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Anti-tumour activity of CS-7017, a selective peroxisome proliferator-activated receptor gamma agonist of thiazolidinedione class, in human tumour xenografts and a syngeneic tumour implant model

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ABSTRACT

The anti-tumour activity of the novel thiazolidinedione class peroxisome proliferator-activated receptor gamma (PPARγ) agonist CS-7017 was investigated. CS-7017 activated PPARγ-mediated luciferase expression with an EC_{50} of 0.20 nM. In addition, CS-7017 was shown to be highly selective for PPARγ amongst other PPAR subfamilies. CS-7017 inhibited the proliferation of the human anaplastic thyroid tumour cell line DRO and the pancreatic tumour cell line AsPC-1 *in vitro* at concentrations as low as 10 nM. In xenograft studies, CS-7017 inhibited the growth of the human colorectal tumour cell line HT-29 in nude mice as well as DRO in nude rats in a dose-dependent manner. At the same dose, an increase in the levels of adiponectin, a surrogate marker for PPARγ activation, was also observed. CS-7017 prolonged the survival of mice inoculated with murine colorectal tumour Colon 38 with marginal tumour growth inhibition. These preclinical results support the potential utility of CS-7017 in a clinical setting.

1. Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to a nuclear hormone receptor superfamily and is thought to be involved in energy homeostasis control.[1] There have been several efforts to identify endogenous ligands for PPARγ and $\Delta^{12,15}$-prostaglandin J_2 and certain fatty acids have been proposed.[2,3] However, their binding constants are relatively weak and whether they physiologically work as PPARγ ligands or not remains in question. Thus, the physiological role of PPARγ-mediated signalling driven by endogenous ligands is not yet clear.

On the other hand, several thiazolidinedione class synthetic agents used in the treatment of type II diabetes to ameliorate sensitivity to insulin, including troglitazone and rosiglitazone, were found to target PPARγ.[4] Since these agents bind to and activate PPARγ more potently than endogenous ligands, they have been used to identify a variety of biological functions of PPARγ. As a transcription factor, PPARγ is known to regulate the transcription of adipose-related genes, includ-

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ing adipsin, adipocyte fatty acid-binding protein 2 (aP2), fatty acid-binding proteins and adiponectin.[5–7] These findings strongly suggest that PPARγ is deeply involved in the differentiation of adipose tissues. Indeed, it has been demonstrated that the activation of PPARγ by its agonists stimulates the differentiation of 3T3-L1 pre-adipocytes to adipocytes.[8]

Since PPARγ is involved in cellular differentiation, there is interest in investigating whether PPARγ also plays a role in carcinogenesis. Studies of clinical tumour specimens have resulted in the discovery of defective mutations of PPARγ, such as PAX8-PPARγ1 chromosomal translocation in follicular thyroid tumours and loss of function mutations in colorectal tumours.[9,10] These findings strongly suggest a role for PPARγ in tumour suppression during carcinogenesis. In addition, some PPARγ agonists demonstrated anti-tumour activity in several preclinical models, including chemically induced carcinogenesis models as well as human tumour xenograft models.[11] Although the mechanism of the anti-tumour activity of PPARγ agonists still remains to be clarified, some reports suggest that PPARγ activation by synthetic ligands induces terminal differentiation in tumour cells.[12] Because this mechanism of action offers a novel approach to the treatment of malignancies, we anticipate that PPARγ agonists will be useful in cancer therapy. Pilot clinical studies utilising other PPARγ agonists have been conducted in cancer patients. Troglitazone was reported to stimulate adipose differentiation in liposarcoma patients as well as to stabilise prostate-specific antigen (PSA) in prostate cancer patients.[13,14] However, trials of troglitazone and rosiglitazone in patients with other tumour types did not show any positive responses.[15,16]

CS-7017 (previously called RS-5444) is an orally active synthetic thiazolidinedione PPARγ agonist that was selected based on its potent anti-tumour activity *in vitro* and *in vivo*. CS-7017 has very high potency in stimulating PPARγ-mediated transcriptional activation. CS-7017 has been shown to have remarkable anti-tumour activity in preclinical models of human anaplastic thyroid tumours, which are known to have high mortality and for which no effective treatment is available.[17] In those studies, CS-7017 showed tumour growth inhibition in a xenograft model when used as a monotherapy and in combination with paclitaxel. In this report, the results of investigations of CS-7017 in several preclinical tumour models are reported.

2. Materials and methods

2.1. Compounds and cell lines

CS-7017 ($C_{27}H_{26}N_4O_4S \cdot 2HCl \cdot H_2O$, previous code name RS-5444) and rosiglitazone maleate were synthesised at the former Process Development Laboratories of Sankyo Co., Ltd. The chemical structure of CS-7017 is shown in Fig. 1. GW7647, a PPARα agonist was purchased from Sigma–Aldrich Co. (St Louis, MO). GW501516, a PPARδ agonist, was purchased from EMD Biosciences Inc. (Madison, WI). GW9662, a PPARγ antagonist was purchased from Calbiochem (San Diego, CA).

Human embryonic kidney cell line 293, human pancreatic tumour cell line AsPC-1 and human colorectal tumour cell line HT-29 were purchased from American Type Culture Collection (Manassas, VA). Human anaplastic thyroid tumour cell line DRO was kindly provided by Dr. G.J. Juillard (University of California-Los Angeles, Los Angeles, CA). Murine colorectal adenocarcinoma Colon 38 was obtained from the Japanese Foundation of Cancer Research (Tokyo, Japan). The 293 cells were cultured in minimal essential medium (MEM, Invitrogen Corp., Carlsbad, CA) containing 10% heat-inactivated foetal bovine serum (HyClone Laboratories, South Logan, UT). The DRO cells were cultured in RPMI 1640 (Invitrogen Corp.) containing 10% heat-inactivated charcoal/dextran-treated foetal bovine serum and 1 mM sodium pyruvate (Invitrogen Corp.). The AsPC-1 cells were cultured in RPMI 1640 containing 10% heat-inactivated foetal bovine serum. The HT-29 and Colon 38 cell lines used in the experiments were maintained by serial passages as a solid tumour subcutaneously implanted into nude mice or syngeneic C57BL/6 mice, respectively.

2.2. Reporter assay

Human PPARγ2 (hPPARγ2) expressing vector pchPPARγ2 or its original empty vector pcDNA 3.1 (+) was co-transfected with pPG2-aP2-TK into the 293 cells at a density of 7.5×10^4 cells per well using 48-well plates. pPG2-aP2-TK is a reporter plasmid containing the sequences of the transcriptional regulatory region of the mouse aP2 gene and the herpes simplex virus thymidine kinase promoter. All the transfections were done using a Lipofectamine 2000 (Invitrogen Corp.). Twenty four hours after the cell inoculation, various concentrations of CS-7017 and rosiglitazone were added to the transfected cells. For the control, dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to a final concentration of 0.01%. After culturing the cells for an additional 24 h, the luciferase activity was measured using Luciferase Assay Reagents (Promega Corp., Madison, WI) and a multilabel counter (Wallac 1420 ARVOsx, Perkin–Elmer Inc., Waltham, MA). The luciferase activity was normalised with the protein concentration of each lysate.

To examine the selectivity to PPARγ, either pM-hPPARα, pM-hPPARδ or pM-hPPARγ was co-transfected with both pFR-Luc (Stratagene, La Jolla, CA), a firefly luciferase reporter vector and phRL-TK (Promega Corp.), a *Renilla* luciferase expression vector, respectively, into 293 cells using a Lipofectamine 2000. pM-hPPARα, pM-hPPARδ and pM-hPPARγ are the

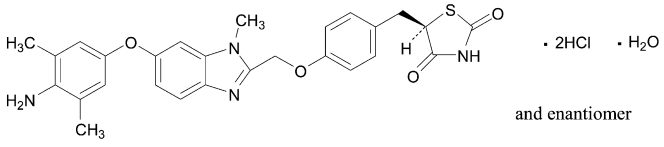

Fig. 1 – Chemical structure of CS-7017. CS-7017 belongs to the thiazolidinedione derivatives in dihydrochloride salt form.

expression vectors encoding a fusion protein with a DNA-binding domain of yeast GAL4 and the ligand-binding domain of each PPAR. The cells were cultured for 24 h after transfection, and then various concentrations of either CS-7017, rosiglitazone, GW7647 or GW501516 were added to the transfected cells. After an additional 24 h of culturing, the firefly and *Renilla* luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corp.). The mean of the firefly luciferase activity was normalised by the *Renilla* luciferase activity and is described as the calibrated light intensity.

To confirm whether DRO and AsPC-1 cells have a functional PPARγ protein, pPG2-aP2-TK was co-transfected with phRL-TK followed by CS-7017 treatment to check PPARγ activation. The luciferase activity was described as the relative luciferase activity in the ratio to the activity without CS-7017.

2.3. Anti-proliferation assay

DRO and AsPC-1 cells were spread into 96-well plates at a density of 1×10^3 cells/well and 2×10^3 cells/well, respectively. CS-7017 was added to the cells at the concentrations from 0.0001 to 10 μM in the presence or absence of 1 μM of GW9662, a PPARγ antagonist. The culture medium was changed to fresh medium containing corresponding concentrations of CS-7017 and GW9662 after 3 d for DRO and 5 d for AsPC-1. The DRO and AsPC-1 cells were then cultured for an additional 3 d and 5 d, respectively. The cellular amount of ATP was measured by a Cell Titer-Glo Luminescent Cell Viability Assay (Promega Corp.) on both Day 0 when the tumour cells were inoculated and on the final day of culturing. The cell proliferation rate (%) for the individual wells was calculated and plotted in a way which displays the drug concentration inhibiting the growth of 50% of the cells (50%), the total growth inhibition (0%) and the drug concentration needed to kill 50% of the cells (–50%).[18]

2.4. Anti-tumour activity in vivo

Specific pathogen-free female nude mice (BALB/cA Jcl-*nu*) and female nude rats (F344/NJcl-*rnu*/*rnu*) were purchased from CLEA Japan Inc. (Tokyo, Japan). Specific pathogen-free female BDF_1 mice were purchased from Charles River Japan Inc. (Yokohama, Japan). CS-7017 was suspended in 0.5% methyl cellulose solution and given daily to the animals by gavage at the volume of 0.1 mL/10 g body weight for mice and 0.2 mL/100 g body weight for rats, respectively. The control animals received 0.5% methyl cellulose solution (vehicle). For the HT-29 study, tumour fragments with a size of approximately $5 \times 5 \times 5$ mm^3 were inoculated subcutaneously into the right axillary region of the nude mice on Day 0. On Day 10, tumour-bearing nude mice were grouped according to tumour volume, after which CS-7017 was administered until Day 44. For the DRO study, 1×10^7 DRO cells were inoculated subcutaneously into the right axillary region of the nude rats on Day 0. On Day 10, tumour-bearing rats were grouped according to tumour volume, and CS-7017 treatment was conducted until Day 32. For the Colon 38 study, tumour fragments of approximately $3 \times 3 \times 3$ mm^3 in size were inoculated subcutaneously into the right axillary region of BDF_1 mice on Day 0. On Day 10, the tumour-bearing mice were grouped according to tumour volume and then CS-7017 treatment was initiated and continued until death.

The tumour volumes, tumour growth inhibition (TGI) and increase in life-span (ILS) were calculated according to the following equations:

$$\text{Tumour volume } (\text{mm}^3) = 1/2 \times (\text{tumour length}) \times (\text{tumour width})^2$$

$$\text{TGI } (\%) = (1 - T/C) \times 100$$

where T is the mean tumour volume of the CS-7017-treated animal, C is the mean tumour volume of the vehicle-treated animal.

$$\text{ILS } (\%) = (T_{\text{survival}}/C_{\text{survival}} - 1)$$

where T_{survival} is the median survival time of the CS-7017-treated mice and C_{survival} is the median survival time for the vehicle-treated mice.

All the animal care and experiments were conducted under the standard operational protocol of the former Sankyo Institutional Animal Care and Use Committee.

2.5. Immunoblotting for PPARγ detection

Tumour cells or tumour fragments excised from the tumour-bearing animals were lysed with lysis buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 10 mM $NaVO_4$, 0.1% SDS, 0.5% deoxycholic acid and 1% IGEPAL CS-630 with proteinase inhibitors. The lysates (50 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then subjected to immunoblotting. Rabbit anti-PPARγ polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as a probe with 500-fold dilution and detection was performed by chemiluminescence (ECL plus, GE Healthcare, Little Chalfont, UK) with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Inc., Beverly, MA). PPARγ-derived band was identified by using the control lysates of 293 cells transfected with pchPPARγ2, a expressing vector that also expresses PPARγ1 from the internal methionine residue.[19] The loading of an equal amount of protein was confirmed by re-probing the membrane with anti-β-actin mouse monoclonal antibody (clone AC-15, Sigma–Aldrich Co.) with 500-fold dilution

2.6. Adiponectin measurement

CS-7017 treatment of the HT-29-bearing nude mice was initiated 10 d after tumour inoculation (Day 10) and continued through Day 17. On Day 10 and Day 17, a plasma sample was obtained from the orbit of each tumour-bearing nude mouse using a heparinised capillary tube to measure adiponectin. The adiponectin concentration was measured by an enzyme-linked immunosorbent assay (ELISA) using a Quantikine Mouse Adiponectin/Acrp 30 Immunoassay (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

3. Results

3.1. PPARγ selective activation by CS-7017

CS-7017 is a novel compound in the class of thiazolidinedione that was originally synthesised at the former Sankyo. Co., Ltd. (Fig. 1). To examine the potency of PPARγ activation by CS-7017, we performed a luciferase assay driven by an enhancer sequence of aP2, an adipocyte specific gene whose transcription is known to be regulated by PPARγ. As Fig. 2A shows, CS-7017 induced PPARγ-mediated luciferase activity with an EC_{50} of 0.20 nM. Rosiglitazone, a reference compound that is a PPARγ activator, also induced luciferase activity, but its activity was less potent than that of CS-7017, with an EC_{50} of 9.3 nM. Comparison of the EC_{50} of both CS-7017 and rosiglitazone indicated that CS-7017 was greater than 40-fold more potent in PPARγ activation than rosiglitazone.

Next, the selectivity of CS-7017 in PPAR activation amongst other PPAR subfamilies was examined. The expression vector for chimeric proteins of a yeast GAL4 DNA-binding domain

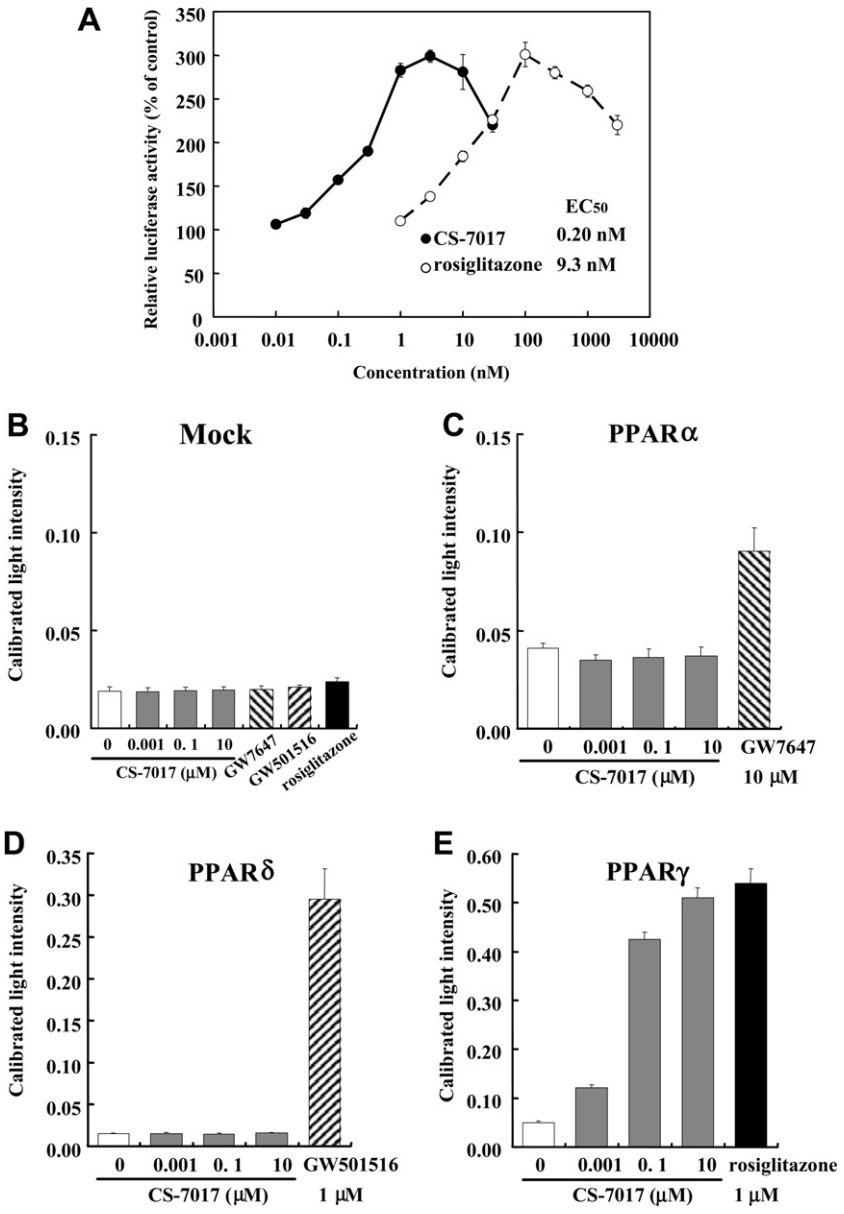

Fig. 2 – Peroxisome proliferator-activated receptor gamma (PPARγ) selective activation by CS-7017. PPARγ activation by CS-7017 and rosiglitazone was evaluated by calculating the relative luciferase activity of each treatment after transfecting a human PPARγ2 (hPPARγ2) expression vector and an acid-binding protein 2 (aP2)-driven luciferase reporter plasmid into 293 cells ($n = 4$, A). Selectivity amongst PPAR families was evaluated by measuring the luciferase activity of each treatment after transfecting pFR-Luc and phRL-TK with pM-GAL4 (B), pM-hPPARα (C), pM-hPPARδ (D) and pM-hPPARγ (E) to 293 cells ($n = 6$). For the mock control, pM-GAL4 was transfected. GW7647, GW501516 and rosiglitazone were used as positive controls for PPARα, PPARδ and PPARγ, respectively. The mean and the standard error for the relative light intensity and calibrated light intensity are displayed in the figures, respectively.

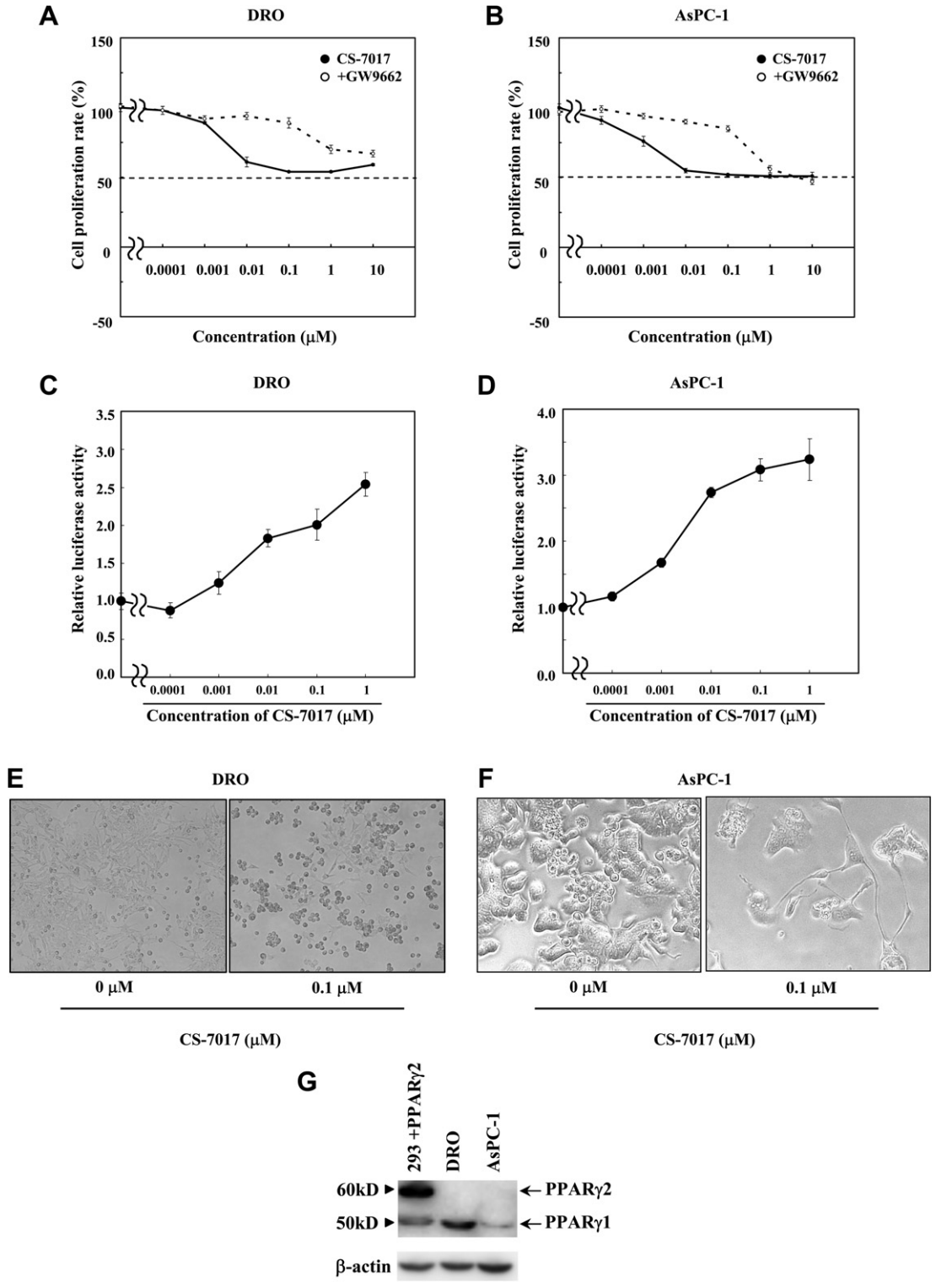

Fig. 3 – The anti-proliferation activity of CS-7017 against human thyroid cancer cell line DRO (A) and human pancreatic cancer cell line AsPC-1 (B) was evaluated *in vitro*. Both the cell lines were treated with various concentrations of CS-7017 (n = 5). The cell viabilities were evaluated by measuring the cellular ATP amount. The cell proliferation rate (%) for the individual wells was calculated and plotted in the way which displays the drug concentration inhibiting the growth of 50% of the cells (50%), the total growth inhibition (0%) and the drug concentration needed to kill 50% of the cells (–50%). PPARγ activation by CS-7017 was confirmed by measuring the luciferase activity after the co-transfection of pPG2-aP2-TK phRL-TK in DRO (C) and AsPC-1 (D) cells, respectively. Standard errors were included in the graphs (A–D). The morphological changes observed in both the cell lines after CS-7017 treatment are shown (DRO, E and AsPC-1, F). PPARγ1 protein expressions were detected in both DRO and AsPC-1 as estimated from the control lane of 293 cell lysate transfected with PPARγ2 expression vector (G). The lower band corresponds to the PPARγ1 protein whose translation was initiated by the internal methionine of PPARγ2.

and each PPAR ligand-binding domain was individually transfected into 293 cells with GAL4-reporter plasmid. A GAL4 system was used because this system facilitates avoiding the effect of endogenous PPAR functions. CS-7017 induced luciferase activity only when the expression vector for a PPARγ fusion protein was transfected (Fig. 2B–E). Since 10 μM of CS-7017 did not induce either PPARα- or PPARδ-mediated transcription and a concentration of CS-7017 as low as 0.001 μM induced PPARγ-mediated transcription, the selectivity of CS-7017 for PPARγ was estimated to be more than 10,000-fold compared with other PPAR receptors.

3.2. *Anti-proliferative activity against human tumour cell lines*

Since several reports suggest that PPARγ agonists appear to have a broad spectrum of anti-tumour activity in preclinical models[11], we first examined the effect of CS-7017 on human tumour cells *in vitro*. CS-7017 inhibited the growth of the human anaplastic thyroid tumour cell line DRO and the human pancreatic tumour cell line AsPC-1 at concentrations of 1–10 nM and higher (Fig. 3A and B). Interestingly, the inhibition of proliferation by CS-7017 seemed to be saturated to a level beyond the level when the tumour cells were spread on Day 0. This finding strongly suggests that CS-7017 would inhibit the proliferation of tumour cells rather than killing them. From this result, the mode of anti-tumour activity of CS-7017 would be cytostatic. In addition, PPARγ antagonist GW9662 cancelled this growth inhibition, suggesting that the growth inhibition by CS-7017 in these tumour cells would be based on a PPARγ-dependent mechanism. To support this hypothesis, CS-7017 was revealed to induce PPARγ-mediated transcriptional activation at a physiologically equivalent concentration to the anti-proliferation activity in each tumour cell line, respectively (Fig. 3C and D). In addition to the effect on proliferation, the morphology of the tumour cells in both the experiments was changed after CS-7017 treatment. After 6 d, cells became rounded and detached from the tissue culture dish (Fig. 3E). These floating cells were still viable, as confirmed by the dye exclusion method. In addition to DRO cells, AsPC-1 cells changed to morphology with long pseudopodia and focal adhesion 10 d after CS-7017 treatment (Fig. 3F). These results suggest that CS-7017 might induce a particular type of differentiation in tumour cells. PPARγ expression in both the cell lines was confirmed by western blotting (Fig. 3G). Both DRO and AsPC-1 expressed PPARγ1 as estimated from the control lane of 293 cell lysate transfected with PPARγ2 expression vector. In this panel, the lower molecule band corresponds to the PPARγ1 protein whose translation was initiated from the internal methionine of PPARγ2.[19]

3.3. *Anti-tumour activity in human tumour xenograft models*

Since we had confirmed the anti-proliferation activity of CS-7017 *in vitro*, we next examined the anti-tumour activity in human tumour xenograft models. Human colorectal tumour HT-29 was subcutaneously inoculated into nude mice and,

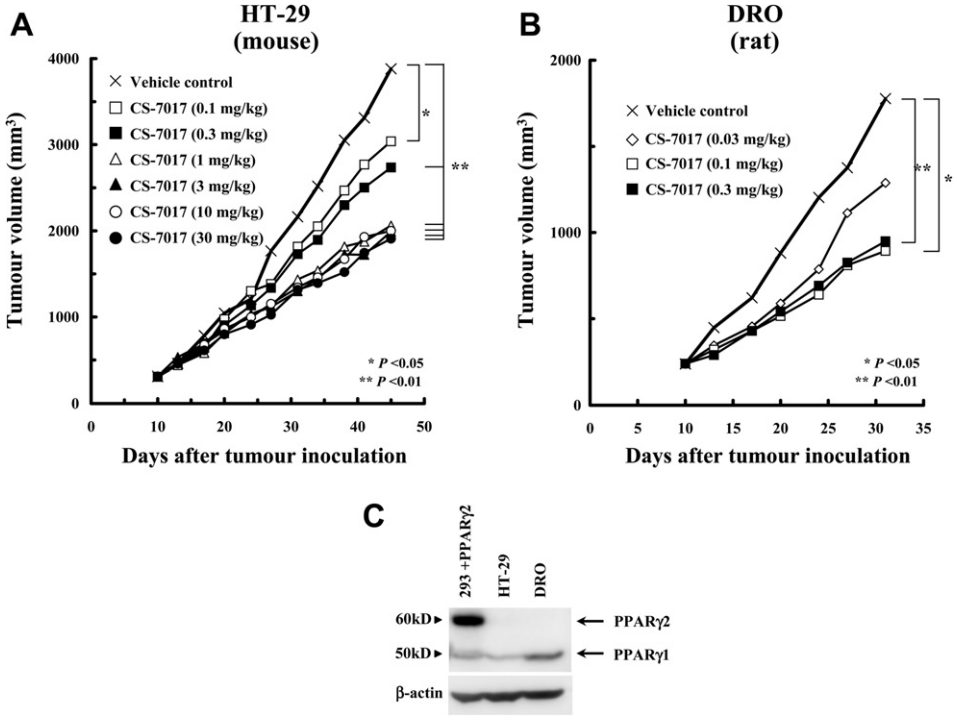

Fig. 4 – The anti-tumour activities of CS-7017 were evaluated in human tumour xenograft models. Human colorectal tumour HT-29 was implanted into nude mice (A) and human anaplastic thyroid tumour DRO was implanted into nude rats (B). In both the studies, the CS-7017 suspension was administered daily and orally at the various doses indicated in the figures from Day 10 when tumours had developed. The mean tumour volume of each group is shown (n = 10 for HT-29 and n = 8 for DRO, respectively). PPARγ protein expression in both tumours was confirmed by immunoblotting (C).

after tumours had developed, CS-7017 was administered orally at daily doses from 0.1 mg/kg to 30 mg/kg. CS-7017 inhibited tumour growth dose-dependently and maximum efficacy was achieved at a dose of 1 mg/kg and above ($p < 0.01$) (Fig. 4A). In that dose range, the tumour growth inhibition was approximately 50%. Body weight loss was not observed at doses up to 30 mg/kg, the highest dose examined. The most common side effect of thiazolidinedione class PPARγ agonists is body fluid retention. In addition, our preliminary examination suggested that rats are more sensitive than mice to fluid retention induced by PPARγ agonists (data not shown). To exclude the possibility of overestimating the safety of PPARγ agonists by testing a less sensitive animal species, we evaluated the anti-tumour activity and safety of CS-7017 in nude rats. DRO cells were subcutaneously inoculated into nude rats, and CS-7017 was orally administered daily at doses from 0.03 mg/kg to 0.3 mg/kg. CS-7017 inhibited tumour growth at doses of 0.1 mg/kg and above with statistical significance ($p < 0.01$) (Fig. 4B). No peripheral oedema was observed in nude rats at any examined doses. These results demonstrate that CS-7017 inhibited the growth of the human tumour xenograft without obvious side effects. PPARγ protein expression in both HT-29 and DRO xenografts was confirmed by immunoblotting (Fig. 4C).

3.4. *Life-prolonging effect in tumour-bearing mice*

Using a mouse syngeneic model, the effect of CS-7017 on the survival of tumour-bearing mice was evaluated. Murine colorectal tumour Colon 38 was inoculated into BDF_1 mice, and CS-7017 was administered orally at daily doses of 3 mg/kg and 10 mg/kg from Day 10 after tumour inoculation. CS-7017 demonstrated only marginal tumour growth inhibition against Colon 38 (Fig. 5A). Tumor-bearing mice in the control group started to die on Day 32, and the median survival was 42.0 d (95% confidence interval (CI): 38.0–48.0). CS-7017 prolonged the median survival by 65% (69.5 d, 95% CI: 61.0–78.0) and 40% (59.0 d, 95% CI: 50.0–73.0) at doses of 3 mg/kg and 10 mg/kg, respectively, despite having minimal effects on tumour size (Fig. 5B). No dose-response relationship was observed between 3 mg/kg and 10 mg/kg, suggesting a potential plateau of the life prolongation effect above 3 mg/kg.

3.5. *Adiponectin induction by CS-7017*

It is well known that PPARγ agonist administration increases plasma adiponectin levels in preclinical and clinical settings.[7] Adiponectin is a cytokine that is selectively and highly secreted by adipose tissues, and there is evidence to suggest a

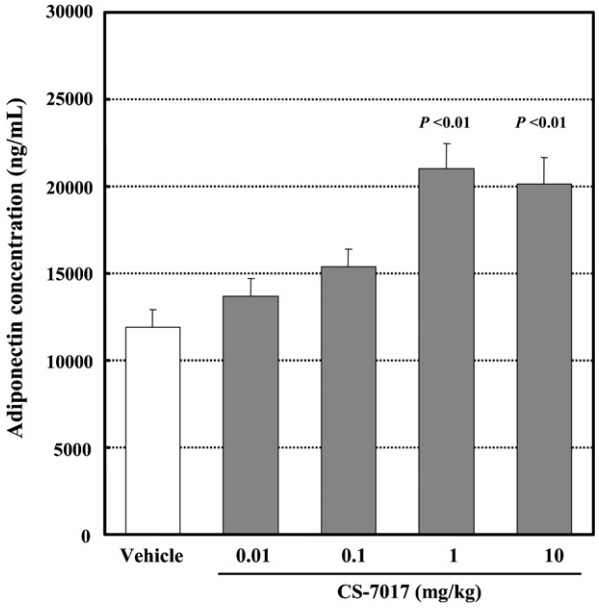

Fig. 6 – Effect of CS-7017 on plasma adiponectin levels in tumour-bearing nude mice. After human colorectal tumour HT-29 was developed in nude mice on Day 10, CS-7017 was administered orally for 7 d at doses of 0.01, 0.1, 1 or 10 mg/kg ($n = 10$). On Day 17, the plasma concentrations of murine adiponectin were measured by an ELISA using a Quantikine Mouse adiponectin/Acrp 30 Immunoassay. Error bars indicate the standard error of each group.

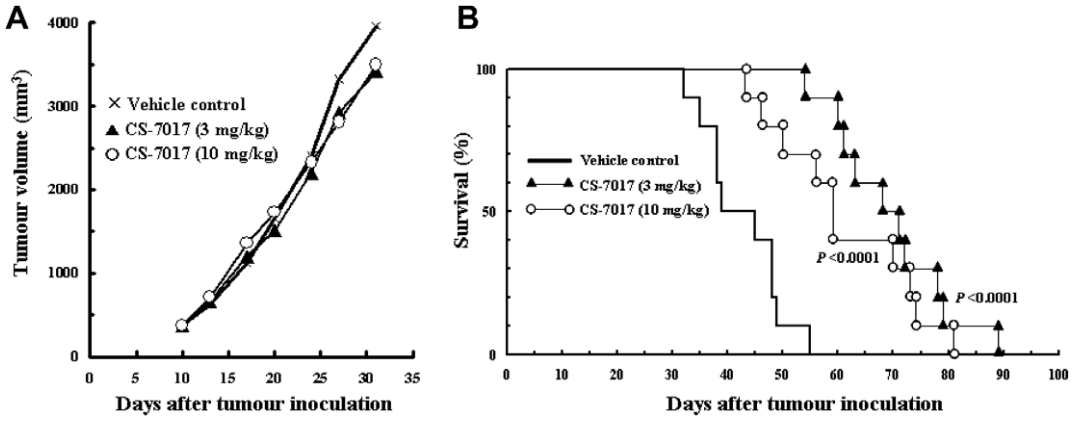

Fig. 5 – The prolongation of the survival of tumour-bearing mice was evaluated. From Day 10 after colon 38 tumour inoculation in BDF_1 mice, CS-7017 was administered orally at 3 mg/kg or 10 mg/kg daily until death. The mean tumour volume of each group ($n = 10$) is shown (A). Kaplan–Meier curves are shown according to the percent of surviving mice in each group (B).

close correlation between low levels of plasma adiponectin and the degree of insulin resistance.[20] Since adiponectin expression is known to be regulated by PPARγ, we examined whether CS-7017 increases adiponectin levels in tumour-bearing mice. CS-7017 was administered to HT-29 tumour-bearing nude mice daily at doses of 0.01, 0.1, 1 and 10 mg/kg for 7 d and the plasma adiponectin levels were measured after the last administration. The mean plasma adiponectin concentration was 11,898 ng/mL in the control group, whereas CS-7017 at the dose of 1 and 10 mg/kg significantly increased the mean adiponectin levels to 21,023 and 20,148 ng/mL, respectively ($p < 0.01$) (Fig. 6). Although a slight increase in the mean plasma adiponectin level was observed in the 0.1 mg/kg administration group, there was no statistical significance. These results combined with those of the anti-tumour dose–response study suggest that the full efficacy of CS-7017 may be achieved at doses of 1 mg/kg and above in murine models.

4. Discussion

CS-7017 belongs to the thiazolidinedione class of compounds and is similar to insulin sensitising agents such as rosiglitazone and pioglitazone. As shown in this report, CS-7017 activates PPARγ-mediated transcription very potently and demonstrates high selectivity for PPARγ amongst the PPAR family when examined in a reporter assay. The potency of PPARγ activation was more than 40-fold stronger than that of rosiglitazone. In this experiment, decreased luciferase activity was observed at high concentrations of both CS-7017 and rosiglitazone. This phenomenon was most likely due to the transcriptional interference (squelching) that resulted from the titration of limited transcriptional co-factors in the forced overexpression of PPARγ.[21] With regard to selectivity, CS-7017 also demonstrated high selectivity for PPARγ amongst PPARα, PPARδ and PPARγ by at least 10,000-fold using a GAL4 system in 293 cells. This selectivity indicates that the anti-tumour activity reported in this paper is likely to be derived from a PPARγ-dependent mechanism. In comparing the two experiments of the reporter assay, inconsistency in the fold activation was observed. In the experiment using a reporter plasmid driven by a P2 enhancer sequence, the level of CS-7017 induced reporter activity was around 3-fold, whilst it was more than 8-fold in the GAL4-driven assay. This difference might be derived from the difference in co-activator recruitment, since the PPARγ fusion protein used in the GAL4 system lacked the N terminus domain of PPARγ, in which the domain contains a phosphorylation residue of Ser118 by MAP kinase, whose phosphorylation plays a critical role in transactivational efficiency by PPARγ.[22]

Although the mechanism by which synthetic PPARγ agonists decrease blood glucose levels in diabetes patients has not yet been completely clarified, these compounds are known to induce terminal differentiation in 3T3-L1 pre-adipocytes to adipocytes via PPARγ activation *in vitro*.[8] Similarly, CS-7017 also causes 3T3-L1 cell differentiation at concentrations lower than approximately 10 nM (data not shown). The retinoid receptor, another nuclear hormone receptor like PPARγ, is also involved in cellular differentiation. Retinoids demonstrate therapeutic benefits in acute promyelocytic leukaemia patients by inducing differentiation in malignant tumour cells.[23] The analogy between retinoid receptor function and that of PPARγ suggests that PPARγ agonists may provide novel intervention in the treatment of other tumour types. In this report, it was demonstrated that CS-7017 induced morphological changes in both DRO and AsPC-1 cells in parallel to its anti-proliferative effects. These morphological changes required a relatively long duration of CS-7017 treatment and could not be observed in short term treatments, such as those for 3 d. In addition, these morphological changes and anti-proliferative effects were observed at physiologically relevant concentrations based on the PPARγ activation by CS-7017 observed in the reporter assay. These morphological effects strongly suggest that CS-7017 might induce a particular type of differentiation in tumour cells. It would be of interest to test whether these morphological changes by CS-7017 were reversible. The investigation of CS-7017 effects on the gene expression profiles in these tumour cells is currently ongoing.

In our *in vitro* studies of anti-proliferative effects of CS-7017, the compound did not appear to induce cytotoxicity, suggesting that the anti-proliferative activity of PPARγ agonists is likely to be cytostatic. Recently, several reports have demonstrated apoptosis induction in many types of tumour cells by troglitazone, another PPARγ agonist, at relatively higher concentrations than those required for PPARγ activation.[24,25] This inconsistency in the concentrations required for PPARγ activation and apoptosis induction is explained by several lines of evidence that suggest that this effect might derive from a PPARγ-independent mechanism.[26] However, the anti-proliferative effects of CS-7017 were demonstrated at concentrations consistent with PPARγ activation in each tumour cell line, suggesting that the anti-tumour activity of CS-7017 is likely to be PPARγ dependent. In addition, we also demonstrated cancellation of the anti-proliferative effects of CS-7017 when co-treated with a PPARγ antagonist, a result consistent with those reported by Copland et al.[17] We concluded that the anti-proliferative effect of CS-7017 in tumour cells that we observed in this report can be explained by a PPARγ-dependent mechanism.

CS-7017 inhibited the growth of HT-29 and DRO xenografts in nude mice and nude rats, respectively. In the HT-29 xenograft study, CS-7017 inhibited tumour growth in a dose-dependent manner with maximum efficacy at doses of 1 mg/kg and above. Since adiponectin, a pharmacodynamic marker of PPARγ, was induced at doses above 1 mg/kg in nude mice, the effective doses for anti-tumour activity and PPARγ activation *in vivo* were consistent. Further investigation is currently ongoing to identify more direct biomarkers in tumours reflecting PPARγ activation. The anti-tumour mode of CS-7017 was tumour growth delay rather than shrinkage, which was also consistent with the observations in the *in vitro* study of its effect on the proliferation of tumour cells.

In a DRO xenograft in nude rats, CS-7017 showed maximum tumour growth inhibition at a dose as low as 0.1 mg/kg. In a previous report, CS-7017 demonstrated anti-tumour activity in DRO-bearing nude mice at the concentration of 0.0025% when administered in the diet, and no efficacy at 0.00025%.[17] These concentrations correspond to approximately 4–5 mg/kg and 0.4–0.5 mg/kg in nude mice, respec-

tively. The difference in effective doses for DRO between nude mice and nude rats may be attributable to a species difference in sensitivity between mice and rats and the effects of CS-7017 on host factors related to tumour progression, for example, inflammation and/or tumour angiogenesis.[27–29] Most interestingly, in addition to tumour growth inhibition in human tumour xenograft models, CS-7017 prolonged the survival of syngeneic tumour-implanted mice without a significant effect on tumour size. The reasons for death in tumour-bearing mice are complicated and are likely to be the integrated results of organ dysfunction due to tumour metastasis, cachexia induction by severe inflammation, etc. PPARγ activation is known to inhibit inflammation.[27] It is possible that the anti-inflammatory effect of CS-7017 may provide a survival benefit in tumour-bearing mice as a part.

In this report, the anti-tumour activity of CS-7017 was demonstrated *in vitro* and *in vivo*. To date, several reports have also shown the preclinical anti-tumour activity of other PPARγ agonists in certain models, including carcinogenesis models and xenograft models.[11] However, pilot clinical trials in cancer patients using PPARγ agonists were not conclusive.[13–16] The issue of whether the optimal doses for diabetes treatment and cancer treatment are similar remains. Further investigation of PPARγ agonists in cancer patients may clarify this issue in the future. The findings described in this report support the administration of CS-7017 in a clinical setting, either alone or in combination with conventional chemotherapy.

Conflict of interest statement

All the authors of this report are employees of Daiichi-Sankyo Co., Ltd. and its subsidiary company.

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