Small Molecule Agonists of PPAR-γ Exert Therapeutic Effects in Esophageal Cancer

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Abstract

The transcription factor PPAR-γ plays various roles in lipid metabolism, inflammation, cellular differentiation, and apoptosis. PPAR-γ agonists used to treat diabetes may have utility in cancer treatment. Efatutazone is a novel later generation PPAR-γ agonist that selectively activates PPAR-γ target genes and has antiproliferative effects in a range of malignancies. In this study, we investigated PPAR-γ status in esophageal squamous cell carcinoma (ESCC) and investigated the antiproliferative effects of efatutazone. PPAR-γ was expressed heterogeneously in ESCC, in which it exhibited an inverse relationship with Ki-67 expression. PPAR-γ expression was associated independently with good prognosis in ESCC. Efatutazone, but not the conventional PPAR-γ agonist troglitazone, inhibited ESCC cell proliferation in vitro and in vivo. Mechanistic investigations suggested that efatutazone acted by upregulating p21Cip1 protein in the nucleus through inactivation of the Akt pathway and dephosphorylation of p21Cip1 at Thr145 without affecting the transcriptional activity of p21Cip1. We also found that treatment with efatutazone led to phosphorylation of the EGF receptor and activation of the mitogen-activated protein kinase (MAPK) pathway. Accordingly, the combination of efatutazone with the anti-epithelial growth factor receptor antibody cetuximab synergized to negatively regulate the phosphoinositide 3-kinase–Akt and MAPK pathways. Together, our results suggest that efatutazone, alone or in combination with cetuximab, may offer therapeutic effects in ESCC. Cancer Res; 74(2): 575–83. ©2013 AACR.

Introduction

PPAR-γ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1). PPAR-γ plays a variety of roles in adipose cell differentiation, modulation of metabolism, and the inflammatory response (2, 3). The protein interacts with and/or regulates multiple signaling pathways, including those associated with p21Cip1 (4, 5) and p27 (6, 7) to regulate the cell cycle, with nuclear factor kappa B (8) to reduce the expression of cytokines such as interleukin-6, and interleukin-8, and with cyclooxygenase-2 (9) to reduce the expression of cytokines such as interleukin-6, and interleukin-8, and with cyclooxygenase-2 (9, 10). Genetic studies have indicated that PPAR-γ functions as a tumor suppressor in a variety of tissues, including the breast (11), prostate (12), and colon (13). Ligands for PPAR-γ include endogenous ligands such as fatty acids, Δ12,15 prostaglandin J2 (14), and exogenous ligands, such as the thiazolidinedione class of antidiabetic drugs including troglitazone, rosiglitazone, and pioglitazone (15, 16). Numerous recent studies have demonstrated that PPAR-γ ligands exert antiproliferative effects. The first-generation thiazolidinedione troglitazone showed antiproliferative effects in hepaticocellular carcinoma (7), colorectal cancer (17, 18), and breast cancer (19). However, these effects were limited, and small clinical trials showed no beneficial effects of these conventional PPAR-γ ligands (20, 21).

Efatutazone is a novel third-generation thiazolidinedione PPAR-γ agonist, which is at least 500-fold more potent than troglitazone in terms of PPAR response–element activation and inhibition of cancer cell growth, rather than inducing apoptosis (4). Furthermore, efatutazone demonstrated antitumor effects in patients with advanced malignancies (22). This agent therefore shows great potential for practical cancer therapy.

Combination therapy using efatutazone with antitumor agents represents an important means of achieving significant tumor regression (22). PPAR-γ agonists have shown activity both in vitro and in vivo in combination with conventional anticancer drugs including platinum-based drugs (23), taxanes (4), and irinotecan (24). However, the pathways affected by

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-13-1836
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treatment with efatutazone, and the most suitable combination therapy (in terms of molecular-targeted agents) based on theoretical signaling mechanisms remain unclear.

Esophageal carcinoma affects more than 450,000 people worldwide, and its incidence is increasing rapidly. SCC is the predominant form of esophageal carcinoma worldwide (25). Furthermore, esophageal carcinoma is an aggressive disease with a propensity to spread both locoregionally and distally, and multidisciplinary therapy has therefore been tested for esophageal squamous cell carcinoma (ESCC; refs. 26, 27). The antiepithelial growth factor receptor (EGFR) antibody, cetuximab, was approved for head and neck SCC (28, 29), and new molecular-targeted therapies have been expected for the treatment of ESCC (30).

The effects of PPAR-γ are tissue and cancer specific. The significance of PPAR-γ expression and antiproliferative effects of PPAR-γ agonists in ESCC have been reported in a few studies, however these studies were limited because they only evaluated the mRNA levels in small number of samples (31), in adenocarcinomas (32), or using conventional PPAR-γ agonists, such as troglitazone (33). Therefore the expression of the PPAR-γ protein in ESCC and the antiproliferative effects of the new generation PPAR-γ agonist remained unclear. In this study, we investigated the relationship between the expression levels of PPAR-γ and Ki-67 in patients with ESCC, and examined the antiproliferative effects and underlying mechanism of efatutazone monotherapy both in vitro and in vivo. We also investigated the effects of combination therapy using efatutazone with molecular-targeted agents, based on signaling analysis. The results of this study may provide a novel therapeutic strategy for ESCC, and suggest that efatutazone, both alone and in combination with an anti-EGFR antibody, may improve the outcomes of patients with ESCC.

Materials and Methods

Chemicals

Efatutazone was kindly provided by Daiichi Sankyo, Inc., Troglitazone, U0126, MK-2206 dihydrochloride, and cetuximab were purchased from Cayman, Wako, Selleck Bio., and Kumamoto University Hospital, respectively. For the in vitro analyses, efatutazone was prepared in dimethyl sulfoxide (DMSO) before addition to cell cultures. Antibody information is provided in the Supplementary Materials and Methods.

Patients

This study involved 145 consecutive patients who underwent surgical resection of ESCC at Kumamoto University Hospital from January 2000 to December 2008 (Supplementary Fig. S1).

Cell lines

ESCC cell lines (TE series) and HT-29 were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, the Riken BioResource Center Cell Bank, and Keio University. The cell lines have been tested and authenticated by Cell ID System in October 2013. Cell lines (TE series and HT-29) were cultured in 5% CO2 at 37°C in RPMI 1640 supplemented with 10% FBS.

Xenograft model

Six-week-old nude mice (Balb-nu/nu sc) were inoculated subcutaneously in the right flank with 5 × 10^6 TE-4 cells in 200 mL PBS containing 50% Matrigel (BD, Recton, Dickinson and Company). When the tumors reached approximately 80 mm^3 in diameter, the mice were randomized into treatment groups. This subcutaneous xenograft model was used to assess the therapeutic effects of efatutazone (10 mg/kg) and troglitazone (10 mg/kg) as single agents. Control animals received 0.5 w/v% methylcellulose solution (vehicle). Efatutazone and troglitazone were suspended in 0.5 w/v% methylcellulose solution and administered to the animals daily by oral gavage in a volume of 0.1 mL/10 g body weight, using an animal-feeding needle. The anti-EGFR antibody, cetuximab (1 mg/injection) or placebo (PBS) was injected intraperitoneally twice a week to assess the effects of combination therapy with efatutazone. Caliper measurements were made twice a week using digital calipers, and the tumor volumes were estimated using the following formula: \( V = \frac{L \times W \times D}{2} \), where \( V \) is the tumor volume, \( L \) is the length, \( W \) is the width, and \( D \) is the depth (30, 34).

Statistical analysis

Comparisons between treatment groups were made using 2-tailed paired or unpaired Student t tests, as appropriate, based on the results of F-tests. The Mann–Whitney U test was used in the event of a nonnormal distribution. The log-rank test was used for survival analysis, and the Kaplan–Meier method was used to assess survival time distribution. For analyses of esophageal cancer-specific mortality, deaths as a result of causes other than ESCC were censored. Univariate Cox regression analysis was also performed. The independent effect of PPAR-γ on mortality was assessed by performing multivariate Cox regression analysis. Statistical significance was defined as a P value <0.05. All data were processed and analyzed using the PASW Statistics 18 software program.

Study approval

All animal procedures and care were approved by the Animal Care and Use Committee of Kumamoto University (approval number H24347). Written informed consent was obtained from the human subjects.

Results

PPAR-γ expression in the normal epithelium and ESCC

We investigated PPAR-γ expression in normal esophageal epithelial samples and tumor lesions from 145 patients with resectable ESCC using immunohistochemical analysis (Supplementary Table S1). PPAR-γ was ubiquitously expressed in the squamous layer of the normal esophageal epithelium in all 145 patients, and there was no variation (Fig. 1A). The positive staining of PPAR-γ was detected in 36 (24.8%) tumors in patients with ESCC. PPAR-γ expression exhibited a significant inverse relationship with Ki-67 expression (\( P < 0.001 \), Mann–Whitney U test; Fig. 1B–D).

A recent study reported on the relationship between PPAR-γ expression and prognosis in colorectal cancer (35), and PPAR-γ mRNA expression has demonstrated prognostic value in ESCC.

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Cancer Res; 74(2) January 15, 2014

Cancer Research

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(n = 55; ref. 31). However, the relation between PPAR-γ protein expression and prognosis in ESCC remains unclear. According to this study, PPAR-γ expression was associated with a good prognosis in terms of disease-free survival (log-rank P = 0.035; Fig. 1E) and esophageal cancer-specific survival (log-rank P = 0.033; Fig. 1F) in the 145 patients with ESCC according to univariate and multivariate Cox regression analysis (Supplementary Table S2).

We therefore hypothesized that PPAR-γ was associated with tumor-suppressive effects against ESCC, and that efatutazone, a novel third-generation PPAR-γ agonist, could be a potentially useful anticancer agent in patients with ESCC.

PPAR-γ expression in ESCC cell lines and antiproliferative effects of efatutazone

PPAR-γ expression was examined in 9 human ESCC cell lines and in the colon cancer cell line HT-29, which was used as a positive control for PPAR-γ expression (36). Various levels of PPAR-γ expression were observed in the ESCC cell lines (Fig. 2A). However, efatutazone demonstrated antiproliferative effects in all 9 ESCC cell lines (Fig. 2B). We investigated antiproliferative effects and underlying mechanism of efatutazone using TE-4, TE-8, and TE-11, TE-6 cells, which express high, medium and low levels of PPAR-γ, respectively.

We used 3 siRNAs for PPARG to confirm that the antitumor effects of efatutazone occurred in a PPAR-γ-dependent manner. We selected TE-8 cells, because the PPAR-γ expression of TE-8 cells was strongly suppressed at both the mRNA and protein levels using siRNAs for PPARG (Fig. 2C and D; Supplementary Fig. S2A and S2B). TE-8 ESCC cells were transfected with PPARG siRNA for 48 hours, followed by treatment with control (DMSO) or 50 μmol/L efatutazone for 48 hours. In the presence of the scrambled siRNA (negative control), efatutazone enhanced the expression of pyruvate dehydrogenase kinase isozyme-4 (PDK4), which is an established marker for the activity of PPAR-γ as a transcriptional factor (37, 38), by 2.5-fold compared with the control level, but this effect was blocked in the presence of PPARG siRNA (Fig. 2E). The inhibition of proliferation by efatutazone was related to the PDK4 mRNA level (Fig. 2F). Efatutazone inhibited the proliferation of the ESCC cell lines in a time-dependent manner (Fig. 2G), and PDK4 levels were also increased in a similarly time-dependent manner and correlated with antiproliferative effect of efatutazone (Fig. 2H). The mRNA level of PDK4 after treatment with 25 μmol/L efatutazone was increased more than after treatment with 25 μmol/L troglitazone (Supplementary Fig. S3A). No significant antiproliferative effects of treatment with troglitazone were detected compared with the
control, however, the proliferation of both TE-4 and TE-8 cells was inhibited by the treatment with efatutazone (Supplementary Fig. S3B). Because the antiproliferative effects of efatutazone were observed in various ESCC cell lines, we investigated the mechanism underlying the antiproliferative effects of efatutazone using TE-4 cells, which highly express PPAR-γ.

**In vitro antiproliferative effects of efatutazone in ESCC cells and dependence on Akt-p21Cip1**

Efatutazone was previously reported to inhibit cell cycle progression via p21Cip1 in anaplastic thyroid carcinoma (4, 5). In order to clarify the effects of efatutazone on the cell cycle in ESCC, we subjected TE-4 and TE-11 cells to cell cycle analysis by flow cytometry. Accumulation of cells in the G1 phase, and reductions in the S and G2/M phases occurred after treatment with efatutazone for 48 hours (Fig. 3A, Supplementary Fig. S1A). In addition, PDK4 mRNA levels were upregulated by treatment with efatutazone for 48 hours, whereas p21Cip1 mRNA levels remained unaffected by the treatment (Fig. 3). However, p21Cip1 protein levels were significantly upregulated in Western blot analysis (Fig. 3C). We therefore focused on the posttranslational modification of the p21 protein (39).

Activation of Akt, which associates with p21Cip1 and phosphorylates it at threonine 145 (Thr145), results in increased cytoplasmic localization of p21Cip1 in breast cancer (40). We therefore stimulated the phosphoinositide 3-kinase (PI3K)–Akt pathway with EGF (100 ng/mL) to investigate these signaling changes in the ESCC cell lines. We found that Akt was activated by treatment with EGF, and p21Cip1 was then gradually phosphorylated at Thr145 in ESCC cells (Fig. 3D).

We treated TE-4 cells with efatutazone for 48 hours, followed by stimulation with or without EGF for 10 minutes to determine if efatutazone was associated with inactivation of Akt. Efatutazone reduced the phosphorylation of both Akt at Ser473 and p21 at Thr145 (Fig. 3E). Localization of p21Cip1 was confirmed by confocal microscopy. Some p21Cip1 protein was detected in the cytoplasm in control cells, but strong staining was detected in the nucleus following treatment with efatutazone for 48 hours (Fig. 3F).

The PPAR-γ expression of TE-6 cells was lower (Fig. 2A) and the antiproliferative effects of treatment with efatutazone were...
lower than those observed in the other ESCC cell lines (Fig. 2B). We showed that the dephosphorylation of both Akt Ser473 and p21 Thr145 in the TE-6 cells was lower than that observed in TE4 cells (Fig. 3E and Supplementary Fig. S4B). Treatment with efatutazone could not dephosphorylate Akt at Ser473 in TE-8 cells after treatment with siRNA for PPARG (Supplementary Fig. 4C). These data indicated that inactivating the Akt pathway was one of the major targeted effects of efatutazone.

Furthermore we used the Akt inhibitor MK-2206 to detect the association between the antiproliferative effects of efatutazone and inactivation of Akt. The antiproliferative effects of efatutazone compared with the control decreased with increasing dose of MK-2206 (Fig. 3G). The antiproliferative effects were correlated with p21 protein levels and were inversely related to phosphorylation of p21 at Thr145 (Fig. 3H).

These data indicate that efatutazone regulates p21Cip1 protein levels in the nucleus by inactivating Akt and dephosphorylating p21 at Thr145, without affecting the transcription of p21Cip1.

**In vivo antiproliferative effects of efatutazone**

To the best of our knowledge, no previous studies have demonstrated the antiproliferative effects of conventional PPAR-γ agonists, such as troglitazone, against ESCC cell lines in vivo. We therefore compared 3 treatments in a mouse xenograft model established using TE-4 ESCC cells. Animals were divided into a control group, a troglitazone group and an efatutazone group. Tumor volume did not differ significantly between the control group and the troglitazone group; however, a 49.6% ± 13.6% (average ± SD) reduction in tumor volume was observed in the efatutazone group compared with the control group (Fig. 4A, Supplementary Fig. S5A). The mRNA expression of PLIN2, one of the markers for the activity of PPAR-γ as a transcriptional factor, was doubled in the efatutazone group compared with the control group. The mRNA expression levels of p21Cip1 and p27 were not increased by efatutazone treatment (Fig. 4B, Supplementary Fig. S5B), but p21Cip1 protein levels were significantly upregulated (Fig. 4C).

We therefore investigated the phosphorylation status of Akt at Ser473 and of p21 at Thr145. Inactivation of Akt and
dephosphorylation of p21 at Thr145 were detected more frequently in the efatutazone group than the control group (Fig. 4C). Immunohistochemical staining also demonstrated dephosphorylation of Akt at Ser473 and p21 at Thr145 in the cytoplasm in the efatutazone group (Fig. 4D). We immunohistochemically examined the expression levels of both p21Cip1 and Ki-67 in the nucleus and found that the rate of positive staining for p21 and Ki-67 in the nucleus of TE-4 xenografts was significantly increased in the efatutazone group compared with both the control and troglitazone groups.

**Efatutazone activates EGFR/MAPK signaling**

The antiproliferative effects of efatutazone were associated with dephosphorylation of Akt at Ser473. Because Akt is downstream of EGFR signaling via the PI3K–Akt pathway, we investigated the phosphorylation of EGFR at tyrosine 1068 (Tyr1068). We confirmed that efatutazone inactivated Akt pathway within 3 hours, whereas efatutazone gradually phosphorylated EGFR at Tyr1068 for 24 hours in TE-4 cells in vitro (Fig. 5A). Furthermore, using conditioned medium (Supplementary Fig. S6), we found that phosphorylation of EGFR was not caused by efatutazone directly, but was induced by medium from efatutazone-treated in TE-4 cells (Fig. 5B). These data indicated that secreted factors after treatment with efatutazone stimulated phosphorylation of EGFR at Tyr1068 in ESCC cells. We confirmed that activated EGFR was detected in 3 ESCC cell lines, TE-4, TE-8, and TE-11 cells after treatment with efatutazone for 48 hours in vitro (Fig. 5C). Tyr1068 of EGFR was significantly phosphorylated following treatment with efatutazone, and extracellular signal–regulated kinase (ERK) 1/2 was phosphorylated at Thr202/Tyr204 in TE-4 xenograft tumor lysates, compared with both control and troglitazone-treated tumors (Supplementary Fig. S7A and S7B). EGFR phosphorylation at Tyr1068 and ERK 1/2 phosphorylation at Thr202/Tyr204 were also detected in xenograft tumor lysates from all 3 cell lines (Fig. 5D). Immunohistochemical staining showed activated EGFR at the membrane in TE-4 xenografts after treatment with efatutazone (Fig. 5E).

**In vitro effects of combination of efatutazone and MEK inhibitor or anti-EGFR antibody**

Importantly, the antiproliferative effects of efatutazone were associated with inactivation of Akt, whereas EGFR/MAPK (mitogen-activated protein kinase) signaling was activated.
The MEK inhibitor U0126 (Supplementary Fig. S8A) was used in combination with efatutazone. Dephosphorylation of ERK 1/2 was detected after treatment with efatutazone combined with U0126. However, EGFR/Akt signaling was activated by combined treatment, compared with treatment with efatutazone alone (Fig. 5F). The growth inhibitory effects of U0126, efatutazone, and the combination of the 2 agents were 37.3 ± 5.0, 31.1 ± 3.6, and 51.6 ± 3.9, respectively, compared with the control (Fig. 5G, Supplementary Fig. S8B).

The anti-EGFR antibody cetuximab (Supplementary Fig. S8C and S8D) was also investigated in combination with efatutazone. Significantly, phosphorylation of EGFR at Tyr1068 caused by treatment with efatutazone was not detected after treatment with the combination of efatutazone and cetuximab. Furthermore, both the PJEK–Akt and MAPK pathways were inactivated by this combination therapy (Fig. 5F). The growth inhibitory effects of cetuximab, efatutazone, and efatutazone combined with cetuximab were 19.7 ± 6.1, 31.1 ± 3.6, and 44.9 ± 0.5, respectively, compared with the control (Fig. 5H, Supplementary Fig. S8E). Interestingly, treatment with efatutazone-conditioned medium for 10 minutes activated EGFR, however EGFR was not phosphorylated at Tyr1068 when TE-4 cells were pretreated with or without cetuximab (300 µg/mL) for 6 hours, followed by stimulation with the conditioned media.

We therefore used molecular-targeted agents in combination with efatutazone to suppress MAPK or EGFR signaling.

The anti-EGFR antibody cetuximab (Supplementary Fig. S8C and S8D) was also investigated in combination with efatutazone. Significantly, phosphorylation of EGFR at Tyr1068 caused by treatment with efatutazone was not detected after treatment with the combination of efatutazone and cetuximab. Furthermore, both the PJEK–Akt and MAPK pathways were inactivated by this combination therapy (Fig. 5F). The growth inhibitory effects of cetuximab, efatutazone, and efatutazone combined with cetuximab were 19.7 ± 6.1, 31.1 ± 3.6, and 44.9 ± 0.5, respectively, compared with the control (Fig. 5H, Supplementary Fig. S8E). Interestingly, treatment with efatutazone-conditioned medium for 10 minutes activated EGFR, however EGFR was not phosphorylated at Tyr1068 when TE-4 cells were pretreated with or without cetuximab (300 µg/mL) for 6 hours, followed by stimulation with the conditioned media.

In vivo effects of combination therapy with efatutazone and cetuximab

Cetuximab exhibited better efficacy than U0126 when used in combination with efatutazone in vitro. We therefore investigated the antiproliferative effects of efatutazone combined with cetuximab in a mouse TE-4 cell xenograft model. Mice were divided into 4 treatment groups: control group, efatutazone group, cetuximab group, and combination group (Fig. 6A). The tumor inhibitory effects after treatments were...
17.6% ± 11.0%, 41.0% ± 8.5%, and 71.7% ± 5.4% (average ± SD) in the cetuximab group, efatutazone group, and combination group, respectively, compared with the control group after 38 days (Fig. 6B).

EGFR was more dephosphorylated in the combination-treatment group than in the control group, and inactivation of both the PI3K–Akt and MAPK pathways was demonstrated (Fig. 6C). Activation of EGFR at the membrane in TE-4 xenografts after treatment with efatutazone was suppressed in tumors of animals treated with efatutazone plus cetuximab, as demonstrated by immunohistochemical staining (Fig. 6D).

The body weights of the mice were measured twice a week. The mice treated with efatutazone alone tended to gain more weight than control mice, but the body weights of mice treated with efatutazone combined with cetuximab showed no significant difference from the controls (Fig. 6E).

**Discussion**

PPAR-γ expression is tissue and cancer specific, and the expression of PPAR-γ protein in patients with ESCC and the antiproliferative effects and mechanism of action of the new generation PPAR-γ agonists in ESCC remain unclear. We therefore investigated the expression of PPAR-γ in 145 patients with ESCC, and clearly demonstrated the antiproliferative effects and mechanism of action of the novel PPAR agonist, efatutazone.

We demonstrated that PPAR-γ was expressed in normal esophageal squamous epithelium, and was expressed heterogeneously in ESCC tumors. It exhibited an inverse relationship with Ki-67 expression, as determined by immunohistochemistry.

To the best of our knowledge, efatutazone is the first PPAR-γ agonist shown to inhibit the proliferation of ESCC cell lines both in vitro and in vivo. Importantly, the results of this study suggest that the novel antiproliferative mechanism of efatutazone involves regulation of p21Cip1 protein levels in the nucleus by inactivating Akt signaling and dephosphorylating p21 at Thr145, without affecting the transcriptional activity of p21Cip1.

The EGFR/MAPK pathway was activated by efatutazone, and combined treatment with efatutazone and cetuximab suppressed both the PI3K–Akt and MAPK pathways, leading to synergistic antiproliferative effects (Fig. 7).

PPAR-γ is affected by endogenous ligands such as Δ12,15-prostaglandin J2, and serves as a transcriptional factor in vivo (14). However, the current investigation demonstrated that PPAR-γ expression seems to be heterogeneous during carcinogenesis. In addition, PPAR-γ expression had an inverse relationship with Ki-67 expression, and may be associated with tumor-suppressive effects. We therefore investigated if activation of PPAR-γ by a high-affinity PPAR-γ ligand induced antitumor effects in ESCC.

The conventional PPAR-γ agonist troglitazone partially inhibited ESCC proliferation in vitro in previous studies (41). However, our results showed no significant antiproliferative
effect of troglitazone in ESCC cell lines, whereas efatutazone caused a 49.6\% reduction in proliferation of xenografted ESCC cells, and the tumor-reduction rate was similar to the maximum effect of efatutazone against HT-29 colon cancer cells in a previous xenograft study (24). This result suggests that efatutazone represents a promising antitumor agent for patients with ESCC. However, the mechanisms underlying the antiproliferative effects of efatutazone are poorly understood.

This study demonstrated that efatutazone upregulated p21Cip1 protein levels in the nucleus without affecting p21Cip1 mRNA. A previous study showed that Akt activation was associated with phosphorylation of p21 at Thr145, which led to p21Cip1 translocation from the nucleus to the cytoplasm (40). Cytoplasmic p21Cip1 has also been associated with survival pathways (42) and a poor prognosis in breast cancer (43). Interestingly, Akt activation by EGF stimulation also led to phosphorylation of p21 at Thr145 in ESCC, whereas Akt inactivation induced by efatutazone led to dephosphorylation of p21 at Thr145, resulting in upregulation of p21Cip1 protein levels in the nucleus in this study.

Significant tumor regression may be typical for treatments combining PPAR-γ agonists with conventional cytotoxic anti-cancer agents (4, 23, 24). We found that additional antiproliferative effects were detected in ESCC cell lines when efatutazone was used in combination with 5-fluorouracil, cisplatin or docetaxel in vitro (data not shown); however, the antiproliferative effects and mechanism of action of efatutazone in combination with molecular-targeted agents are unclear. Importantly, EGFR/MAPK signaling was activated by efatutazone. EGFR was gradually activated after treatment with efatutazone and the cause of the phosphorylation of EGFR Tyr1068 was a secreted protein (factors) produced by the efatutazone-treated cells. Activation of these pathways may limit the antiproliferative effects of efatutazone, and the addition of molecular-targeted agents to efatutazone may help to suppress these growth pathways. We used U0126 in combination with efatutazone, but the additive effect was small. Recent studies reported ERK-dependent negative feedback in several cancers (44, 45). This ERK-dependent negative feedback was lost after treatment with a MEK inhibitor, and the ability of receptor tyrosine kinase ligands to activate growth signaling was markedly enhanced (44, 46). Similar processes may have been responsible for the effects observed when efatutazone was combined with U0126. ERK-dependent negative feedback was lost after treatment with U0126, and the secreted factors produced by the efatutazone-treated cells stimulated EGFR, followed by the remarkable activation of Akt pathway.

The activation of EGFR induced by the secreted factors after treatment with efatutazone was not detected after pretreating ESCC cells with cetuximab. We therefore used cetuximab in combination with efatutazone, and demonstrated a notable synergistic effect based on signal analysis. This combination therapy may have other benefits. Although the tolerability of efatutazone has been demonstrated in a clinical study, more than half of the patients (51.6\%) suffered from peripheral edema, which is a recognized adverse effect of efatutazone treatment (22). In our study, mice treated with efatutazone alone also tended to gain more weight than control mice, although this adverse weight gain was reduced in mice treated with efatutazone combined with cetuximab. A recent study showed that PPAR-γ agonists rapidly stimulated sodium-coupled bicarbonate absorption from the renal proximal tubule, followed by plasma volume expansion. The PPAR-γ agonist–induced transport stimulation was dependent on PPAR-γ–Src–EGFR–ERK (47). In our study, inactivation of MAPK pathway by efatutazone combined with cetuximab may have suppressed the sodium-coupled bicarbonate absorption from the renal proximal tubule and led to a reduction in the adverse weight gain caused by treatment with efatutazone alone.

**Figure 7.** Effects of treatment with efatutazone alone and in combination with cetuximab. A, no treatment. EGFR is phosphorylated by EGF stimulation, and both the PI3K-Akt and MAPK pathways are activated. Activated Akt phosphorylates p21 Thr145 and leads to p21Cip1 translocation from the nucleus to the cytoplasm. Cytoplasmic localization of p21Cip1 is associated with survival pathways. B, efatutazone inactivates Akt, and p21 Thr145 is dephosphorylated. This leads to an increase in the p21Cip1 protein level in the nucleus and G1 arrest. However, the EGFR/MAPK pathways are activated. C, the combination of efatutazone with cetuximab suppresses both the PI3K-Akt and MAPK pathways and leads to synergistic antiproliferative effects.
In conclusion, this study demonstrated the tumor-suppressive effects and major underlying mechanism of efatutazone involving inactivation of the Akt pathway. Treatment with efatutazone combined with cetuximab produced a synergistic effect by negatively regulating both the PI3K–Akt and MAPK pathways. These results suggest that efatutazone could be used both alone and in combination with cetuximab as a potential therapeutic approach for ESCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Sawayama, T. Ishimoto, J. Kurashige, Y. Shiose, Hideo Baba
Development of methodology: H. Sawayama, T. Ishimoto, J. Kurashige, Hirohisa Motomura
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Sawayama, T. Ishimoto, M. Watanabe, N. Yoshida
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Sawayama, T. Ishimoto, M. Watanabe

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Small Molecule Agonists of PPAR-γ Exert Therapeutic Effects in Esophageal Cancer

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doi:10.1158/0008-5472.CAN-13-1836

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