hyde (CNA), methyl cinnamate, 2-aminoethoxydiphenyl borate (2-APB), 4α-phorbol 12,13-didecanoate (4α-PDD), H-89, calphostatin C, LY294002, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Aldrich (St. Louis, MO). HAS and hydroxy-β-sanshool (HBS) were extracted from Japanese pepper at Tsumura and Co. with purities greater than 97.9%. Xanthoxylin (Tokyo Chemical Industry, Tokyo), butorphanol (Bristol-Myers Squibb, New York), HC-030031 (Biomol International, Plymouth Meeting, PA), and *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)-tetrahydropyrazine-1(2H) carboxamide (BCTC; Biomol International) purchased for the study as well the other reagents used for analysis were the highest purity commercially available.

Animals. Seven-week-old male Sprague-Dawley rats weighing 210–230 g were purchased from Japan SLC (Shizuoka, Japan). The animals were allowed free access to water and standard laboratory food, and housed at a temperature of 23 ± 2°C with relative humidity of 55 ± 10%, and a 12:12-h light/dark cycle with lights on from 0700–1900 daily. All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Asahikawa Medical University or Tsumura and Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Asahikawa Medical University or Tsumura and Co. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurement of intestinal blood flow. Jejunal blood flow was measured by a laser-Doppler flowmeter (ALF21N, Advance, Tokyo) as previously described (30). Briefly, rats were anesthetized with urethane (900 mg/kg ip), α-chloralose (45 mg/kg ip), and butorphanol (1 mg/kg im). A tracheotomy was performed and the rats were artificially ventilated. The left cervical artery was cannulated and connected to a transducer (P23XL, Nihon Kohden, Tokyo) to monitor systemic arterial blood pressure (AP) and heart rate (HR). Body temperature was maintained at 37 ± 0.5°C by a heating pad. After exposing the small intestine by a midline laparotomy, a cannula was inserted into the duodenum to facilitate injection of the test sample. A fiber optic probe was positioned 4 mm above the surface of the midjejunum. Vascular conductance (VC), calculated as the quotient of mean blood flow divided by mean AP, was used as an index of IBF.

Antagonist and antibody studies in vivo. Rabbit polyclonal IgG (50 μg/kg) against rat ADM (Peninsula Laboratory, Belmont, CA), rabbit IgG as an isotype-matched control (Abcam, Cambridge, UK), or the TRPV1 antagonist BCTC (10 mg/kg) was injected at a volume of 1 ml/kg through a polyethylene tube cannulated into the right jugular vein after confirming stable blood flow. TU-100 or a related vasodilator was administered intraduodenally 15 min later. The TRPA1 antagonist HC-030031 prepared in 1% DMSO was administered into the lumen at 1 mg·5 ml $^{-1}$·kg $^{-1}$ together with the test sample.

Quantitation of ADM. Plasma ADM levels were assayed using enzyme immunoassay (EIA) kits specific for rat ADM according to the procedure provided by the manufacturer (Phoenix Pharmaceuticals, Burlingame, CA). Briefly, 5 ml blood was collected from the portal vein at 15, 30, 60, and 120 min after administration of TU-100 (2,700 mg/kg), and plasma was separated immediately. The plasma was then applied to ADM extraction using a C18 Sep-Column. The detection limit for ADM was 10 pg/ml. ADM release was assayed using an IEC-6 rat intestinal epithelial cell line (DS Pharmaceuticals, Osaka, Japan). IEC-6 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mmol/l HEPES. Cells between the 30th and 37th passage were plated in 96-well flat-bottom microtiter plates at 2 × 10^4 cells/well in DMEM supplemented with the same additives as described above, allowed to settle overnight, and then culture fluids were replaced with HBSS containing 0.1% BSA, 0.1–0.3% DMSO. TU-100 was added to the culture after being passed through a 0.45-μm filter. Cells were incubated for 6 h, and ADM in the culture fluids was quantified using EIA kits specific for rat ADM. To investigate functional expression of TRPA1 in IEC-6 cells, the cell was exposed to the TRPA1-selective antagonist HC-030031 (100 μmol/l) 30 min before addition of TRPA1 activators.

Preparation of IE cells from small intestine. Segments of the small intestine were everted, end-ligated, and preincubated in HBSS containing 1 mmol/l DTT and 10% FBS to remove mucus. The sacs were then incubated for 10 min at 37°C in chelating digestive buffer (70 mmol/l NaCl, 5 mmol/l KCl, 20 mmol/l $NaHCO_3$, 0.5 mmol/l NaH_2PO_4, 1 mmol/l Na_2HPO_4, 50 mmol/l HEPES, 11 mmol/l glucose, 1 mmol/l EDTA, 0.5% BSA, and 0.05 mmol/l DTT), followed by collection of the supernatant. The incubation was repeated twice, and the supernatants of each were pooled. The cell pellets obtained by centrifugation at 300 *g* for 10 min were suspended in 0.1% BSA HBSS and passed through a nylon mesh filter. The cell suspension was applied to a 25% gradient of Percoll (GE Healthcare, Piscataway, NJ). After centrifugation at 710 *g* for 30 min, the interface containing enriched IE cells was collected. IE cells were separated into negative fractions using a BD IMag cell separation system (BD Biosciences, San Jose, CA) with rabbit anti-nerve growth factor receptor p75 antibody (Millipore, Bedford, MA), followed by biotinylated anti-rabbit Ig (BD Bioscience) and biotinylated anti-CD45 antibody (clone, OX-1; BD Bioscience), and thereafter incubated with streptavidin-labeled magnetic beads. Further, purified IE cells were stained with various cell-marker antibodies following a cytospin. Antibodies and positive cell percentages were wide cross-reactivity anti-cytokeratin (DAKO, Carpinteria, CA) at ≥90%, and anti-E-cadherin (clone, 36/E-cadherin; BD Bioscience) at ≥95%. Positive staining with anti-CD45 (clone, OX-1; BD Bioscience), anti-PGP9.5 (clone, 13C4/I3C4; Abcam), or anti-GFAP (clone, GF12.24; Progen, Heidelberg, Germany) was not detected.

Gene expression. The pellets of IEC-6 cells, enriched IE cells obtained from the small intestines, and L1 to L6 dorsal root ganglia (DRG) isolated from normal rats were homogenized in QIAzol reagent (Qiagen, Valencia, CA), and total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's recommendations. The respective cDNA was prepared using a high-capacity RT kit (Applied Biosystems, Warrington, UK). The sequences of the sense and antisense primers for rat TRPA1 were 5′-TTTGCCGCCAGCTATGGGCG-3′ and 5′-TGCTGCCAGATGGAGAGGGGT-3′ to obtain a 117-bp product. Those for rat TRPV1 were 5′-GGTGTGCCTGCACCTAGC-3′ and 5′-CTCTTGGGGTGGGGACTC-3′ to obtain a 107-bp product. Those for rat ADM were 5′-CTCGACACTTCCTCGCAGTT-3′ and 5′-GCTGGAGCTGAGTGTGTCTG-3′ to obtain a 446-bp product. Those for rat β-actin were 5′-CCTGGGTATGGAATCCTGTGGCAT-3′ and 5′-GGAGCAATGATCTTGATCTTC-3′ to obtain a 198-bp product. An aliquot of the RT reaction product served as a template in 30 cycles with 10 s of denaturation at 98°C, 30 s of annealing at 60°C, and 30 s of extension at 68°C using the DNA polymerase KOD FX (TOYOBO, Osaka, Japan). A portion of the PCR mixture was electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer (pH 8.0), and the gel was stained with ethidium bromide and imaged on a Typhoon 9410 imager (GE Healthcare). Sample-to-sample variation in RNA loading was controlled by comparison with β-actin.

Flow cytometry. Single cells were suspended in Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C, washed, and then preincubated for 5 min at 4°C with goat polyclonal IgG antibody (Abcam) to reduce nonspecific binding of antibodies. Next, cells were incubated for 20 min at 4°C with rabbit polyclonal IgG antibody (4 μg/ml) against rat ADM, rat TRPA1 (Abcam), TRPV1 (Alomone Labs, Jerusalem, Israel), or isotype control IgG (Abcam). Cells were washed, incubated for 20 min with the Alexa Fluor 488-labeled goat polyclonal antibody against rabbit IgG (Invitrogen, Carlsbad, CA), and subjected to flow cytometry analysis using a FACScalibur analyzer and CellQuest Pro software (BD Biosciences). In some experiments, a control peptide for TRPA1 or TRPV1 (Abcam) was added at 4 μg/ml with antigen-specific antibody.

Calcium influx in rat TRPA1-transfected cells. A rat TRPA1-expressing cell line was generated using a tetracycline-inducible T-Rex expression system (Life Technologies, Grand Island, NY). T-Rex293 cell (Life Technologies) was transfected stably with plasmids encoding rat TRPA1 (pcDNA4/TO-rat TRPA1) using FuGENE HD Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. Control cell was transfected with the pcDNA4/TO vector alone. Intracellular calcium was measured 1 day after induction with tetracycline (1 μg/ml). Cells were washed with an assay buffer (115 mmol/l NaCl, 5.4 mmol/l KCl, 13.8 mmol/l glucose, 2.5 mmol/l probenecid, 20 mmol/l HEPES, pH 7.6) and then loaded with Fluo-4 dye (Dojindo, Kumamoto, Japan). After 30 min incubation, cells were washed with the assay buffer. Then the test compound was added to each well. Fluorescence intensity was measure by FlexStation3 (Molecular Devices, Sunnyvale, CA). Concentration-response curves were fitted using Prism 3.0 with a Hill equation model.

Statistical analysis. All values are expressed as means ± SE. The statistical significance was evaluated by one- or two-way analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test. A probability of less than 0.05 was considered significant.

RESULTS

Upregulation of IBF by TRPV1 and TRPA1 stimulation. We first investigated the vasoactive effect of TRPV1 and TRPA1 agonists administered into the lumen of the small intestine. The TRPV1 agonist CAP (3 mg/kg) caused a rapid increase in IBF, which peaked 15 min after administration and remained at high levels throughout data acquisition (Fig. 1*A*). The TRPA1 agonist AITC (0.002 mg/kg) produced a gradual increase in vasodilatation which peaked at 120 min or later (Fig. 1*B*). Neither of the agonists influenced systemic circulation (data not shown), and therefore, the effects were limited to the local microcirculation. The TRPV1-selective antagonist BCTC and the TRPA1-selective antagonist HC-030031 diminished the vasodilatory effect of CAP and AITC, respectively. Both antagonists had no effect by themselves (data not shown).

Involvement of TRPA1 and ADM in the vasodilatory effect of TU-100. Past studies have shown that TU-100 increases blood flow in the small intestine of normal rats (38) and potentiates the production of vasoactive ADM by IE cells (27, 30). Accordingly, we sought to identify the TRP channel involved in the vasodilatory effect of TU-100. IBF increase by administration of TU-100 (2,700 mg/kg) was largely attenuated by pretreatment with HC-030031, but BCTC showed no effect (Fig. 2, *A* and *B*). We next addressed whether ADM is critical for the vasodilatory effect of TU-100. As shown in Fig. 2*C*, vascular conductance at 90, 105, and 120 min was decreased significantly ($P < 0.01$) by pretreatment with antibody against ADM. In accordance with the above findings, ADM concentrations in plasma of the portal vein (Fig. 2*D*) were elevated significantly at 15, 30, and 60 min by administration of TU-100 (2,700 mg/kg). Finally, the vasodilatory effect by AITC was also abrogated by anti-ADM treatment (Fig. 2*E*).

Expression of TRPA1 and ADM in IEC-6 and purified IE cells. We previously reported immunohistochemical identification of ADM in the mucosal epithelium of the small and large intestines of SD rats, the same strain used in the present study (30). Here we examined the expression of TRPA1 and TRPV1 mRNAs in IEC-6 cells and purified IE cells obtained from the intestines. The expression of TRPA1 mRNA was clearly detected in these cells, as was DRG (Fig. 3*A*), while gene expression of TRPV1 was below the detection limit. TRPA1 protein levels in these cells were evaluated by flow cytometric analysis. As shown in Fig. 3*B*, the fluorescence intensities for anti-TRPA1 and anti-ADM antibody were higher than those of the subtype-control antibody. Marked reduction of fluorescence intensity by coexistence of the epitope peptide of TRPA1 antigen indicated that both of these cells types expressed TRPA1 protein.

ADM releasing activity of TRPA1 agonists and TU-100. Considering the expression of TRPA1 and ADM in IE cells, we investigated the ability of TRP channel agonists to release ADM. Samples tested were CAP, AITC, and CNA (TRPA1 agonists), 2-APB (agonist of TRPV1, TRPV2, and TRPV3), and 4α-PDD (TRPV4 agonist). As shown in Fig. 4*A*, the ADM concentrations in the culture fluids from rat IEC-6 cells treated with AITC (3–30 μmol/l) or CNA (100 μmol/l) were several times greater than control. On the other hand, CAP, 2-APB, and 4α-PDD were inactive in the test. As for TU-100 (Fig. 4*B*), the ADM concentrations in the culture fluids from IEC-6 cells with 270, 900, or 2,700 μg/ml of TU-100 were 16 ± 1, 17 ± 1, and 19 ± 1 pg/mL, respectively. These concentrations were 1.44, 1.60, and 1.74 times greater than control (11 ± 1), respectively. We then sought to identify the active ingredients responsible for the enhancement of ADM release. Twelve main ingredients were tested (Fig. 4, *C–E*). 6SG at concentrations of 10 and 30 μmol/l dramatically increased ADM release (2.27 and 8.30 times greater than control, respectively) with no cytotoxic effects. HAS significantly enhanced ADM release at concentrations of 30 and 100 μmol/l (1.49 and 1.83 times, respectively), although its activity was weaker than that of 6SG. 6-Gingerol was inactive in this test. Considering the intensity of ADM release activity and the high 6SG content in TU-100, 6SG appears to be the main active ingredient responsible for the vasodilatory effect of TU-100.

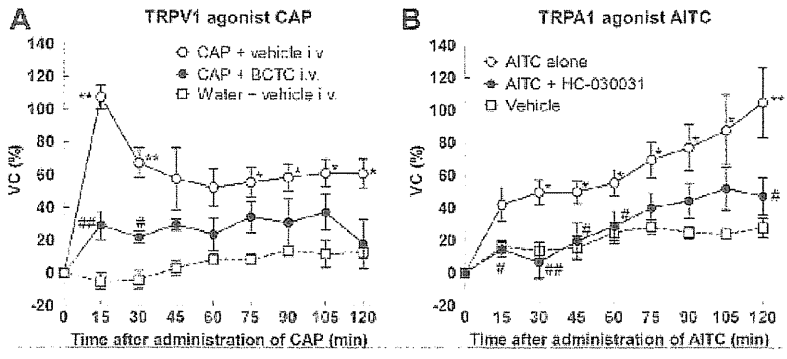

Fig. 1. Intraluminal transient receptor potential (TRP) vanilloid type 1 (TRPV1) and TRP ankyrin 1 (TRPA1) agonists increase blood flow in the small intestine. Capsaicin (CAP, 3 mg/kg body wt) or allyl isothiocyanate (AITC, 0.002 mg/kg body wt) was administered intraduodenally, and vascular conductance (VC) in the midjejunum was monitored. *A*: the TRPV1 antagonist *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC) (10 mg/kg) was given intravenously 15 min before CAP administration; $N = 3$; *B*: TRPA1 antagonist HC-030031 (1 mg/kg) was administrated intraluminally together with AITC; $N = 5$–6. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. water + vehicle (*A*) or vehicle (*B*). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. agonist alone, respectively.

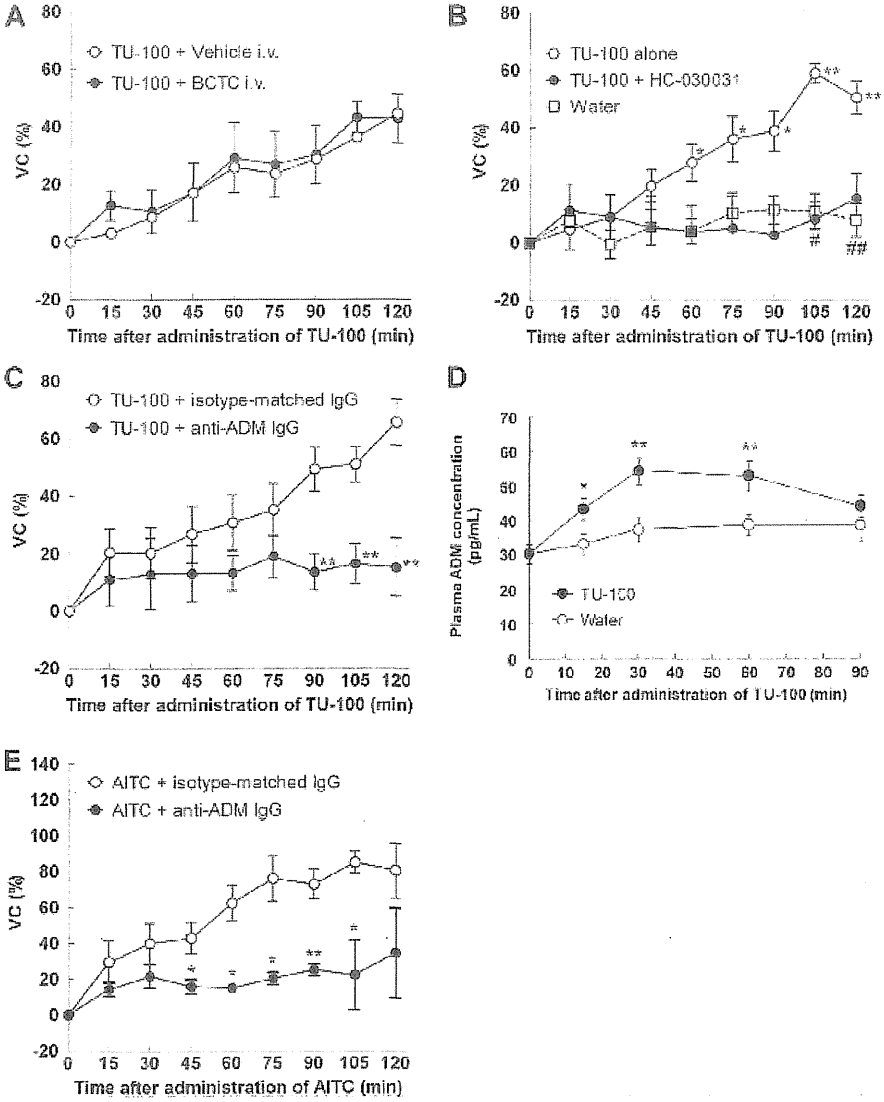

Fig. 2. TU-100 (daikenchuto) increases blood flow via the TRPA1-adrenomedullin cascade. TU-100 was administered intraduodenally at a dose of 2,700 mg/kg. Blood flow was monitored every 15 min after administration of TU-100. *A*: BCTC (10 mg/kg) was given intravenously 15 min before TU-100 administration; $N = 3$–5; *B*: HC-030031 (1 mg/kg) was administrated intraluminally together with TU-100; $N = 6$–7. *C*: antibody to adrenomedullin (ADM) was injected intravenously at a dose of 50 μg/kg 15 min before the administration of TU-100; $N = 7$–8. *D*: ADM content in the portal veins was measured using an EIA kit after purification on a C18 Sep-column; $N = 16$. *E*: AITC (0.002 mg/kg body wt) was administrated intraduodenally and anti-ADM antibody was injected as described above; $N = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. water (*B*), no-antibody control (*C*), water (*D*), or no-antibody control (*E*). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. TU-100 alone, respectively.

Investigation of signal pathways linking TRPA1 to ADM release. The functional interaction of TRPA1 activators intrinsic to TU-100 with the TRPA1 molecule was investigated in two assays: blockage of ADM release using HC-030031 in IEC-6 cells and calcium influx in TRPA1-transfected cells. The influence of coaddition of HC-030031 was first examined with respect to ADM-releasing activity of TU-100, AITC, and 6SG. As shown in Fig. 5*A*, ADM release by these activators was significantly abolished by HC-030031. In addition, the ADM-releasing activity of these activators was not detected in calcium-free buffer (data not shown). T-Rex293 cells stably expressing rat TRPA1 were incubated with various concentrations of AITC and 6SG (Fig. 5*B*). Calcium influx was clearly evoked after their addition, while mock-transfected cells showed no response (data not shown). Finally, the involvement of the kinase pathway in ADM release by TRPA1 activators was examined. This was accomplished by evaluating the effects of the cAMP-dependent protein kinase (PKA) inhibitor H-89, the protein kinase C (PKC) inhibitor calphostin C, and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 in an ADM release test of AITC and 6SG. As shown in Fig. 5*C*, ADM-releasing activity of AITC and 6SG was reduced by the addition of calphostin C. On the other hand, the activity of 6SG but not AITC was enhanced by the addition of H-89, while LY294002 had no effect. Moreover, the PKC-specific activator PMA significantly augmented ADM release (Fig. 5*D*).

Vasodilatory effect of 6SG. After confirming that 6SG was the main active ingredient of TU-100 that stimulates TRPA1 and ADM release, we evaluated its effect on IBF. As shown in Fig. 6*A*, the dose-dependent vasodilatory effect by 6SG was quantified using the area under curve of vascular conductance from 0 to 120 min. The effect of 6SG was completely abolished by pretreatment with HC-030031 (Fig. 6*B*).

DISCUSSION

In this study we demonstrated that *1*) freshly purified rat IE cells and the rat intestinal epithelial cell line IEC-6 expressed

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ORIGINAL ARTICLE

Goshajinkigan oxaliplatin neurotoxicity evaluation (GONE): a phase 2, multicenter, randomized, double-blind, placebo-controlled trial of goshajinkigan to prevent oxaliplatin-induced neuropathy

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Abstract
Purpose Oxaliplatin-induced peripheral neurotoxicity (OPN) is frequent and potentially severe, but successful treatment of this condition is still an unmet clinical need. We aimed to determine whether treatment with goshajinkigan (TJ-107), a traditional Japanese medicine, is better than placebo in preventing OPN in patients with advanced or recurrent colorectal cancer patients treated with standard FOLFOX regimens.
Methods In this phase 2, randomized, double-blind, placebo-controlled study, patients undergoing oxaliplatin-based chemotherapy were randomized to receive either oral TJ-107 (7.5 g) or matching placebo daily. The severity of OPN was assessed according to the Common Toxicity Criteria for Adverse Events at baseline, every 2 weeks until the 8th cycle, and every 4 weeks thereafter until the 26th week. The primary endpoint was the incidence of grade 2 or greater OPN until the 8th cycle of chemotherapy.
Results Analyses were done by intention to treat. Eighty-nine patients were randomly assigned to receive either TJ-107 ($n = 44$) or placebo ($n = 45$) between May 2009 and March 2010. The incidence of grade 2 or greater OPN until the 8th cycle was 39 and 51 % in the TJ-107 and placebo groups, respectively (relative risk (RR), 0.76; 95 % CI, 0.47–1.21). The incidence of grade 3 OPN was 7 % (TJ-107) vs. 13 % (placebo) (0.51, 0.14–1.92). No concerns regarding toxicity emerged with TJ-107 treatment.
Conclusions TJ-107 appears to have an acceptable safety margin and a promising effect in delaying the onset of grade 2 or greater OPN without impairing FOLFOX efficacy.

Keywords Goshajinkigan · Peripheral neuropathy · Double-blind randomized trial · Oxaliplatin · Colorectal cancer

Findings from this study have been partially presented at the 36th European Multidisciplinary Cancer Congress (EMCC), September 23–27, 2011, Stockholm, Sweden; Multinational Association of Supportive Care in Cancer/International Symposium on Supportive Care in Cancer 2011 (MASCC/ISOO 2011), June 23–25, 2011, Athens, Greek; 13th World Congress on Gastrointestinal Cancer, June 22–25, 2011, Barcelona, Spain; and American Society of Clinical Oncology (ASCO) 47th Annual Meeting 2011, June 1–15, 2011, Chicago, USA.

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Introduction

Oxaliplatin is considered one of the gold standard chemotherapeutic agents for advanced colorectal cancers and for adjuvant chemotherapy. However, oxaliplatin-induced peripheral neurotoxicity (OPN) is extremely common with the incidence varying from 82 to 98 % [1–3]. Severe OPN occurs in 10 to 20 % of patients [1, 4], and some may require dose reductions and discontinuation of treatment, potentially reducing the efficacy of chemotherapy and survival [5–7]. Despite considerable efforts to discover neuroprotective agents to prevent OPN [8], the best pharmacologic strategy for the management of OPN remains controversial [9–11].

In Japan, TJ-107 (goshajinkigan), a traditional Japanese medicine (Kampo) [12], has been frequently prescribed to alleviate symptoms of diabetic peripheral neuropathy such as numbness, cold sensation, and paresthesias/dysesthesias [13–15]. We hypothesized that TJ-107 might be effective against OPN and retrospectively investigated its use in a pilot study of 90 patients with advanced colorectal cancer undergoing FOLFOX therapy [16]. Patients were treated with TJ-107, calcium (Ca) gluconate and magnesium (Mg) sulfate infusion, combination of TJ-107 and Ca/Mg infusion, or chemotherapy alone. Our results showed that the group receiving TJ-107 with FOLFOX regimen experienced significant improvement in OPN and showed a favorable safety profile. We subsequently conducted a small, single-arm prospective study in 45 patients who were treated with modified FOLFOX6 for advanced colorectal cancer, in which 22 patients receiving oral TJ-107 reported lower incidence of grades 2 and 3 OPN than that in the control group [17]. Hence, this phase 2, randomized, double-blind, placebo-controlled, exploratory trial was initiated to investigate the neuroprotective effect of TJ-107 for OPN.

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Methods

Study design

This was an exploratory, randomized, phase 2 trial to evaluate the efficacy of TJ-107 for preventing OPN in the Goshajinkigan Oxaliplatin Neurotoxicity Evaluation (GONE) study group conducted at 20 institutions in Japan [18].

Eligibility criteria

Patients were eligible if they had histologically confirmed colorectal cancer and were scheduled to undergo chemotherapy with infusional 5-fluorouracil (5-FU), leucovorin (*l*-LV), and oxaliplatin (either FOLFOX4 or modified FOLFOX6 regimen). Patients had to have a good performance status (ECOG 0–1), adequate bone marrow function (WBC $\geq$ 3,000 and $\leq$12,000/mm^3, neutrophil count $\geq$ 1,500/mm^3, platelet count $\geq$100,000/mm^3), renal function (serum creatinine level less than the institutional upper limit of normal), and hepatic function (bilirubin $\leq$ 1.5 times institutional normal, aspartate aminotransferase, and alanine aminotransferase levels less than 2.5 times the institutional upper limit of normal), life expectancy $\geq$12 weeks, without evidence of clinical infection, and without preexisting peripheral neuropathy from any cause. Exclusion criteria included prior exposure to chemotherapy, except for oral fluorinated pyrimidine derivatives or 5-FU/*l*-LV in an adjuvant setting, use of other Kampo medicines, history of severe hypersensitivity (allergy) to any medications, other active malignancies or a history of other malignancies within the past 5 years, congestive heart failure, diabetes, or a history of a hemorrhagic stroke. Patients who were pregnant or nursing, taking a neuropathic pain medication, or receiving radiation were deemed ineligible.

This study was conducted in accordance with the Declaration of Helsinki and the Japanese Ministry of Health, Labour and Welfare guidelines, and informed consent was obtained from all participants. The study protocol was approved by the local Institutional Review Board at each participating institution.

Randomization and masking

Eligible patients were centrally randomized by a computer-generated allocation sequence in a 1:1 ratio to either TJ-107 group or placebo group. Information regarding the necessary follow-up tests was then sent to the registration center at the non-profit organization Epidemiological and Clinical Research Information Network (ECRIN). Patients, investigators, and data collectors were all blinded to treatment allocation.

Study medications

TJ-107 is a mixture of aqueous extracts from 10 crude herbs in fixed proportions (Rehmanniae Radix, 10.7 %; Achyranthis Radix, 6.4 %; Corni Fructus, 6.4 %; Moutan Cortex, 6.4 %; Alismatis Rhizome, 6.4 %; Dioscoreae Rhizome, 6.4 %; Plantaginis Semen, 6.4 %; Poria (*Poria cocos* Wolf), 6.4 %; processed Aconiti Tuber, 2.1 %; and Cinnamomi Cortex, 2.1 %). The extract powder of TJ-107 is commercially available in Japan and was obtained from Tsumura & Co. (Tokyo, Japan). Matching placebo (Tsumura & Co.) was specifically manufactured for this clinical trial. The appearance, color, and odor of the placebo were well controlled that both patients and clinicians were unable to distinguish this placebo from the original TJ-107. Dried powder (2.5 g) of TJ-107 or placebo was administered orally three times a day before each meal (7.5 g/day).

Treatment

Patients were randomly assigned to receive TJ-107 or placebo with either FOLFOX4 or mFOLFOX6 therapy. This treatment was initiated at the first delivery of FOLFOX and continued throughout the administration of chemotherapy and for 26 weeks beyond the completion of chemotherapy. Cycles of chemotherapy were given every 2 weeks until progressive disease or unacceptable toxicity occurred.

TJ-107 was given orally for 26 weeks starting on the day of oxaliplatin infusion. To avoid any possible influence on the assessment of neurotoxicity, Ca/Mg infusion was prohibited only during the 26-week administration of TJ-107 and not throughout the chemotherapy regimen. FOLFOX4 therapy consisted of infusion of *l*-LV at 100 mg/m^2 over 2 h followed by 5-FU as a bolus (400 mg/m^2) and a 22-h infusion of 5-FU (600 mg/m^2) on day 1 and day 2, with infusion of oxaliplatin at 85 mg/m^2 over 2 h on day 1. This regimen was repeated every 2 weeks. mFOLFOX6 therapy consisted of infusion of *l*-LV at 200 mg/m^2 over 2 h followed by 5-FU as a bolus (400 mg/m^2) and a 46-h infusion of 5-FU (2,400 mg/m^2) with an infusion of oxaliplatin at 85 mg/m^2 over 2 h on day 1. This regimen was repeated every 2 weeks.

Adverse reactions including OPN were assessed at baseline (prior to starting FOLFOX + TJ-107 or FOLFOX + placebo), every 2 weeks until the 8th cycle and every 4 weeks thereafter until the 26th week according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE v3.0). The severity of neurotoxicity was assessed by the study clinicians who were blinded to treatment allocation and used the sensory neuropathy items in NCI-CTCAE v3.0, which describe the four grades as follows: grade 1, loss of deep tendon reflexes or paresthesia, including tingling, but not interfering with function; grade 2, objective sensory alteration or paresthesia, including tingling, interfering with function, but not interfering with activities of daily living (ADL); grade 3, sensory alteration or paresthesia interfering with ADL; and grade 4, permanent sensory losses that are disabling. In addition, patients rated their symptoms on a 0–4 scale using the Functional Assessment of Cancer Therapy/Gynecological Oncology Group-Neurotoxicity (FACT/GOG-Ntx-12) score at screening, at baseline, and before each chemotherapy treatment. These subjective ratings were independently evaluated from the clinician-rated CTCAE grading. The follow-up period was 1 year after registration of the last patient.

Statistical analysis

The primary endpoint of this study was the incidence of grade 2 or greater OPN after 8 cycles of chemotherapy as assessed by the clinical investigators. The rate of incidence was compared between evaluable patients randomized to either the TJ-107 or the placebo group. The rate of occurrence of grade 2 or greater OPN was calculated for each group and compared using the chi-square test.

Secondary endpoints included the incidence and grading of OPN after each cycle, FACT/GOG-Ntx-12 score, time to occurrence of OPN, response rate to chemotherapy, and toxicity.

Previously, we found that the incidence of grade 2 or greater OPN was 15 and 45 % (TJ-107 vs placebo) from the start of oxaliplatin treatment until the completion of cycle 8 [16]. On the basis of this data, we determined that in order to detect with 80 % power while maintaining a significance level of 10 % in a two-sided test, 35 patients per group would be required to compare the two treatment groups with a chi-square test. To account for possible dropouts, a minimum of 40 patients were enrolled in each group (80 in total). Randomization was achieved by using three strata: use of bevacizumab, the institution, and the presence of target lesions evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. This trial is registered with the UMIN Clinical Trials Registry in Japan (UMIN000002211).

Results

A total of 93 patients were enrolled from May 1, 2009, to March 31, 2010. Of the 93 patients, 47 were assigned to the TJ-107 group and 46 to the placebo group. Four patients (3 receiving TJ-107 and 1 control) were withdrawn before initiation of treatment and included in the intention-to-treat set analysis (Fig. 1). The remaining 89 (96 %) patients were consequently assessable for efficacy

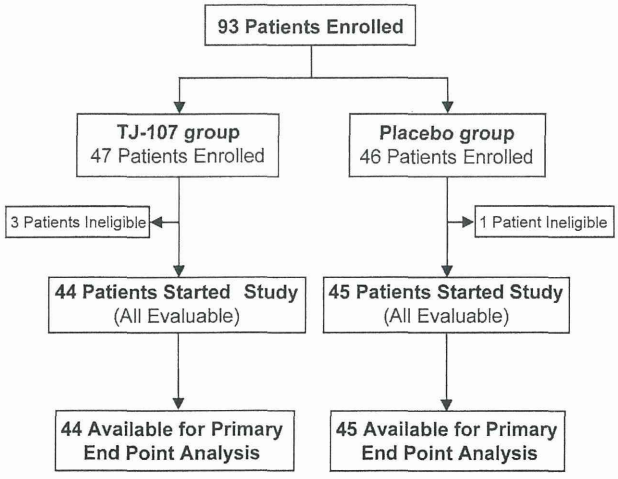

Fig. 1 CONSORT diagram

Table 1 Patient characteristics

	TJ-107 ($N = 44$)	Placebo ($N = 45$)	P value
Sex			
Male	23	25	0.833
Female	21	20	
Age			
Median	67	61	0.215
Range	40–88	36–82	
Performance status			
0	40	44	0.203
1	4	1	
Primary tumor			
Colon	28	30	0.826
Rectum	16	15	
Chemotherapy			
First-line	36	35	0.793
Adjuvant	8	10	

and toxicity. Reasons for premature withdrawal were disease progression (6 patients), adverse events (4), medical reasons (4), patient request (2), and complete response, operation, or death (1 each). Generally, demographic and background characteristics of the patients were well balanced between the TJ-107 group ($n = 44$) and placebo group ($n = 45$) (P values ranging from 0.203–0.833) (Table 1).

Incidence of OPN

Data on the incidence of grade 2 or greater OPN and grade 3 OPN until the 8th cycle are summarized (Table 2). The incidence of grade 2 or greater OPN until the 8th cycle was 39 % in the TJ-107 group and 51 % in the placebo group (relative risk (RR) 0.76; 95 % confidence interval (CI) 0.47–1.21). Similarly, the incidence of grade 3 OPN until the 8th cycle was 7 % in the TJ-107 group and 13 % in the placebo group (RR 0.51; 95 %CI 0.14–1.92).

Table 2 Oxaliplatin-induced peripheral neurotoxicity until the 8th cycle

Oxaliplatin-induced peripheral neurotoxicity until the 8th cycle			
	TJ-107 ($N = 44$) (%)	Placebo ($N = 45$) (%)	Relative risk [95 %CI]
Grade ≥2	39	51	0.76 [0.47–1.21]
Grade 3	7	13	0.51 [0.14–1.92]

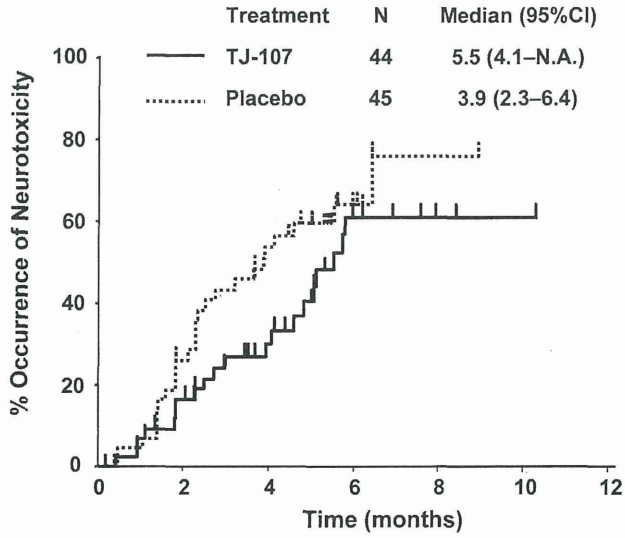

Fig. 2 Time to occurrence of grade 2 or greater neurotoxicity. *Solid line* TJ-107, *broken line* Placebo

The time to occurrence of grade 2 or greater OPN is shown in Fig. 2. In patients who developed grade 2 or greater OPN, the median time to occurrence was 5.5 months (95 %CI 4.1–N.A.) in the TJ-107 group and 3.9 months (95 % CI 2.3–6.4) in the placebo group (RR 0.65; 95 % CI 0.36–1.17). The median time to occurrence of grade 3 neurotoxicity was better controlled in the TJ-107 group (RR 0.71; 95 % CI 0.29–1.77). The median frequency of occurrence of OPN at 26 weeks was 54.1 and 62.5 % (RR 0.86) in the TJ-107 and placebo groups, respectively.

When we stratified the patients based on FOLFOX regimen (i.e., FOLFOX4 vs. mFOLFOX6) and performed a subanalysis to determine whether there was a difference in the effects of TJ-107 on the occurrence of OPN, we found that there was no significant difference between

Table 3 Median FACT/GOG-Ntx-12 score

	TJ-107 ($N = 44$)	Placebo ($N = 45$)	P value
8 weeks	6.0	9.0	0.421
26 weeks	7.0	10.5	0.151

* FACT/GOG-Ntx-12: Functional Assessment of Cancer Therapy/Gynecological Oncology Group-Neurotoxicity-12 score

Table 4 Tumor response rate

Overall	TJ-107 ($N = 27$)	Placebo ($N = 23$)	P value
Complete response (CR)	1	1	
Partial response (PR)	14	10	
Stable disease (SD)	9	11	
Progression disease (PD)	3	1	
Not evaluable (NE)	0	0	
CP + PR 95 % CI	15 (56 %) 0.37–0.74	11 (48 %) 0.27–0.68	0.777
CR + PR + SD 95 % CI	24 (89 %) 0.77–1.00	22 (96 %) 0.87–1.00	0.614

treatment groups albeit the small sample size in each group.

The median FACT scores of the TJ-107 and placebo groups were 6.0 vs. 9.0 ($P = 0.421$) at 8 weeks and 7.0 vs. 10.5 ($P = 0.151$) at 26 weeks (Table 3). Although the differences were statistically unremarkable, patients receiving TJ-107 tended to show milder symptoms of neurotoxicity than those who received placebo.

Tumor response rate

The anti-tumor effect was assessed in 27 (TJ-107) and 23 (placebo) patients who had a target lesion at the time of enrollment. Bevacizumab was administered in 74 % (20/27) and 74 % (17/23) of patients in the TJ-107 group and the placebo group, respectively (Table 4).

The overall chemotherapy response rates were 56 % in the TJ-107 group and 48 % in the placebo group. In addition, 89 % (TJ-107) and 96 % (placebo) of patients demonstrated disease control (complete response, partial response, or stable disease) (Table 4).

Toxicity assessment

TJ-107 used in this study appeared to be well tolerated. There were no significant differences between the two groups in terms of toxicity. The most common adverse events likely caused by the chemotherapy were anorexia, fatigue, nausea, and stomatitis, which were reported at similar rates from patients of both groups (Table 5). Vomiting was significantly suppressed in patients on TJ-107 compared with controls (9 vs. 29 %, $P = 0.029$). In the context of systemic chemotherapy, most of these events were likely related to chemotherapy-induced toxicity, yet none of them were considered TJ-107 related. Eighteen hematologic toxicity events of grade 3 or greater (15 neutropenia) in the placebo group were reported, and 12 events (10 neutropenia) were noted in the TJ-107 group (Table 6). One patient in the placebo group died as a direct result of progressive disease.

Table 5 Incidence of adverse events

All grades	TJ-107 ($N = 44$) (%)	Placebo ($N = 45$) (%)	P value
Fatigue	25 (57)	26 (58)	1.000
Anorexia	27 (61)	22 (49)	0.289
Nausea	20 (45)	28 (62)	0.139
Vomiting	4 (9)	13 (29)	0.029
Stomatitis	19 (43)	16 (36)	0.519
Diarrhea	15 (34)	10 (22)	0.245
Hand-food syndrome	11 (25)	7 (16)	0.302
Allergic reaction	8 (18)	4 (9)	0.230
Febrile neutropenia	0 (0)	2 (4)	0.494
Constipation	2 (5)	2 (4)	1.000
Ileus	0 (0)	2 (4)	0.494
Total Bilirubin	4 (9)	4 (9)	1.000
AST	13 (30)	23 (51)	0.052
ALT	10 (23)	19 (42)	0.070
ALP	13(30)	19(42)	0.271

Table 6 Incidence of hematologic toxicity events

	TJ-107 ($N = 44$) (%)	Placebo ($N = 45$) (%)	P value
All grades			
Leukopenia	21 (48)	27 (60)	0.291
Neutropenia	15 (34)	21 (47)	0.282
Anemia	30 (68)	31 (69)	1.000
Thrombocytopenia	12 (27)	15 (33)	0.646
Grade ≥ 3			
Leukopenia	1 (2)	2 (4)	1.000
Neutropenia	10 (23)	15 (33)	0.347
Anemia	1 (2)	1 (2)	1.000
Thrombocytopenia	0 (0)	0 (0)	1.000

Discussion

This is the first placebo-controlled, randomized, exploratory study that assessed the efficacy of oral TJ-107 in the treatment of OPN in colorectal cancer patients undergoing oxaliplatin-based chemotherapy.

Our randomized, phase 2, exploratory trial using a placebo was designed to assess the potential success of oral TJ-107 in the phase 3 setting, rather than provide solid data on its efficacy. Results of this study showed promising relative risk that support the clinical activity of oral TJ-107 against OPN, particularly acute cold-associated neuropathy, in patients who received FOLFOX therapy (FOLFOX4 or mFOLFOX6) for colorectal cancer without imposing negative impact on oxaliplatin-based anti-tumor effect. Additionally, TJ-107 did not cause any adverse effects during the trial. Our findings, which showed improvement in both CTCAE grades and patient-rated FACT/GOG-Ntx scores, suggest that TJ-107 delays the occurrence of grade 2 or greater OPN during active treatment although its therapeutic effect may plateau after 6.5 months of continuous administration and that the development of neurotoxicity was not correlated with the completion of oxaliplatin treatment. Given the difficulty of generating statistically robust data with a small sample size, we surmised that our data warrant further investigation in a large phase 3 setting.

As an exploratory, phase 2 trial of patients with advanced or relapsed colorectal cancer (some with unresectable cancer), we conducted the endpoint assessment after 8 cycles rather than at treatment completion on the basis of a previous study that reported the high likelihood of detecting the side effects of oxaliplatin after 8 cycles [19], suggesting that this time point may be critical for deciding whether to continue oxaliplatin-based chemotherapy. Furthermore, a postmarketing drug surveillance of TJ-107 in Japan has shown that the median time to occurrence of grade 3 neuropathy with motor disorder was after 8 cycles. Taken together, these data suggest the evaluation of neuropathy at its peak incidence after 8 cycles to be clinically more meaningful than delaying the evaluation until treatment completion.

TJ-107 is a complex drug containing 10 medicinal herbs with a wide spectrum of pharmacologic actions [16]. Experimental studies have shown that TJ-107 relieves neurologic symptoms of diabetic peripheral neuropathy such as cold hyperalgesia and mechanical allodynia [13–15] primarily by the action of its analgesic component, detoxified Aconiti Tuber. The purported mechanisms by which this component works in concert with the other components of TJ-107 to exert a neuroprotective effect include (1) evoking the release of dynorphin and activating endogenous κ-opioid receptors to improve numbness or paresthesia [20, 21], (2) decreasing the release of transmitter proteins and sensory receptors associated with C-fiber nociceptor activation [22, 23], and (3) promoting nitric oxide production to improve blood supply to the nerves [24]. Furthermore, a recent experimental study has demonstrated that TJ-107 ameliorates the pain associated with OPN in rats without affecting the anti-tumor activity of oxaliplatin [25], which is in line with our findings. Interestingly, we also found that TJ-107 significantly decreased vomiting compared with placebo. The precise mechanism remains unclear but one of the components of TJ-107, Poria (*Poria cocos* Wolf), has shown an antiemetic effect through 5-HT3A receptor inhibition [26, 27].

According to the Multicenter International Study of Oxaliplatin, 5-Fluorouracil and Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) study, the reported incidences of OPN were as follows: grade 1 (48 %), grade 2 (32 %), and grade 3 (12 %) [1]. In the present study, the incidence of grade 2 and grade 3 OPN in the placebo group until the 8th cycle was 51 % and 13 %, respectively, which is consistent with the MOSAIC study [1]. There was no marked difference in time to treatment failure in this study. One possible explanation for this is that even if greater than grade 2 OPN had occurred once, oxaliplatin administration was continued so long as OPN was downgraded to grade 2 on the day of administration. Another possibility is that patients may not acknowledge or report neuropathic symptoms for fear of missing out on an effective cancer treatment. Thus, it seems imperative to discover an agent that has sufficient evidence to decrease OPN development.

Many agents have been tested, in both humans and experimental animals, to ameliorate OPN [28]. Recently, the antidepressant drug venlafaxine, which is also used to manage pain associated with diabetic peripheral neuropathy, has been reported to significantly decrease the incidence of acute OPN in a placebo-controlled, randomized, phase 3 trial; however, grade 1–2 vomiting was observed more frequently in patients who received venlafaxine [29]. TJ-107 has also been used to treat painful diabetic peripheral neuropathy [13–15], but our study showed that TJ-107 significantly decreased vomiting compared to placebo, and other common adverse events due to chemotherapy did not worsen with TJ-107 treatment. Effective preventive treatments must not only mitigate neurotoxicity but must also preserve the antineoplastic effect of chemotherapeutic drugs. In this study, no between-group differences were found in response rates to chemotherapy or in the survival rates, suggesting that TJ-107 had no influence on FOLFOX therapy. Moreover, TJ-107 is an easily administered alternative that does not produce serious adverse effect, rendering it conducive to increasing compliance among patients and health care practitioners in a cancer treatment setting.

This is the first, randomized, phase 2, exploratory trial of TJ-107 whose study design itself is comparable to that

of an older phase 3 trial design. The primary objective of this phase 2 trial study was to determine whether our findings would warrant validation in a phase 3 setting and not necessarily to obtain concrete data that show statistical significance. In other words, our aim was to define the characteristics of TJ-107 against OPN relative to placebo in order to refine the design of a phase 3 trial. Through this study, we were able to obtain a more accurate estimation of sample size and confirm that TJ-107 was similar to placebo in terms of toxicity despite the small sample size, and TJ-107 prevented the progression and development of severe neurotoxicity, one of the primary dose-limiting factors of oxaliplatin-based chemotherapy. Taken together, our findings suggest that this trial served as an effective platform for testing the efficacy of a novel agent like TJ-107 in oncology and for designing and accelerating the transition from phase 2 to a large phase 3 trial that employs objective measures.

Conclusions

Findings from this phase 2, exploratory trial suggest that oral TJ-107 has acceptable margins of safety and tolerability and a promising effect in delaying the onset of grade 2 or greater OPN in colorectal cancer patients treated with oxaliplatin.

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Conflict of interest None.

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