pretreatment and control (DMSO-pretreatment) (B). C and D, mice were treated with 100 mg/kg of TAE226 before and after the intraperitoneal inoculation of untreated HCT116 cells and peritoneum tissues were collected 48 h after inoculation. The disseminated cancer cells were detected by H-E staining (C) and were quantified to compare the ability of HCT116 cells to attach on the peritoneum between TAE226 administration and control (methylcellulose administration) (D). Arrows in pictures A and C indicate the peritoneum-attached cancer cells. Scale bar: 200 μm. Each column in B and D is given as a percentage of attached cells (control was set as 100%), by which the numbers of

attached cells were counted in 10 randomly selected high power fields (40x) per slide. Error bars represent the mean ± SD. *$p < 0.005$ vs. control.

Fig.4. Oral administration of TAE226 contributes to reducing peritoneal dissemination and the prolonged survival of tumor-bearing mice. Nude mice were orally administrated with TAE226 (60 mg/kg) or methylcellulose after intraperitoneal inoculation with HCT116 cells. A, Macroscopic view of the peritoneal cavities at the time of sacrifice was take after a 14-day administration of TAE226. Arrows indicate disseminated tumors. B, Collected peritoneum tissues were subjected to H-E staining. Scale bar: 500 μm. C, The expression of phosphorylated FAK (green) was detected in the tissues taken from the mice treated with either methylcellulose (left panel) or TAE226 (right panel). Propidium iodide staining (red) was given as a counterstain for the nuclei. Scale bar: 200 μm. D, The area of disseminated tumor in 5 randomly selected fields of H-E stained section from each mouse was measured using computer software (Image J). The variance of tumor area between the control and TAE226 treated group was statistically analyzed by drawing histogram. Each column is given as a percentage of tumor area comparing with the control, which was set as 100%. Error bars represent the mean ± SD. *p=0.000678. E, Survival periods were tracked to examine the advantage of TAE226 treatment and a survival curve was drawn by the Kaplan-Meier method.

Supplementary Fig. S1. Oral administration of TAE226 represents a similar inhibitory effect on the development of peritoneal dissemination using other colorectal cancer cells. Mice were treated with 100 mg/kg of TAE226 before and after intraperitoneal inoculation of untreated RPMI4788, LoVo and DLD-1 cells and peritoneum tissues were collected 48 h after inoculation. The disseminated cells were detected by H-E staining (A) and were quantified to compare the ability for cancer cells to attach on the peritoneum between TAE226 administration and methylcellulose only as a control (B-D). Arrows in the H-E staining pictures indicate peritoneum-attached cancer cells. Scale bar: 200 μm. Each column in B, C and D is given as a percentage of attached cells (control was set as 100%), by which the numbers of attached cells were counted in 10 randomly selected high power fields (40x) per slide. Error bars represent the mean ± SD. The reduction of peritoneum-attached cells from control was 68% (RPMI, *p=0.004064, B), 41% (LoVo, **p=0.011297, C) and 51% (DLD-1, *+p=0.020808, D).

Figure 1


A
TAE226 (μM) 0 0.1 0.5 1 5 10
pFAK(Tyr397)
FAK
β - actin
B
Cell viability (% of control)
100
80
60
40
20
0
0 0.01 0.05 0.1 0.5 1 5 10
TAE226 (μM)
C
Migrated cells (% of control)
100
80
60
40
20
0
*
0 5 10
TAE226 (μM)
D
Attached cells (% of control)
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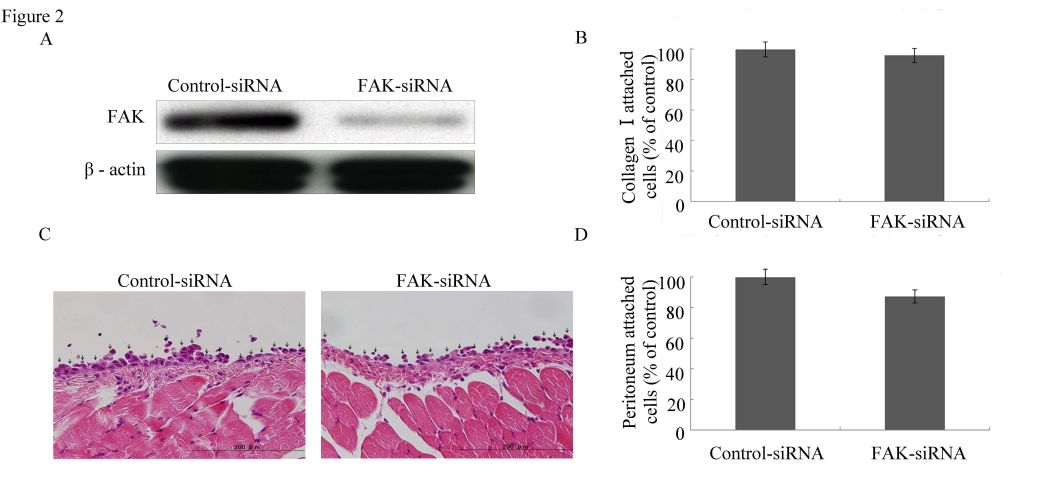
80
60
40
20
0
Collagen I
Fibronectin
0 5 10
TAE226 (μM)

Figure 2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Control-siRNA
FAK-siRNA
FAK
β - actin
B
Collagen I attached cells (% of control)
100
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Control-siRNA
FAK-siRNA
C
Control-siRNA
FAK-siRNA
D
Peritoneum attached cells (% of control)
100
80
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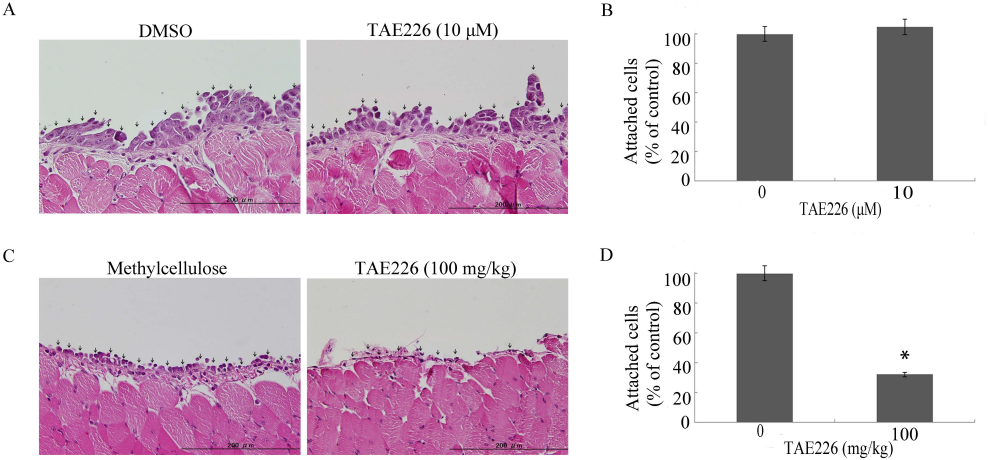
Control-siRNA
FAK-siRNA

Figure 3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DMSO
TAE226 (10 μM)
B
Attached cells
(% of control)
100
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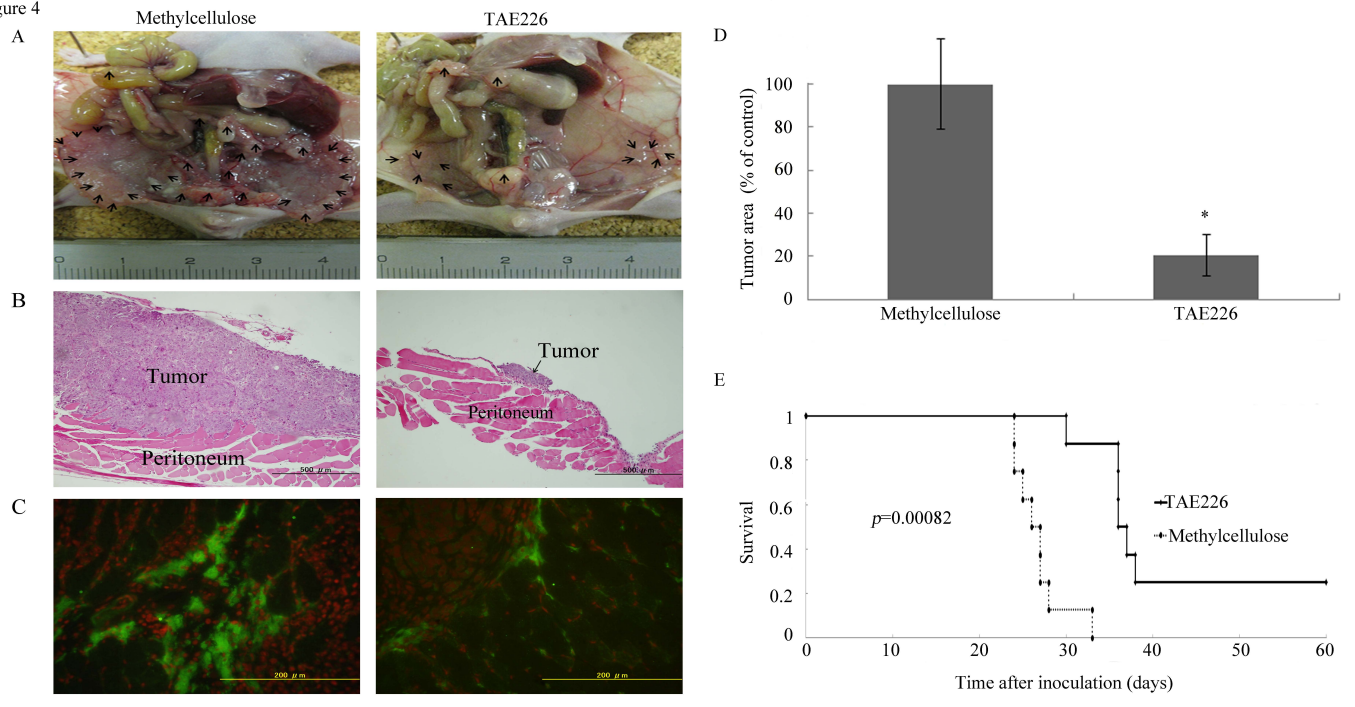
Methylcellulose
TAE226 (100 mg/kg)
D
Attached cells
(% of control)
100
80
60
40
20
0
*
0
100
TAE226 (mg/kg)

Figure 4


A
Methylcellulose
TAE226
B
Tumor
Peritoneum
500 μm
Tumor
Peritoneum
500 μm
C
200 μm
200 μm
D
Tumor area (% of control)
100
80
60
40
20
0
*
Methylcellulose
TAE226
E
Survival
1
0.8
0.6
0.4
0.2
0
p=0.00082
TAE226
Methylcellulose
0
10
20
30
40
50
60
Time after inoculation (days)

Supplementary Fig.S1