



# Is Genotoxicity of Peroxisome Proliferator-Activated Receptor Agonists Due to Oxidative Stress Via Agonistic Pathways?

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## Research Article

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## Abstract

In order to investigate the relationship between genotoxicity and peroxisome proliferator-activated receptor (PPAR) agonistic effects, we conducted two kinds of comet assays (cellular and acellular), a micronucleus (MN) test, and a TK mutation assay with and without PPAR antagonists using human lymphoblastoid cells. PPAR $\alpha$  agonist clofibrate (CLF) and PPAR $\gamma$  agonists indomethacin (IND) and pioglitazone (PGZ) showed positive responses in the cellular comet assay, TK mutation assay, and detection of intracellular reactive oxygen species (ROS), but not in the acellular comet assay and MN test. PPAR $\alpha$  antagonist (GW6471) suppressed the induction of ROS, DNA damage, and TK mutation by CLF. PPAR $\gamma$  antagonist (BADGE) suppressed the induction of ROS, DNA damage, and TK mutation by IND and PGZ. Therefore, CLF and two PPAR $\gamma$  agonists (PGZ and IND) show genotoxicity by oxidative stress via PPAR $\alpha$  and PPAR $\gamma$  agonistic pathways, respectively. Considering that some unrepaired DNA lesions induced by them persist to form gene mutations but not chromosome aberrations, there is a possibility that their genotoxic potential is due to mutagenic but not clastogenic potential by the production of ROS via agonistic pathway.

**Keywords:** PPAR Agonist; PPAR Antagonist; Genotoxicity; DNA Damage; TK Mutation; ROS; PPAR Agonistic Pathway

## Introduction

Several proliferator-activated receptors (PPAR) agonists have tumor-promoting activity in rodent liver [1] and are classified as "possible human carcinogens" by the Environmental Protection Agency (EPA) [2]. We have previously investigated the genotoxicity of PPAR $\alpha$  agonists perfluoroalkyl acids (PFAAs), using two kinds of comet assays (cellular and acellular), a micronucleus (MN) test, and a TK mutation assay with and without PPAR $\alpha$  antagonists in human lymphoblastoid TK6 cells. In that study, PFAAs at

125-1000  $\mu\text{g}/\text{mL}$  showed positive responses in the cellular comet assay but not in the MN test and TK mutation assay. Based on the findings that PPAR $\alpha$  antagonist GW6471 reduced PFAA-induced DNA damage (in the cellular comet assay) and abolished PFOA-induced intracellular reactive oxygen species (ROS) formation, PFAA-induced DNA damage is partly related to the oxidative stress via PPAR $\alpha$ , without manifestation of chromosome aberration and point mutation [3]. Various PPAR agonists, such as clofibrate, simfibrate ciprofibrate, and phthalate ester plasticizers, increase levels of 8-OHdG [4-6] and ciprofibrate has been shown to induce

DNA adducts in rat liver in the  $^{32}\text{P}$ -postlabeling studies [7]. Furthermore, indomethacin induced gene mutations in *Salmonella* [8]. *In vivo* clonogenicity of indomethacin was also shown in male mice [9]. Although PPAR agonists are considered to be non-genotoxic rodent carcinogens [1], therefore, it remains possible that PPAR agonists are genotoxic. In the present study, we investigated whether DNA damages is induced by PPAR $\alpha$  and PPAR $\gamma$  agonists via PPAR agonistic pathways. For this purpose, we conducted

comet assay, MN test, and *TK* mutation assay using human lymphoblastoid cells.

## Materials and Methods

### Chemicals

Studied PPAR agonists and antagonists are shown in Table 1.

Agonists, antagonists, and CAS No	Abbreviation	Mode of action	Source
Perfluorooctanoic 335-67-1	PFOA	PPAR $\alpha$ agonist	W
Clofibrate 637-07-0	CLF	PPAR $\alpha$ agonist	T
Indomethacin 53-86-1	IND	PPAR $\gamma$ agonist	T
Pioglitazone 111025-46-8	PGZ	PPAR $\gamma$ agonist	S
GW6471 880635-03-0		PPAR $\alpha$ antagonist	To
Bisphenol A 1675-54-3	BADGE	PPAR $\gamma$ antagonist	A

**Table 1:** PPAR agonists and antagonists examined in this study

W: Wako Pure Chemical Industries, Ltd., Osaka, Japan; T: Tokyo Kasei Kogyo; S: Sigma-Aldrich; TO: TOCRIS Bioscience, Minneapolis, MN, USA; A: Abcam Co, Cambridge, UK.

### Cells

The TK $^{+/-}$  heterozygote of the WTK1 human lymphoblastoid cells (kindly provided by Dr. Honma, National Institute of Health Sciences) and L5178Y mouse lymphoblastoid cells were maintained in culture using RPMI1640 medium (Nissui Pharmaceutical Co., Ktd.) supplemented with 10% horse serum (SAFC Biosciences), 200 $\mu\text{g}/\text{mL}$  sodium pyruvate, and 200 $\mu\text{g}/\text{mL}$  streptomycin at 37°C under 5% CO $_2$  atmosphere. Cells were maintained in logarithmic growth. Cells at 5 x 10 $^5$  cells/mL were exposed to each PPAR agonist for 4h with and without PPAR antagonists. After exposure, the cell cultures were centrifuged, washed twice with Hanks' BSS, and re-suspended in the fresh medium. The cell cultures were used for the comet assay, MN test, and *TK* gene mutation assay.

### Comet Assay

Both cellular and acellular comet assays were conducted. The cellular comet assay was generally conducted as previously described [10], immediately after chemical treatment, with or without the combination of DNA repair inhibitors of cytosine-1- $\beta$ -D-arabinofuranoside (araC) (1.8 mM) and hydroxyurea (HU) (10 mM). The two inhibitors were used at concentrations that did not induce significant reductions in cell viability [10]. Immediately after chemical treatment, cell viability was also measured using the trypan blue exclusion test. Relative survivals (survivals of treated cells compared with untreated control cells) were obtained. The cells were embedded in LGT agarose (Nakalai Tesque,

Kyoto, Japan) dissolved in saline at 1%. Then, slides were placed in a chilled lysing solution (2.5M NaCl, 100mM Na $_4$ EDTA, 10 mM Tris-HCl, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 0°C in the dark for >60 min, then the slides were placed on a horizontal gel electrophoresis platform, and covered with pH>13 alkaline solution comprised of 300mM NaOH and 1mM Na $_2$ EDTA for 20 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 20min at 25V (0.96V/cm, approximately 300 mA). In the acellular comet assay, slides embedded with untreated WTK1 cells were lysed as indicated above. The lysed slides were then neutralized in 400mM Tris HCl buffer (pH 7.5) for 15 min, and then treated with PPAR agonists in the buffer for 4h at room temperature in the dark. After treatment, the slides were rinsed three times for 5min by immersing in cold distilled water, placed on a horizontal gel electrophoresis platform, and covered with pH >13 alkaline solution comprised of 300 mM NaOH and 1 mM Na $_2$ EDTA, then electrophoresed as described above. For both the cellular and acellular comet assays, neutralized slides were stained with 50 $\mu\text{L}$  of 2  $\mu\text{g}/\text{mL}$  ethidiumbromide. Photographs of comet images were taken using Fuji Neopan Presto 400 Black & White film and tail length was measured manually using a scale for 50 nuclei for each dose.

### MN Test

WTK1 cells were exposed to each PPAR agonist for 4 h as described above. At the end of the treatment period, the cells were washed with Hanks' BSS, cultured for 24h in medium containing 3  $\mu\text{g}/\text{mL}$  cytochalasin B, and then

sampled. The collected cells were suspended in 0.075M KCl hypotonic solution for 15 min, and the cell suspension was concentrated to the volume of 1 mL, mixed with 1 mL of 10% neutral buffered formalin solution, and then concentrated to a volume of 100 $\mu$ L. The cell suspension was further mixed with 100  $\mu$ L of 0.05 w/v% aqueous solution of acridine orange, and then 50  $\mu$ L of cell suspension was put onto a slide glass and mounted with a 24 x 48 mm cover slip. Bi-nuclei cells with micronuclei (MNBNC) at 1000 bi-nuclei cells (BNC) and BNC at 1000 cells were scored with the aid of a fluorescence microscope (Olympus at 600x magnification) equipped with a B filter.

### TK mutation assay

To evaluate cytotoxicity, cells treated for 4 h were seeded onto 96-well cluster dishes at 1.6 cells/well and cultured for 12 days, and then the numbers of wells with colonies were recorded. To detect *TK* mutations, cells treated for 4 h were cultured in fresh medium for 3 days, and then cells were seeded onto 96-well cluster dishes at 2000 cells/well in fresh medium with 3.0  $\mu$ g/mL trifluorothymidine (TFT) and 1.6 cells/well in fresh medium without TFT. For cells cultured with TFT, the numbers of wells with normally growing (NG) and slowly growing (SG) colonies were recorded to evaluate TFT resistant mutation frequency after 12 and 30 days, respectively.

### Detection of Intracellular Reactive Oxygen Species (ROS) Formation

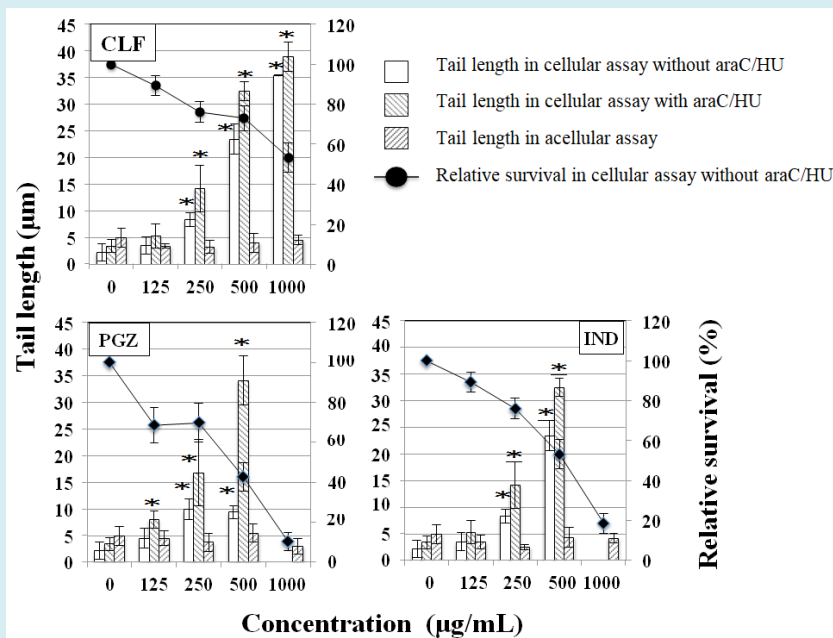
The intracellular ROS was detected as previously described [3]. WTK1 cells were cultured for 1 h with 50  $\mu$ M BES-H<sub>2</sub>O<sub>2</sub>-Ac (Wako Pure Chemical Industries, Ltd.), exposed to each PPAR agonist or H<sub>2</sub>O<sub>2</sub> for 30 min with and without PPAR antagonists, and then the frequency of fluorescent cells was scored with the aid of a fluorescence microscope (Olympus at 600x magnification) equipped with a B filter [3].

### Statistics

Differences between the means of triplicated studies in treated and control groups were compared with the Dunnett test after one-way ANOVA. For each pairwise comparison, Student's t-test was used. A p-value less than 0.05 was considered significant.

### Results

In the cellular comet assay, the studied PPAR agonists at >250 $\mu$ g/mL significantly increased tail length in the presence and absence of a combination of DNA repair inhibitors of araC and HU, and tail length was longer with than without DNA repair inhibitors. In the acellular comet assay, PPAR agonists did not significantly increase tail length (Figure 1).

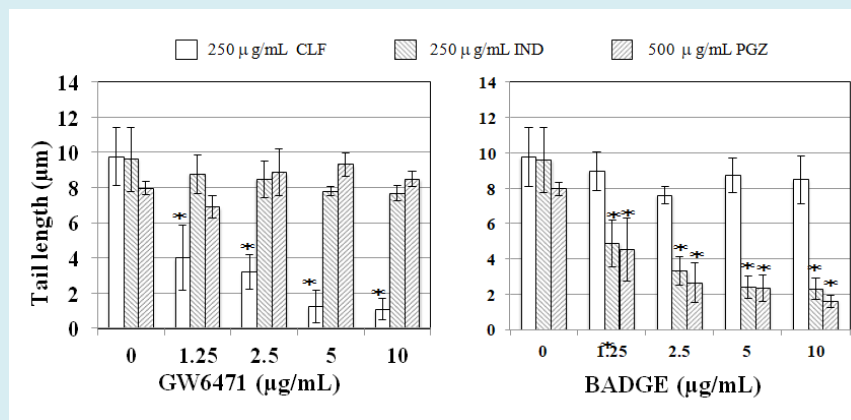


**Figure 1:** Tail length and relative survival by CLF, IND, and PGZ in the acellular and cellular comet assay. Tail length with and without araC/HU and relative survival without araC/HU (% survival compared with the untreated control) were obtained immediately after exposure to each chemical for 4 h. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

\*Significantly higher than untreated cells:  $p < 0.05$ .

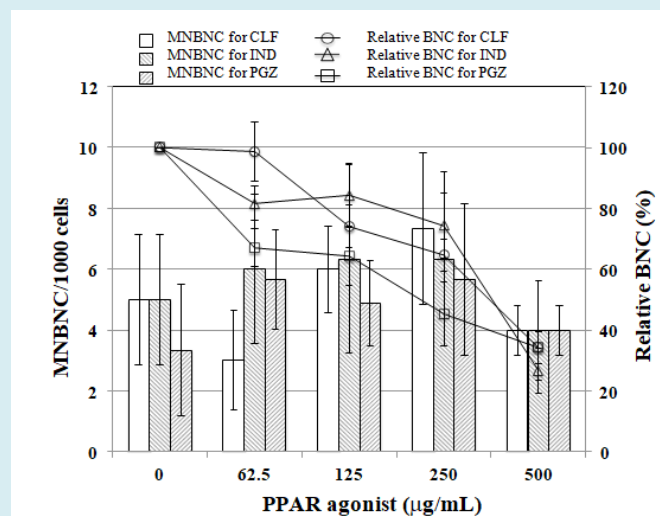
PPAR $\alpha$  antagonist GW6471 reduced tail length induced by CLF but not by IND and PGZ. On the contrary, PPAR $\gamma$  antagonist BADGE reduced tail length induced by IND and PGZ but not by CLF (Figure 2). In the MN test, no significant increase in the frequency of MNBNCs was observed in cells treated with CLF, IND, and PGZ for 4 h at the concentration range where remarkable reduction in relative BNC was not observed (Figure 3). In the *TK* mutation assay with WTK1 cells (Figure 4), CLF, IND, and PGZ increased the frequency of NG but not SG mutant colonies. The ratio of SG mutant colony to total mutant colony (%SG) decreased dose-dependently. PFOA increased the frequency of NG mutant colonies in WTK1 and L5178Y cells (Figure 5). The frequencies of SG

mutant colonies were increased by 62.5 and 125  $\mu\text{g/mL}$  PFOA in WTK1 and L5178Y cells. Although %SG decreased dose-dependently at >15.6  $\mu\text{g/mL}$  PFOA and reached to plateau (about 30%) at >31.3  $\mu\text{g/mL}$  in L5178Y cells, it increased dose-dependently and reached >50% at 125  $\mu\text{g/mL}$  in WTK1 cells. The frequency of total mutant colony was about 8-times higher in L5178Y cells than in WTK1 cells. The induction of mutant colony by CLF was observed with PPAR $\gamma$  antagonist BADGE but reduced with PPAR $\alpha$  antagonist GW6471 (Figure 6). On the contrary, the induction of mutant colonies by IND and PGZ was observed with PPAR $\alpha$  antagonist GW6471 but was reduced with PPAR $\gamma$  antagonist BADGE (Figure 6).

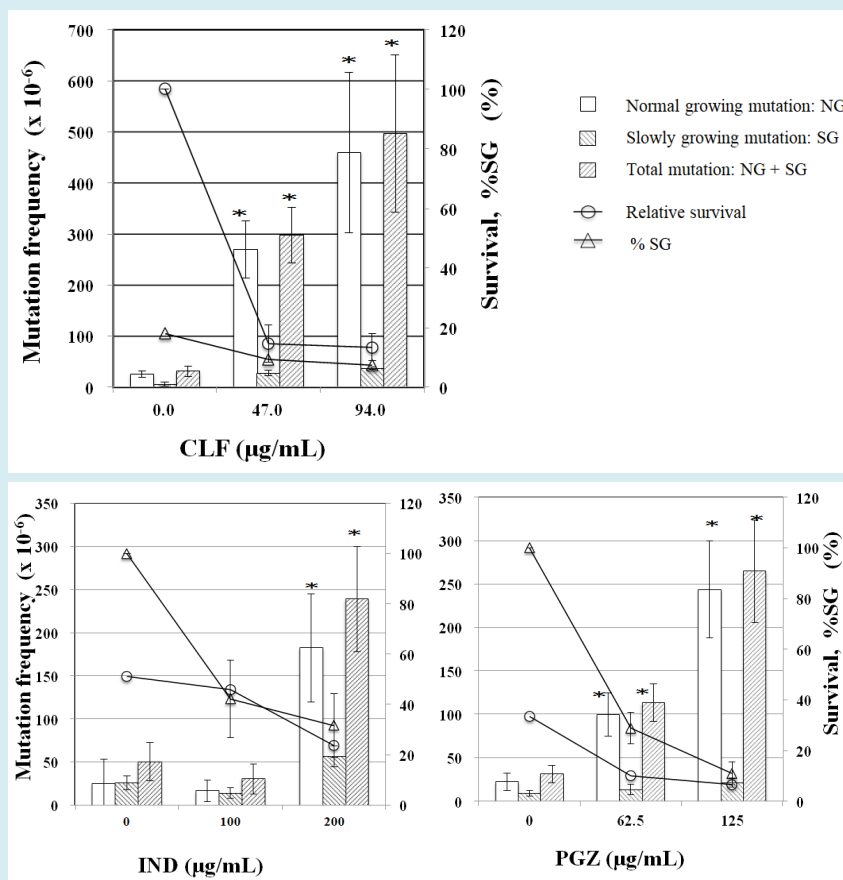


**Figure 2:** Effect of PPAR antagonists on DNA migration by CLF, IND, and PGZ in the cellular comet assay. Tail length was obtained immediately after the exposure to 250  $\mu\text{g/mL}$  CLF, 250  $\mu\text{g/mL}$  IND, or 500  $\mu\text{g/mL}$  PGZ for 4 h with a PPAR antagonist without araC/HU. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

\*Significantly lower than without PPAR antagonist:  $p < 0.05$ .

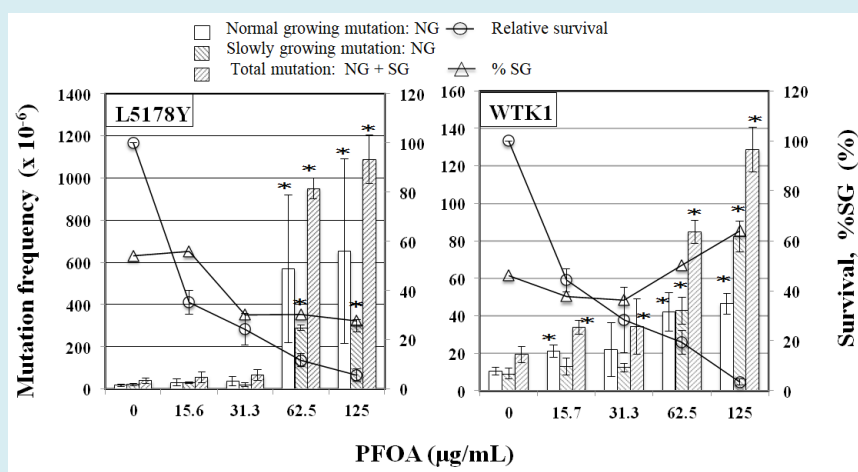


**Figure 3:** Results of MN test with CLF, IND, and PGZ. The frequencies of MNBNC and BNC were obtained after 24 h cultivation with cytochalasin B following exposure to CLF, IND, and PGZ for 4h. The mean of three trials is shown. The error bars indicate standard deviation of the mean of three independent trials.



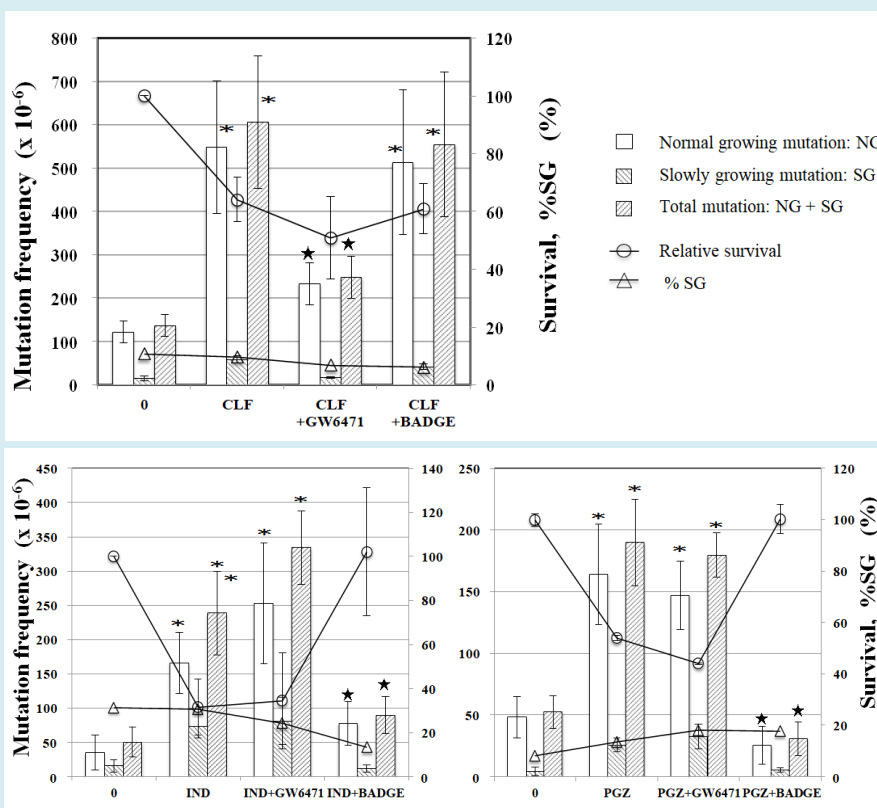
**Figure 4:** TK mutation induction by CLF, IND, and PGZ. The frequency of TK mutation was obtained after a 3-day expression period following exposure to each chemical for 4h. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

\*Significantly higher than untreated cells:  $p < 0.05$ .



**Figure 5:** TK mutation induction by PFOA in WTK1 and L5178Y cells. The frequency of TK mutation was obtained after a 3-day expression period following exposure to each chemical for 4h. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

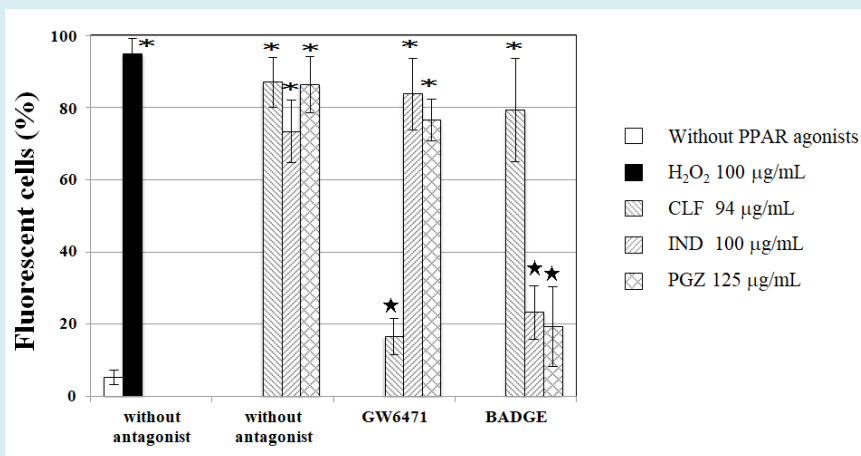
\*Significantly higher than untreated cells:  $p < 0.05$



**Figure 6:** Effect of PPAR antagonists on TK mutation induction by CLF, IND, and PGZ. The frequency of TK mutation was obtained after a 3-day expression period following exposure to 94  $\mu\text{g/mL}$  CLF, 100  $\mu\text{g/mL}$  IND, or 125  $\mu\text{g/mL}$  PGZ with or without 4  $\mu\text{g/mL}$  PPAR antagonists. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

\*Significantly higher than untreated cells:  $p < 0.05$ .

★ Significantly lower than without PPAR antagonist:  $p < 0.05$ . 30



**Figure 7:** Effects of PPAR antagonists on CLF-, IND-, and PGZ-induced intracellular ROS. Fluorescent cells were scored immediately after 30 min exposure to CLF, IND, or PGZ with and without 4  $\mu\text{g/mL}$  PPAR antagonist GW6471 or BADGE after 1h incubation with 50  $\mu\text{M}$  BES- $\text{H}_2\text{O}_2$ -Ac. Mean of three trials are shown. The error bars indicate standard deviation of the mean of three independent trials.

\*Significantly higher than untreated cells:  $p < 0.05$ .

★ Significantly lower than without PPAR antagonist:  $p < 0.05$ .

The induction of intracellular ROS is shown in Figure 7. Not only H<sub>2</sub>O<sub>2</sub> but also 3 PPAR agonists (CLF, IND, and PGZ) without PPAR antagonists increased the frequency of fluorescent cells, showing the induction of intracellular ROS. Significant differences in the frequencies of fluorescent cells by CLF were not observed between with and without PPAR $\gamma$  antagonist BADGE, but the frequency of fluorescent cells by CLF was significantly lower with than without PPAR $\alpha$  antagonist GW6471. Significant differences in the frequencies of fluorescent cells by IND and PGZ were not observed between with and without PPAR $\alpha$  antagonist GW6471, but the frequencies of fluorescent cells by IND and PGZ were significantly lower with than without PPAR $\gamma$  antagonists BADGE.

## Discussion

In the acellular assay, since lysed cells are exposed to test compounds, it detects only single strand breaks (SSBs) as initial lesions produced by the direct reaction between DNA and test compounds without any biological events [11]. Previously, PFOA, a PPAR $\alpha$  agonist, showed positive response in the acellular comet assay where there is no cellular function, such as PPAR, from which PFOA-induced DNA damage was considered to be caused by two distinct pathways: (1) oxidative stress via PPAR $\alpha$  agonistic pathway and (2) direct reaction of PFOA with DNA or remaining cellular components such as proteins [3]. The induction of ROS by PPAR $\alpha$  agonist CLF and PPAR $\gamma$  agonists (IND and PGZ) was suppressed by PPAR $\alpha$  and PPAR $\gamma$  antagonists but not by PPAR $\gamma$  and PPAR $\alpha$  antagonists, respectively, supporting that PPAR agonists induce ROS via PPAR agonistic pathways. Furthermore, the PPAR agonists did not show positive responses in the acellular comet assay, which supports the ruling out of genotoxic mechanisms through the induction of SSBs as initial damage without any cellular functions. For SSBs as initial lesions and DNA lesions that may be repaired by excision repair, tail length is greater with than without DNA repair inhibitors [10]. Tail length by CLF, IND, and PGZ was greater with than without araC/HU, supporting that they induced DNA lesions that can be repaired by the excision repair. Considering that oxidized bases are removed by excision repair [11] and that CLF, IND, and PGZ were shown to induce ROS in the present study, it is possible that DNA damages induced by CLF, IND, and PGZ through PPAR agonistic pathways are oxidized bases, which coincides with that CLF increased levels of 8-OHdG [4].

The comet assay detects not only SSBs as initial DNA damage but also SSBs that are developed from alkali-labile sites under alkaline conditions [12]. The MN test detects structural chromosome aberrations and/or numerical chromosome aberrations due to aneugenic effects [13]. The *TK* mutation assay detects point mutations and

gross structural changes that may result in chromosome aberrations [14]. CLF, IND, and PGZ showed positive responses in the cellular comet and *TK* mutation assays but not in the acellular comet assay and MN test, indicating that they induced DNA damages that can form point mutations but not chromosome aberrations. PPAR $\alpha$  antagonist GW6471 reduced the induction of DNA damage and *TK* mutation by CLF in the cellular comet assay and *TK* mutation assay, but did not affect the induction of DNA damage and *TK* mutation by IND and PGZ. PPAR $\gamma$  antagonist BADGE reduced the induction of DNA damage and *TK* mutation by PGZ and IND but not by CLF. Therefore, PPAR $\alpha$  agonist (CLF) and PPAR $\gamma$  agonists (PGZ and IND) may have mutagenic potential via PPAR $\alpha$  agonistic and PPAR $\gamma$  agonistic pathways, respectively.

Considering the carcinogenic process, it is important to inspect whether DNA primary lesions are repaired, un-repaired DNA lesions result in cytotoxicity, or un-repaired DNA lesions form chromosome aberrations and/or gene mutations. In the present *in vitro* study, we compared the induction of DNA lesions, chromosome aberrations (micronuclei), and *TK* gene mutations. Both CLF and IND led to positive responses in the cellular comet assay and *TK* gene mutation assay but not in the MN test, suggesting that un-repaired DNA lesions induced by them persist to form gene mutations but not chromosome aberrations. Therefore, it is possible that genotoxicity of PPAR agonists via PPAR dependent pathways may be correlated with carcinogenicity.

In this study, the PPAR $\alpha$  agonist PFOA induced *TK* mutation in both WTK1 and L5178Y cells, which seems to contradict our previous findings that PFOA induced DNA damage but not *TK* mutation in human lymphoblastoid TK6 cells [3]. The results suggest that PFOA-induced DNA damage persist to form point mutations in WTK1 but not in TK6 cells. Although TK6 have a functional *p53*, WTK1 and L5178Y cells have a mutated *p53*. Therefore, the discrepancy between WTK1 and TK6 cells in the induction of *TK* mutation by PFOA is most likely attributed to *p53* functionality rather than cell origin (human vs mice). Although PFOA induced *TK* mutation in both WTK1 and L5178Y cells, mutation frequency is about 8-times higher in L5178Y than in WTK1 cells, which is concordant with the finding that PPAR $\alpha$  expression in rodents is about 10 times higher than that in humans [2].

In TK6 and WTK1 cells, NG mutants are produced mainly as a result of point mutations in the *TK* locus, whereas SG mutants are induced by gross structural changes outside the *TK* locus [15]. In the present study, CLF, IND, and PGZ increased the frequency of NG but not SG mutants, suggesting that observed gene mutations are due to point mutations in the *TK* locus but not gross structural changes. CLF, IND, and PGZ did not induce MNBNC, which coincides with the finding that they did not induce SG mutants. Therefore, our

results suggest that the genotoxicity of these agonists is due to mutagenic but not clastogenic potential. On the other hand, PFOA increased both NG and SG mutants, suggesting that the genotoxicity of PFOA is due to both mutagenic and clastogenic potential.

## Conclusion

The genotoxicity of PPAR agonists except for PFOA is due to mutagenic but not clastogenic potential by the production of ROS via agonistic pathway.

## Conflicts of Interest

The authors declare that the research was conducted in the absence of any intellectual commercial or financial relationships that could be construed as potential conflicts of interest.

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