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著者	Shakoori Abbas, Mai Wei, Miyashita Katsuyoshi, Yasumoto Kazuo, Takahashi Yutaka, Ooi Akishi, Kawakami Kazuyuki, Minamoto Toshinari
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# Inhibition of GSK-3 $\beta$ activity attenuates proliferation of human colon cancer cells in rodents

Abbas Shakoori,<sup>1,3,7,8</sup> Wei Mai,<sup>1,3,7</sup> Katsuyoshi Miyashita,<sup>1,3,4</sup> Kazuo Yasumoto,<sup>2,3</sup> Yutaka Takahashi,<sup>2,3</sup> Akishi Ooi,<sup>5</sup> Kazuyuki Kawakami<sup>1,3</sup> and Toshinari Minamoto<sup>1-3,6</sup>

Divisions of <sup>1</sup>Translational and Clinical Oncology and <sup>2</sup>Surgical Oncology, <sup>3</sup>Molecular and Cellular Targeting Translational Oncology Center, Cancer Research Institute, and Departments of <sup>4</sup>Neurosurgery and <sup>5</sup>Molecular and Cellular Pathology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

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The authors' recent discovery that glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) participates in colon cancer cells' survival and proliferation prompted us to investigate whether GSK-3 $\beta$  inhibition alters proliferation of colon cancer cells *in vivo*. Groups of four or five athymic mice (Balb/c, nu/nu) with subcutaneous xenografts of SW480 human colon cancer cells were treated with dimethyl sulfoxide (DMSO) or different doses (1, 2 and 5 mg/kg body weight) of either small-molecule GSK-3 $\beta$  inhibitor (SB-216763 and AR-A014418) by intraperitoneal injection three times per week for 5 weeks. Compared with DMSO (a diluent of the GSK-3 $\beta$  inhibitors) as a control, either GSK-3 $\beta$  inhibitor significantly inhibited proliferation of cancer cell xenografts in the rodents in a dose-dependent manner. Histochemical and immunohistochemical analysis of tumor xenografts demonstrated a significant, dose-dependent decrease in fractions of proliferating cells and an increase in the incidence of apoptosis of cancer cells in mice treated with either GSK-3 $\beta$  inhibitor. No adverse events or effects were observed in the rodents during the course of treatment, except for rare lethal accidents due to intraperitoneal injection. Morphological examination showed no apparent pathologic changes in major organs including the lungs, liver, pancreas, kidneys, spleen and large bowel of rodents treated with DMSO and the GSK-3 $\beta$  inhibitors. The results indicate that the GSK-3 $\beta$  inhibitors would be a novel class of therapeutic agent for colon cancer. (*Cancer Sci* 2007; 98: 1388–1393)

A striking contribution toward understanding the molecular basis of cancer has been the identification of targets for diagnosis and treatment of this disease. A target of great interest among the molecules altered in cancer is a set of protein kinases, most proto-oncogene products, that mediate complex cellular signaling networks in physiological conditions and evoke oncogenic signaling in cancer development.<sup>(1,2)</sup> Among the novel therapeutic strategies that led to a variety of therapeutic agents under clinical development, one approach utilizes humanized monoclonal antibodies directed against the extracellular domains of transmembrane receptor-type protein kinases. Another approach is the generation of small-molecule analogs of ATP targeting the kinase domain. Many small-molecule inhibitors of these kinases are being developed for target-directed therapies of cancer.<sup>(1-3)</sup>

GSK-3 $\beta$ , a molecular target of interest in this study, is a multifunctional serine/threonine protein kinase that regulates fundamental cellular pathways, depending on its substrates for phosphorylation and its subcellular localization.<sup>(4-6)</sup> On the basis of its primary pathologic activities in NIDDM,<sup>(7-9)</sup> and AD,<sup>(10,11)</sup> GSK-3 $\beta$  has emerged as a promising target in relation to generating a novel strategy and developing new drugs for treatment of adult-onset chronic and progressive diseases.<sup>(12-14)</sup> Because a variety of transcription factors (e.g. c-Jun, c-Myc), cell cycle regulators (e.g. cyclin D1) and proto-oncoproteins (e.g.  $\beta$ -catenin, Gli2) are GSK-3 $\beta$  substrates for phosphorylation-dependent protein degradation in the ubiquitin–proteasome system, GSK-3 $\beta$  is recognized as a putative suppressor of cellular neoplastic

transformation and tumor development (reviewed in<sup>(15)</sup>). Another line of study, however, indicates that GSK-3 $\beta$  plays a crucial role in cell survival signaling under physiological conditions, presumably by stimulating NF- $\kappa$ B-mediated gene transcription.<sup>(16,17)</sup> These conflicting notions as to the biological properties of GSK-3 $\beta$  in cells have promoted investigation of the pathologic roles of the kinase in cancer, which are characterized by irreversible deregulation in cell survival, proliferation and differentiation.<sup>(18)</sup>

Under normal conditions, GSK-3 $\beta$  is constitutively active in cells, and its activity is regulated by the balance between the levels of phosphorylation at its S9 and Y216 residues.<sup>(4-6)</sup> Recently, the authors found that increased GSK-3 $\beta$  expression and activity and deregulation of its activity by impairment in differential phosphorylation in S9 and Y216 residues are characteristic of both colon cancer cell lines and clinical CRC, which was unrelated to  $\beta$ -catenin activation in tumor cells. Functional analysis by genetic depletion of expression and pharmacological inhibition of activity of the kinase demonstrated the novel pathologic roles of GSK-3 $\beta$  in promoting cancer cell survival and proliferation and in interfering with those undergoing apoptosis in CRC, which contradicts its predicted role as a tumor suppressor.<sup>(19)</sup> By developing and using the non-radioisotopic *in vitro* kinase assay (NRKA), the authors detected increased activity of GSK-3 $\beta$  in colon, stomach, pancreas and liver cancer cells in comparison with HEK293, where the kinase activity is seemingly regulated by differential phosphorylation; similar pathologic properties of GSK-3 $\beta$  were also observed in cancer cells of these types.<sup>(20)</sup> On this basis, the authors warrant proposing this kinase as a potential therapeutic target in gastrointestinal cancer (PCT/JP2006/300160).

Similar observations in colon and pancreas cancer cells have been reported,<sup>(21,22)</sup> in parallel with the authors' earlier studies.<sup>(19,20)</sup> These studies raise an important concern, namely, whether inhibition of GSK-3 $\beta$  activity by small-molecule inhibitors alters proliferation of human colon cancer cells xenografted into rodents. The present study was undertaken to address this question. The pathological effects of GSK-3 $\beta$  inhibition in the major organs of the rodents were also monitored.

<sup>6</sup>To whom correspondence should be addressed.

E-mail: minamot@kenroku.kanazawa-u.ac.jp

<sup>7</sup>These authors contributed equally to this study.

<sup>8</sup>Present affiliation: Laboratory of Population Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892-5060, USA.

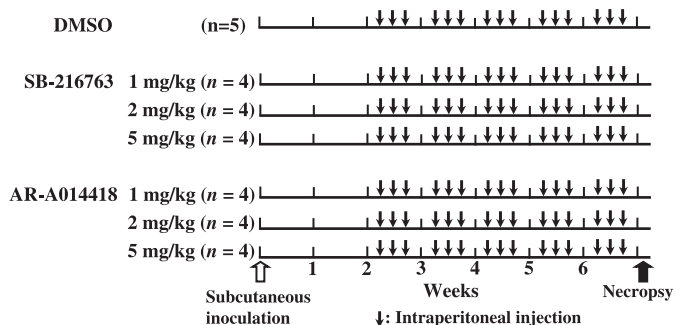
Abbreviations: AD, Alzheimer's neurodegenerative disease; APC, adenomatous polyposis coli; BSA, bovine serum albumin; COX2, cyclooxygenase 2; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetra-acetic acid; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; HEK293, human embryonic kidney cells; HMEC, human mammary epithelial cells; NIDDM, non-insulin-dependent diabetes mellitus; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; S9, serine 9; SD, standard deviation; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Y216, tyrosine 216.

## Materials and Methods

**Rodents.** Pathogen-free 6-week-old female athymic nude mice (Balb/c, nu/nu) were supplied by Japan SLC (Hamamatsu, Japan). After quarantine for 2–3 weeks in pathogen-free conditions at the Animal Experiment Facility in the Advanced Science Research Center of the University, these mice were subjected to inoculation of colon cancer cells and subsequent treatment. All experiments were conducted under strict controls according to the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University and in accordance with the national guidelines for animal usage in research in Japan.

**Inoculation of cancer cells and treatment.** Cells from the human colon cancer cell line SW480 (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco). Cells for inoculation were harvested from subconfluent cultures by brief treatment with 0.25% trypsin and 0.02% EDTA (Gibco). After inactivation of trypsin in the cells' suspension by adding fresh medium, the cells were resuspended in PBS and used for subcutaneous inoculation of  $1 \times 10^6$  cells in 200  $\mu$ L PBS into each of 35 mice. Two weeks after inoculation, subcutaneous tumors that were formed and visible in all mice were size-matched (the diameter of each tumor was approximately 2 mm), and the mice were randomly assigned to seven groups (five mice each) for treatment with DMSO (a diluent of the GSK-3 $\beta$  inhibitors) or either of two small-molecule GSK-3 $\beta$  inhibitors, SB-216763 (Sigma-Aldrich, St Louis, MO, USA) and AR-A014418 (Calbiochem, San Diego, CA, USA).

All mice were given intraperitoneal injections of a 200- $\mu$ L aliquot of 75% DMSO or either GSK-3 $\beta$  inhibitor dissolved in 200  $\mu$ L of DMSO at different doses (1 mg/kg, 2 mg/kg or 5 mg/kg body weight), respectively, three times a week for 5 weeks (Fig. 1). The doses of GSK-3 $\beta$  inhibitors corresponded to the concentrations of the respective inhibitors in culture media used in the treatment of cells *in vitro* in the authors' earlier studies.<sup>(19,20)</sup> Assuming that 60% of body weight is accounted for by body fluid in each mouse, the doses of inhibitors of 1 mg/kg, 2 mg/kg and 5 mg/kg (body weight) correspond approximately to concentrations of 5  $\mu$ M, 10  $\mu$ M and 25  $\mu$ M in culture media, respectively. Throughout the experiment, all mice were carefully observed for adverse events every day, and tumors were measured



**Fig. 1.** Design and protocol of the animal experiment. At week 0,  $1 \times 10^6$  SW480 human colon cancer cells were subcutaneously inoculated into each mouse. Two weeks later subcutaneous tumors that were formed in all mice were size-matched and the mice were randomly assigned to seven groups for treatment with dimethyl sulfoxide (DMSO, a diluent of the GSK3 $\beta$  inhibitors) or either of two small-molecule GSK-3 $\beta$  inhibitors, SB-216763 and AR-A014418. DMSO and indicated doses of the GSK-3 $\beta$  inhibitors were given to mice by intraperitoneal injections three times per week. After 5 weeks' treatment, all mice were euthanized for necropsy. At the beginning of treatment, one mouse in each of the groups treated with the GSK-3 $\beta$  inhibitors died because of a lethal accident (i.e. bleeding in the peritoneal cavity) due to the intraperitoneal injection.

in two dimensions every week. Tumor volume ( $\text{cm}^3$ ) was calculated using the formula:  $0.5 \times a^2 \times b$ , where  $a$  is the smallest tumor diameter (cm) and  $b$  is the largest.<sup>(23)</sup>

**Necropsy and histopathologic examination.** Following treatment, all mice were euthanized. At necropsy, tumor and the major organs (lungs, liver, pancreas, spleen, kidney and large bowel) were removed, fixed in 10% neutral-buffered formalin and embedded in paraffin for histopathologic and immunohistochemical examination. Paraffin sections of these organs and tumors were stained with HE for histopathologic examination by a certified pathologist (A.O.). Tumors removed from all rodents at necropsy were weighed before fixation.

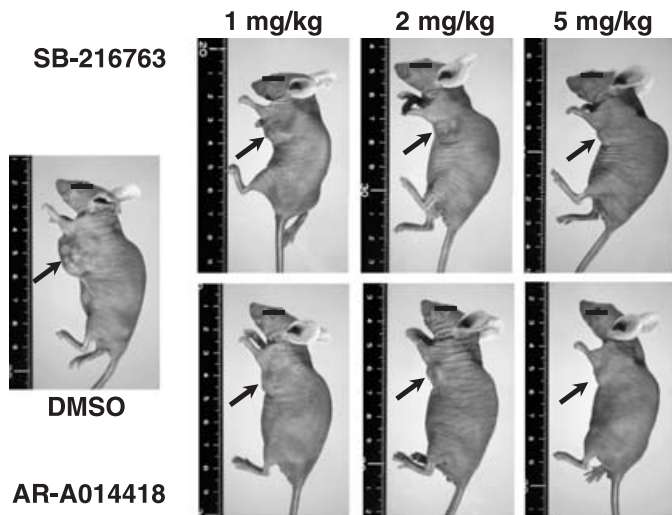
**Histochemical and immunohistochemical analysis.** Expression and localization of GSK-3 $\beta$ ,  $\beta$ -catenin and PCNA in tumor tissues were immunohistochemically examined using the avidin–biotin–peroxidase complex method as described in the authors' previous studies,<sup>(24,25)</sup> with certain modifications. Representative paraffin sections placed on silanized slides (Dako, Glostrup, Denmark) were treated by microwaving in citrate buffer to unmask antigens and incubated with 0.3%  $\text{H}_2\text{O}_2$  in methanol and then with 10% normal goat serum to block non-specific immunohistochemical reactions. The pretreated paraffin sections were incubated with the rabbit monoclonal antibody to human GSK-3 $\beta$  (diluted 1:100; Epitomics, Burlingame, CA, USA), polyclonal antibody to  $\beta$ -catenin (diluted 1:100; Cell Signaling Technology, Beverly, MA, USA) or PCNA (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with either antibody, the sections were incubated with the biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) diluted 1:200 in PBS containing 1% BSA and 10% normal mouse serum (DakoCytomation, Glostrup, Denmark) to prevent cross-reactions with endogenous mouse IgG.

Cancer cells undergoing apoptosis were histochemically detected in the representative section of tumor tissues using the TUNEL method,<sup>(26)</sup> with the *in situ* apoptosis detection TUNEL kit (Takara, Kusatsu, Japan). The frequency of proliferating cells and cells undergoing apoptosis in the tumors was calculated by counting PCNA-positive cells and TUNEL-positive cells and debris (apoptosis changes), respectively. In each tumor, a total of more than 200 nuclei per high-power microscopic field was counted and scored for PCNA-positive nuclei and apoptosis changes, respectively. The mean scores for PCNA-positive nuclei and apoptosis changes in five high-power fields were then calculated with SD.

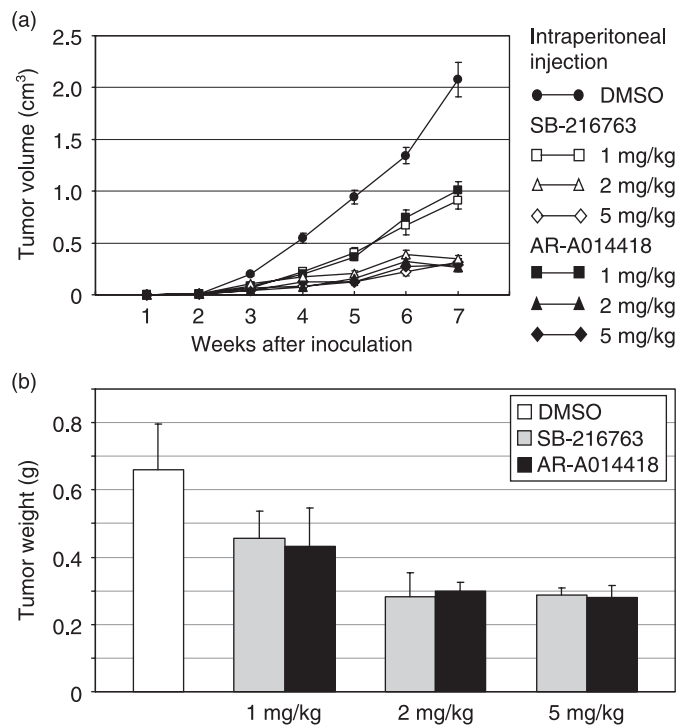
**Statistical analysis.** Body weight of mice, tumor volume, tumor weight (at necropsy) and scores of PCNA-positive cells and TUNEL-positive cells in each treatment group were expressed as means  $\pm$  SD. The statistical significance of differences among the data was determined with the one-way ANOVA followed by Fisher's PLSD *post hoc* test. Values of  $P < 0.05$  were considered significant.

## Results

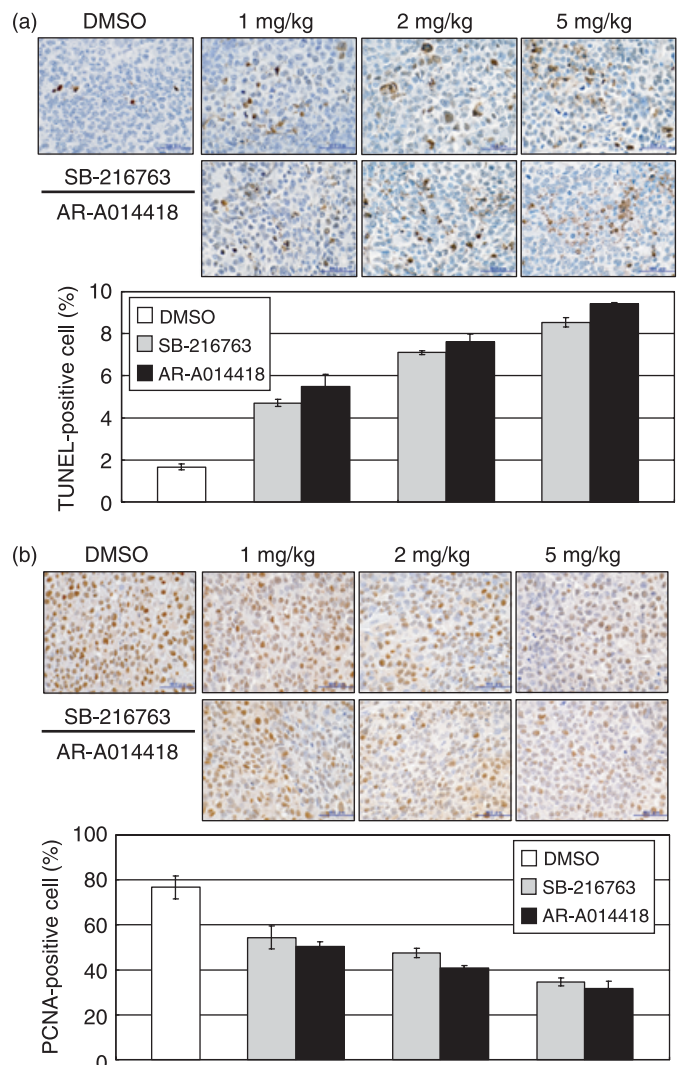
Representative rodents in the respective groups of mice treated with DMSO or different doses of GSK-3 $\beta$  inhibitors are shown in Fig. 2. In all mice treated with DMSO, SW480 colon cancer cells formed subcutaneous tumors with a mean volume of  $2.07 \pm 0.17 \text{ cm}^3$ . After 5 weeks' treatment with either GSK-3 $\beta$  inhibitor, there was a significant and dose-dependent decrease in tumor volume in mice treated with different doses (1 mg/kg, 2 mg/kg, and 5 mg/kg body weight) of inhibitor, in comparison with those treated with DMSO. As shown in Fig. 3a, the effect of GSK-3 $\beta$  inhibitors against tumor proliferation increased as treatment duration grew longer. Following 3 weeks' treatment (i.e. as of week 5 in Fig. 3a), Fischer's PLSD *post hoc* test showed significant differences in tumor volume between mice treated with DMSO and those treated with either inhibitor, and between



**Fig. 2.** Representative rodents in groups of mice treated with dimethyl sulfoxide (DMSO) or indicated doses of GSK-3 $\beta$  inhibitors, SB-216763 and AR-A014418. After 5 weeks' treatment with either inhibitor, there was significant dose-dependent decrease in tumor size in mice treated with different doses (1 mg/kg, 2 mg/kg, and 5 mg/kg body weight, shown at the top of the panels) of the inhibitors, in comparison with those treated with DMSO. Arrows point to subcutaneous tumors formed by inoculated SW480 colon cancer cells.



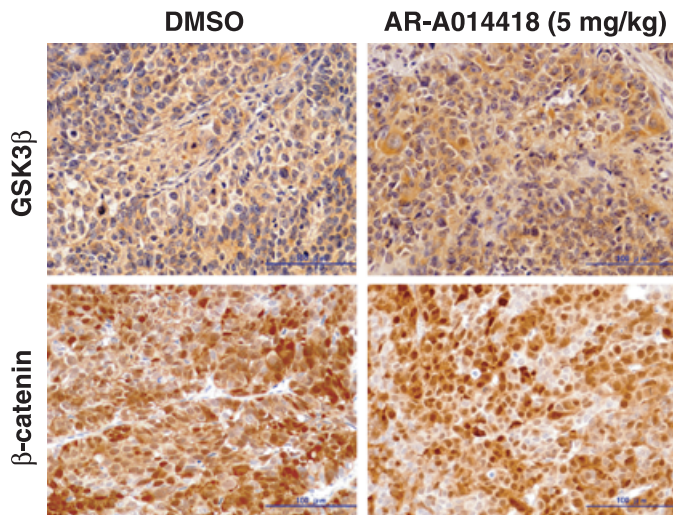
**Fig. 3.** (a) Effect of intraperitoneal injection of GSK-3 $\beta$  inhibitors on proliferation of SW480 xenografts and (b) comparison of xenograft weights in rodents treated with dimethyl sulfoxide (DMSO) and indicated doses of the kinase inhibitors. (a) Tumor sizes were measured weekly and their volumes calculated. Administered agents and their doses are indicated on the right together with corresponding symbols. There was a dose-dependent decrease in tumor volume both with SB-216763 and AR-A014418 from week 5 and beyond ( $P < 0.05$ ). (b) Mean weights  $\pm$  SD of the tumors removed at necropsy from the respective groups of mice with indicated treatments. Statistically similar differences were found in tumor weights between mice with the respective treatments. (a,b) There were no statistically significant differences in tumor volume or weight between mice treated with any dose of SB-216763 and AR-A014418, or between mice treated with 2 mg/kg and 5 mg/kg of the inhibitors, respectively.



**Fig. 4.** Scores of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)- and proliferating cell nuclear antigen (PCNA)-positive cells, respectively, in tumor tissues removed from mice at necropsy after 5 weeks' treatment with dimethyl sulfoxide (DMSO) and indicated doses (1 mg/kg, 2 mg/kg, and 5 mg/kg body weight) of GSK-3 $\beta$  inhibitors. Representative sections of histochemical (TUNEL) and immunohistochemical (PCNA) staining are shown in the upper panels. There were (a) dose-dependent increases in TUNEL-positive cell rates and (b) decreases in PCNA-positive cell rates in tumors treated with SB-216763 or AR-A014418 ( $P < 0.05$ ), compared with those treated with DMSO.

mice treated with 1 mg/kg of inhibitors and those treated with 2 mg/kg or 5 mg/kg of them. Statistically similar differences were found in tumor weights at necropsy between mice with the respective treatments (Fig. 3b). There were no statistically significant differences in tumor volume or weight between mice treated with any doses of SB-216763 and AR-A014418, or between those treated with 2 mg/kg and 5 mg/kg of inhibitor, respectively.

Histological examination of the tumors showed medullary proliferation of oval and polyhedral cancer cells in all cases (upper panels in Fig. 4a,b and Fig. 5). Little prominent host reaction, such as inflammation and fibrosis, was associated with the tumors. The effects of the GSK-3 $\beta$  inhibitors on tumor cells' survival and proliferation were investigated using histochemical (TUNEL) and immunohistochemical (PCNA) staining of the



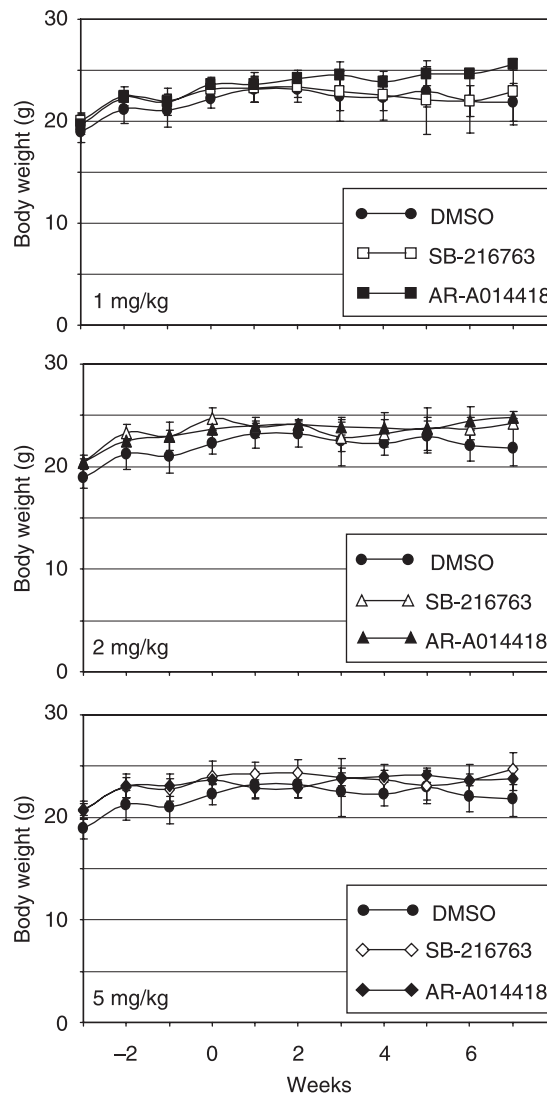
**Fig. 5.** Expression and subcellular localization of GSK-3 $\beta$  and  $\beta$ -catenin in xenografts of SW480 human colon cancer cells in mice treated with dimethyl sulfoxide (DMSO) and 5 mg/kg of AR-A014418, respectively. GSK-3 $\beta$  expression was found in the cytoplasm of cancer cells. Cytoplasmic and nuclear accumulation of  $\beta$ -catenin was observed in most cancer cells. No differences were found in intensity of expression or subcellular localization of these molecules between the tumor cell xenografts in mice treated with DMSO and AR-A014418. Nuclei were counterstained with hematoxylin. The scale bar in each panel indicates 100  $\mu$ m.

representative paraffin sections, respectively (upper panels in Fig. 4a,b). There were dose-dependent increases in TUNEL-positive cell rates and decreases in PCNA-positive cell rates in tumors treated either with SB-216763 or AR-A014418 ( $P < 0.05$ ; lower panels in Fig. 4a,b). Immunohistochemical examination showed cytoplasmic expression of GSK-3 $\beta$  and cytoplasmic and nuclear expression of  $\beta$ -catenin in tumor cells. No differences were found in intensity of expression or subcellular localization of these molecules between the tumor cell xenografts in mice treated with DMSO and different doses of the small molecule GSK-3 $\beta$  inhibitors (Fig. 5).

During treatment, all mice tolerated DMSO and increasing doses of the GSK-3 $\beta$  inhibitors well. There were no adverse events involving general appearance, body weight (Fig. 6) or food intake (data not shown); as noted, at the beginning of the treatment, one mouse in each of the groups treated with the respective doses of the GSK-3 $\beta$  inhibitors died because of an acute, lethal accident (i.e. intraperitoneal bleeding) due to intraperitoneal injection. At necropsy, gross observation and histopathologic examination showed no apparent pathological findings, neoplastic lesions or metastatic tumors in the lungs, liver, pancreas, kidneys, spleen or large bowel in all mice (not shown).

## Discussion

The present study demonstrated a dose-dependent effect *in vivo* of GSK-3 $\beta$  inhibitors against survival and proliferation of human cancer cells xenografted into rodents. The effect was associated with no apparent adverse events in general appearance, body weight or food intake of the rodents. Unlike the report that disruption of the murine *GSK-3 $\beta$*  gene results in embryonic lethality caused by severe hepatocyte degeneration,<sup>(16,17)</sup> there were no pathologic lesions in major organs, in particular, the liver of rodents treated with the GSK-3 $\beta$  inhibitors. In a recent series of studies the authors have demonstrated that the pathologic roles of GSK-3 $\beta$  are definite and common in various types of gastrointestinal cancer by analysis of expression, phosphorylation, activity and function of the kinase in multiple cell lines



**Fig. 6.** Effect of intraperitoneal injection of GSK-3 $\beta$  inhibitors on body weights of animals during the course of treatment. Body weights of the mice were measured weekly for 3 weeks before inoculation of SW480 colon cancer cells, as indicated with a minus number on the week axis, and over the treatment. The results are displayed for respective groups of mice with indicated doses (1 mg/kg, 2 mg/kg, and 5 mg/kg body weight) of GSK-3 $\beta$  inhibitors. All panels include the data of body weights of mice treated with dimethyl sulfoxide (DMSO; indicated by closed circles) as a control. No significant differences in body weight were observed between the groups.

derived from colon, stomach, pancreas and liver cancers.<sup>(19,20)</sup> Following these studies, the authors have monitored and found *in vivo* effects of the GSK-3 $\beta$  inhibitors against xenografts of a colon cancer cell line other than SW480 and a pancreas cancer cell line in rodents by treatment with the kinase inhibitors longer than 5 weeks; the treatment was associated with no apparent adverse events (unpublished observations, 2007). The results of the authors' studies indicate that the GSK-3 $\beta$  inhibitors would be a novel class of therapeutic agents for colon cancer in the clinical setting. The dose-dependency of the effects of the inhibitors with no adverse effects supports the hypothesis of a specific inhibitory effect but not of a non-specific cytotoxic effect of these compounds against cancer cell survival and proliferation *in vivo*. Importantly, there was no detectable tumor other than colon cancer cells xenograft in rodents treated with the GSK-3 $\beta$  inhibitors, as discussed below.

The molecular mechanisms underlying the putative pathologic roles of GSK-3 $\beta$  in cancer and the effect of GSK-3 $\beta$  inhibition against cancer are of particular interest and under investigation. Depending on the variety of its substrates for phosphorylation, it is assumed that GSK-3 $\beta$  exerts disparate functions in cell survival and proliferation that depend on cell type and context in both physiological cells,<sup>(4-6)</sup> and cancer cells.<sup>(15)</sup> Earlier studies have indicated that GSK-3 $\beta$  is active against cancer, mimicking a tumor suppressor, on the basis of its reported role as a repressor in Wnt/ $\beta$ -catenin signaling,<sup>(27)</sup> expression of cyclin D1, c-Myc and COX2,<sup>(28-30)</sup> and EMT by targeting snail.<sup>(31)</sup> Contrarily, since around the time of the authors' discovery of a Wnt signal-independent novel pathologic role of GSK-3 $\beta$  in gastrointestinal cancer,<sup>(19,20)</sup> an increasing number of reports have shown that active GSK-3 $\beta$  exerts tumor-promoting functions. Such reports include the participation of GSK-3 $\beta$  in increased resistance to apoptosis caused by TRAIL,<sup>(32)</sup> p53,<sup>(21,33)</sup> and c-Myc,<sup>(34)</sup> NF- $\kappa$ B-mediated survival pathway,<sup>(22)</sup> induction of cyclin D1 expression,<sup>(35)</sup> and regulation of cell adhesion and migration.<sup>(36)</sup> The reported results of the conflicting roles of GSK-3 $\beta$  in regulation of the same molecules, such as cyclin D1,<sup>(17,35)</sup> and c-Myc,<sup>(29,34)</sup> are intriguing. All these reports concern only the molecules and pathways known to be involved in cancer, and accordingly a systemic and comprehensive investigation is necessary to clarify the unknown role of GSK-3 $\beta$  in the pathology of cancer and its relevance to the fate of cancer cells.

Because of the nature of GSK-3 $\beta$  as a multitasking kinase,<sup>(4-6)</sup> the systemic inhibition of GSK-3 $\beta$  seems to lead to some detrimental effects in the mouse body due to disturbing the normal metabolism. However, no apparent detrimental effect was actually observed in rodents treated by intraperitoneal injection of the GSK-3 $\beta$  inhibitors in the present study. Prior to the present study the authors showed that, unlike the effects against various types of cancer cells, there was no or little effect of inhibition of activity or expression of GSK-3 $\beta$  on cell survival, proliferation or apoptosis in HEK293 that was considered and used as a non-neoplastic cell line.<sup>(19,20)</sup> This observation is supported by subsequent studies showing that neither GSK-3 $\beta$  inhibitors affected cell survival or proliferation in cultured HMEC, embryonic lung fibroblasts (WI38) or mouse embryonic fibroblasts (NIH-3T3).<sup>(22,37)</sup> The authors' previous study showing the presence of both GSK-3 $\beta$  fractions phosphorylated at S9 and Y216 residues in HEK293 cells,<sup>(19)</sup> suggest that the kinase activity is seemingly regulated by differential phosphorylation in these key residues depending on stimuli in cells of non-neoplastic origin. This post-translational modification of the kinase would underlie the putative mechanism by which physiological cells in the major organs of rodents are protected from detrimental effects by GSK-3 $\beta$  inhibition. In contrast to these cells with a non-neoplastic nature, the authors found overexpression and increased activity of GSK-3 $\beta$  and deregulation in the balance between the levels of phosphorylation at its S9 and Y216 residues in colon,<sup>(19,20)</sup> stomach, pancreas and liver cancer cells (unpublished observation, 2007), indicating selective and common effects of GSK-3 $\beta$  inhibition against cancer cells. Consequently, different effects of GSK-3 $\beta$  inhibition in non-neoplastic and neoplastic cells would depend on the differences in biological properties and functions of the kinase in these cells.

The molecular pathways and mechanisms that include GSK-3 $\beta$  as a crucial mediator have been implicated in the development

of diseases other than cancer, represented by NIDDM,<sup>(7-9)</sup> and AD.<sup>(10,11)</sup> Apart from the molecular basis of the diseases, epidemiological studies indicate an association in risk between cancer and NIDDM,<sup>(38-41)</sup> or AD.<sup>(42)</sup> More recently, a line of studies showing that Wnt/ $\beta$ -catenin signaling participates in bone formation (reviewed in<sup>(43,44)</sup>) suggests that GSK-3 $\beta$  would be a therapeutic target in osteoporotic bone disorders as a negative regulator of the canonical Wnt signal pathway. Accordingly, GSK-3 $\beta$  is expected to be a promising therapeutic target for common adult-onset diseases (NIDDM, AD, bone disorders and cancer) that share the characteristics of chronic and progressive clinical course and resistance to standard treatments presently available. In the present study, the authors treated tumor-burdened mice with small-molecule GSK-3 $\beta$  inhibitors by means of repeated intraperitoneal injections. However, a less invasive method, namely oral administration, is required for long-term treatment of chronic diseases such as these. With regard to the feasibility of and compliance with the requirements of the drug delivery system, recent efforts are being directed to the development of an orally bioavailable GSK-3 $\alpha/\beta$  dual inhibitor as a new drug for osteoporosis.<sup>(45)</sup>

Clinical application of GSK-3 $\beta$  inhibitors to the treatment of chronic diseases requires heightened awareness of safety considerations. Given the primary roles of GSK-3 $\beta$  in regulating its substrates, inhibition of GSK-3 $\beta$  results in up-regulation of various proto-oncoproteins (e.g.  $\beta$ -catenin, cyclin D1, c-Myc, c-Jun), leading to concern that long-term inhibition of GSK-3 $\beta$  may increase the risk of carcinogenesis.<sup>(12)</sup> To date, however, the investigators of a cohort study reported that long-term use of lithium, the classical but non-specific GSK-3 $\beta$  inhibitor, increases neither cancer morbidity nor mortality but rather is associated with reduced overall mortality in patients with bipolar disorder.<sup>(46)</sup> Similarly, although safety issues involving mutagenicity and carcinogenicity have not been available for lithium,<sup>(47)</sup> it has been reported that lithium treatment did not significantly increase the number of tumors in genetically predisposed APC mutant mice.<sup>(48)</sup> This observation is supported by the present study's finding of no detectable tumor, other than the xenograft, in the major organs, including the large bowel, after 5 weeks' treatment with either SB-216763 or AR-A014418, even at the maximum dose of 5 mg/kg. Furthermore, it has become evident that inhibition of GSK-3 $\beta$  by itself might not be sufficient to elevate the level of  $\beta$ -catenin in primary cells; and this might only occur in cells in which one or more transforming events such as APC protein truncation have already taken place.<sup>(49)</sup> In all, the evidence at hand suggests that we can prevent cancer risk caused by long-term GSK-3 $\beta$  inhibition if we generate a new class of compounds which can appropriately control GSK-3 $\beta$  expression and activity.

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## References

- 1 Morin MJ. From oncogene to drug: development of small molecule tyrosine kinase inhibitors as anti-tumor and anti-angiogenic agents. *Oncogene* 2000; **19**: 6574-83.
- 2 Diehl KM, Keller ET, Ignatoski KMW. Why should we still care about oncogenes? *Mol Cancer Ther* 2007; **6**: 418-27.
- 3 Fischer OM, Streit S, Hart S, Ullrich A. Beyond herceptin and gleevec. *Curr Opin Chem Biol* 2003; **7**: 490-5.
- 4 Harwood AJ. Regulation of GSK-3: a cellular multiprocessor. *Cell* 2001; **105**: 821-4.
- 5 Doble BW, Woodgett JR. GSK3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 2003; **116**: 1175-86.
- 6 Jope RS, Johnson GVW. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 2004; **29**: 95-102.
- 7 Eldar-Finkelman H, Krebs EG. Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci USA* 1997; **94**: 9660-4.
- 8 Cohen P. Identification of a protein kinase cascade of major importance in insulin signal transduction. *Phil Trans R Soc Lond Biol Sci* 1999; **354**: 485-95.

- 9 Nikouline SE, Ciaraldi TP, Mudaliar S, Mohideen P, Carter L, Henry RR. Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes* 2000; **49**: 263–71.
- 10 Annaert W, De Strooper B. A cell biological perspective on Alzheimers disease. *Annu Rev Cell Dev Biol* 2002; **18**: 25–51.
- 11 Bhat RV, Budd SL. GSK3 $\beta$  signalling: casting a wide net in Alzheimers disease. *Neurosignals* 2002; **11**: 251–61.
- 12 Cohen P, Goedert M. GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* 2004; **3**: 479–87.
- 13 Meijer L, Flajolet M, Greengard P. Pharmacological inhibitors of glycogen synthase kinase 3. *Trends Pharmacol Sci* 2004; **25**: 471–80.
- 14 Kypta RM. GSK-3 inhibitors and their potential in the treatment of Alzheimers disease. *Expert Opin Ther Patents* 2005; **15**: 1315–31.
- 15 Manoukian AS, Woodgett JR. Role of GSK-3 in cancer: regulation by Wnts and other signaling pathways. *Adv Cancer Res* 2002; **84**: 203–28.
- 16 Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3 $\beta$  in cell survival and NF- $\kappa$ B activation. *Nature* 2004; **406**: 86–90.
- 17 Schwabe RF, Brenner DA. Role of glycogen synthase kinase-3 in TNF-induced NF- $\kappa$ B activation and apoptosis in hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G204–11.
- 18 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
- 19 Shakoori A, Ougolkov A, Yu ZW *et al*. Deregulated GSK3 $\beta$  activity in colorectal cancer: its association with tumor cell survival and proliferation. *Biochem Biophys Res Commun* 2005; **334**: 1365–73.
- 20 Mai W, Miyashita K, Shakoori A *et al*. Detection of active fraction of GSK3 $\beta$  in cancer cells by non-radioisotopic *in vitro* kinase assay. *Oncology* (in press).
- 21 Ghosh JC, Altieri DC. Activation of p53-dependent apoptosis by acute ablation of glycogen synthase kinase-3 $\beta$  in colorectal cancer cells. *Clin Cancer Res* 2005; **11**: 4580–8.
- 22 Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD. Glycogen synthase kinase-3 $\beta$  participates in nuclear factor  $\kappa$ B-mediated gene transcription and cell survival in pancreatic cancer cells. *Cancer Res* 2005; **65**: 2076–81.
- 23 Sun F-X, Tohgo A, Bouvet M *et al*. Efficacy of camptothecin analog DX-8951f (exatecan mesylate) on human pancreatic cancer in an orthotopic metastatic model. *Cancer Res* 2003; **63**: 80–5.
- 24 Ougolkov AV, Yamashita K, Mai M, Minamoto T. Oncogenic  $\beta$ -catenin and MMP-7 cosegregate in late-stage clinical colon cancer. *Gastroenterology* 2002; **122**: 60–71.
- 25 Ougolkov A, Zhang B, Yamashita K *et al*. Associations among  $\beta$ -TrCP, an E3 ubiquitin ligase receptor,  $\beta$ -catenin, and NF- $\kappa$ B in colorectal cancer. *J Natl Cancer Inst* 2004; **96**: 1161–70.
- 26 Tornusciolo DR, Schmidt RE, Roth KA. Simultaneous detection of TDT-mediated dUTP-biotin nick end-labeling (TUNEL)-positive cells and multiple immunohistochemical markers in single tissue sections. *Biotechniques* 1995; **19**: 800–5.
- 27 Liu C, Li Y, Semenov M *et al*. Control of  $\beta$ -catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 2002; **108**: 837–47.
- 28 Hamelers IHL, van Schaik RFMA, Sipkema J, Sussenbach JS, Steenbergh PH. Insulin-like growth factor I triggers nuclear accumulation of cyclin D1 in MCF-7S breast cancer cells. *J Biol Chem* 2002; **277**: 47645–52.
- 29 Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-Myc proteolysis and subnuclear localization. *J Biol Chem* 2003; **278**: 51 606–12.
- 30 Thiel A, Heinonen M, Rintakaha J *et al*. Expression of cyclooxygenase-2 is regulated by glycogen synthase kinase-3 $\beta$  in gastric cancer cells. *J Biol Chem* 2006; **281**: 4564–9.
- 31 Zhou BP, Deng J, Xia W *et al*. Dual regulation of Snail by GSK-3 $\beta$ -mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 2006; **6**: 931–40.
- 32 Liao X, Zhang L, Du Thrasher JBJ, Li B. Glycogen synthase kinase-3 $\beta$  suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. *Mol Cancer Ther* 2003; **2**: 1215–22.
- 33 Kulikov R, Boehme KA, Blattner C. Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance. *Mol Cell Biol* 2005; **25**: 7170–80.
- 34 Rottmann S, Wang Y, Nasoff M, Deveraux QL, Quon KC. A TRAIL receptor-dependent synthetic lethal relationship between *Myc* activation and GSK3 $\beta$ /FBW7 loss of function. *Proc Natl Acad Sci USA* 2005; **102**: 15 195–200.
- 35 Cao Q, Lu X, Feng Y-J. Glycogen synthase kinase-3 $\beta$  positively regulates the proliferation of human ovarian cancer cells. *Cell Res* 2006; **16**: 671–7.
- 36 Kobayashi T, Hino S-I, Oue N *et al*. Glycogen synthase kinase 3 and h-prune regulate cell migration by modulating focal adhesions. *Mol Cell Biol* 2006; **26**: 898–911.
- 37 Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3 $\beta$ , a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. *Mol Cancer Ther* 2007; **6**: 1151–8.
- 38 Hu FB, Manson JE, Liu S *et al*. Prospective study of adult onset diabetes mellitus (type 2) and risk of colorectal cancer in women. *J Natl Cancer Inst* 1999; **91**: 542–7.
- 39 Komninou D, Ayonote A, Richie JP Jr, Rigas B. Insulin resistance and its contribution to colon carcinogenesis. *Exp Biol Med* 2003; **228**: 396–405.
- 40 Jee SH, Ohrr H, Sull JW, Yun JE, Ji M, Samet JM. Fasting serum glucose level and cancer risk in Korean men and women. *JAMA* 2005; **293**: 194–202.
- 41 Inoue M, Iwasaki M, Otani T, Sasazuki S, Noda M, Tsugane S. Diabetes mellitus and the risk of cancer: results from a large-scale population-based cohort study in Japan. *Arch Intern Med* 2006; **166**: 1871–6.
- 42 Heflin LH, Meyerowitz BE, Hall P *et al*. Cancer as a risk factor for long-term cognitive deficits and dementia. *J Natl Cancer Inst* 2005; **97**: 854–6.
- 43 Hartmann C. A Wnt canon orchestrating osteoblastogenesis. *Trends Cell Biol* 2006; **16**: 151–8.
- 44 Krishnan V, Bryant HU, MacDougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest* 2006; **116**: 1202–9.
- 45 Kulkarni NH, Onyia JE, Zeng Q *et al*. Orally bioavailable GSK-3 $\alpha$ / $\beta$  dual inhibitor increases markers of cellular differentiation *in vitro* and bone mass *in vivo*. *J Bone Miner Res* 2006; **21**: 910–20.
- 46 Cohen Y, Chetrit A, Cohen Y, Sirota P, Modan B. Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. *Med Oncol* 1998; **15**: 32–6.
- 47 Léonard A, Hanston Ph, Gerber GB. Mutagenicity, carcinogenicity and teratogenicity of lithium compounds. *Mut Res* 1995; **339**: 131–7.
- 48 Gould TD, Gray NA, Manji HK. Effects of a glycogen synthase kinase-3 inhibitor, lithium, in adenomatous polyposis coli mutant mice. *Pharmacol Res* 2003; **48**: 49–53.
- 49 Yuan H, Mao J, Li L, Wu D. Suppression of glycogen synthase kinase activity is not sufficient for leukemia enhancer factor-1 activation. *J Biol Chem* 1999; **274**: 30 419–23.