Peroxisome Proliferator-Activated Receptor \( \gamma \) Ligands Improve the Antitumor Efficacy of Thrombospondin Peptide ABT510

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Abstract
An expanding capillary network is critical for several pathologic conditions. In cancer, the decrease of antiangiogenic thrombospondin-1 (TSP1) often enables an angiogenic switch, which can be reversed with exogenous TSP1 or its peptide derivative ABT510. TSP1 acts by inducing endothelial cell apoptosis via signaling cascade initiated at CD36, a TSP1 antiangiogenic receptor. Here, we show that the ligands of nuclear receptor peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)), 15-deoxy-\( \Delta^{12,14} \)-prostaglandin J2, troglitazone, and rosiglitazone increased PPAR\( \gamma \) and CD36 expression in endothelial cells and improved the efficacy of TSP1 and ABT510 in a CD36-dependent manner. The ABT510 and PPAR\( \gamma \) ligands cooperatively blocked angiogenic endothelial functions in vitro and neovascularization in vivo. In tumor xenografts, 15-deoxy-\( \Delta^{12,14} \)-prostaglandin J2 and troglitazone synergistically improved antiangiogenic and antitumor effects of ABT510. Our data provide one mechanism for the in vivo angioinhibitory effect of PPAR\( \gamma \) ligands and show fine-tuning of the antiangiogenic efficacy via targeted up-regulation of the endothelial receptor.

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Introduction
Angiogenesis is controlled via balance between angiogenic inhibitors and stimuli in the tissue environment where subtle disturbances of the balance set off neovascularization and allow exponential progression of cancer, atherosclerosis, arthritis, or retinopathy (1-3). Loss or down-regulation of thrombospondin-1 (TSP1), a potent angiogenesis inhibitor, drives the angiogenic switch in fibrosarcoma, glioblastoma, and carcinomas of breast and bladder (4). TSP1 expression can be repressed by Ras-dependent Myc activation, leading to angiogenesis and tumor expansion (5). Reintroduction of TSP1 or its peptide derivative ABT510 reverses the angiogenic switch and blocks tumor progression (6, 7). TSP1 also inhibits angiogenesis in experimental models of retinopathy (8). Increased TSP1 expression is responsible for the effect of low-dose metronomic treatment with cyclophosphamide (9).

TSP1 blocks angiogenesis by targeting endothelial cells (EC) for apoptosis via CD36, one of many TSP1 receptors (10-13). In addition, soluble TSP1 and its \( \alpha_\gamma \beta_1 \) integrin-binding peptide inhibit proliferation of cultured EC independent of CD36 (14). ABT510, a short peptide from TSP1 type 1 repeats, binds CD36 and activates the same antiangiogenic cascade as nascent TSP1 (15). It is an efficient inhibitor of angiogenesis and tumor growth in mouse models of bladder cancer (7, 15). However, CD36 expression by human endothelium is nonuniform (16) and low CD36 presentation on the surface of EC limits their ability to respond to TSP1 in chemotaxis and morphogenesis assays (10). Moreover, CD36-/- mice develop angiogenic response despite TSP1 or ABT510 treatment (11).

In macrophages, CD36 expression is controlled by peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)), a transcription factor of a nuclear hormone receptor superfamily. PPAR\( \gamma \) is activated by endogenous and synthetic ligands, including polyunsaturated fatty acids, 15-deoxy-\( \Delta^{12,14} \)-prostaglandin J2 (15d-PGJ2), and metabolic products of the oxidized low-density lipoprotein (oxLDL), and by antidiabetic thiazolidinediones (TZD; ref. 17). 15d-PGJ2 blocks corneal angiogenesis (18), troglitazone and rosiglitazone decrease choroidal neovascularization (19), and rosiglitazone has been recently shown to inhibit EC proliferation and tumor angiogenesis (20).

Seeking means to improve the antiangiogenic efficacy of TSP1, we examined the effect of PPAR\( \gamma \) ligands on CD36 expression by ECs and the link between CD36 expression and the ability of TSP1/ABT510 to block neovascularization and tumor growth. We found that moderately antiangiogenic doses of 15d-PGJ2, rosiglitazone, and troglitazone augmented CD36 surface display and improved EC response to TSP1 and ABT510. This cooperative effect was CD36-dependent as it was ablated with CD36 neutralizing antibodies. In vivo, troglitazone, rosiglitazone, or 15d-PGJ2 used in concert with...
ABT510, inhibited angiogenesis and induced EC apoptosis at doses marginally effective for each individual compound. We thus show for the first time that targeted up-regulation of an antiangiogenic receptor dramatically improves the sensitivity of remodeling vasculature to a specific inhibitor and thus enhances inhibitors’ efficacy.

Results

PPARγ Ligands Increased CD36 on Microvascular EC

Seeking factors to augment TSP1 antiangiogenic effect, we chose PPARγ ligands, the molecules known to block angiogenesis and to induce CD36 expression in non-ECs. In human microvascular EC (HMVEC), 48-hour treatment with a natural PPARγ ligand, 15d-PGJ2, induced a dose-dependent increase of surface CD36 as was determined by flow cytometry (Fig. 1A and B). PPARα ligand WY14643 had no effect on CD36 display by HMVECs. oxLDL induces CD36 in macrophages and regulates vascular endothelial growth factor (VEGF) expression in monocytes and human coronary artery ECs via PPARγ activation. Endothelial CD36 was also up-regulated by oxLDL but not by control LDL (data not shown).

Troglitazone and rosiglitazone, synthetic ligands with pronounced specificity for PPARγ, also strongly induced CD36 display by HMVECs (Fig. 1C and D). GW9662, an inhibitor of PPARγ, reduced basal CD36 levels in untreated cells and blocked up-regulation by troglitazone and rosiglitazone (Fig. 1C and D). The effective concentrations of PPARγ ligands in HMVECs were similar to those in monocytes (21). Treatment with rosiglitazone induced PPARγ and CD36 message in HMVECs (Fig. 1E). In CD36-negative bladder carcinoma 253J B-ν, rosiglitazone increased PPARγ mRNA but not CD36 expression (Fig. 1F).

Nuclear extracts from the cells treated with 15d-PGJ2 showed increased binding with the oligonucleotide containing PPAR response element (PPRE) from human CD36 promoter. This binding was disrupted by a 40-fold excess of nonlabeled oligonucleotide containing PPRE but not the AP2 binding site (Fig. 2).

PPARγ Ligands Restored CD36 Expression and EC Response to TSP1 and ABT510

CD36 levels suffer dramatic decrease in primary HMVEC cultures at passages above 5. These cells show minimal or no response to the inhibitory TSP1 and its peptides (Fig. 3). To examine if PPARγ ligands can restore CD36 expression and TSP1 inhibitory effect, HMVECs were treated with 15d-PGJ2 (3 μM), LDL (10 μg/mL), or oxLDL (10 μg/mL) and subsequently tested in EC chemotaxis assay. 15d-PGJ2 and oxLDL restored the responsiveness of the late-passage HMVECs to TSP1, whereas LDL had no significant effect (Fig. 3A). 15d-PGJ2 reduced the ED50 of ABT510 of the late-passage HMVECs to 0.03 nM from ~10 nM for untreated late-passage HMVECs (Fig. 3B). Troglitazone, rosiglitazone, and pioglitazone restored inhibition of basic fibroblast growth factor (bFGF)–induced HMVEC migration by ABT510 (Fig. 3C).

Rosiglitazone inhibited the proliferation of HMVECs with ED50 of ~5 μM. ABT510 weakly inhibited HMVEC proliferation (ED50 ~ 50 nM) and was noninhibitory at 1 nM/L. However, the addition of rosiglitazone (1 μM/L) to ABT510 (1 nM) inhibited proliferation by >75% (Fig. 3D). Single or combined rosiglitazone and ABT510 had no effect on the proliferation of 253J B-ν cells (Fig. 3E).

Cooperative Antiangiogenesis by PPARγ Ligands and ABT510 In vivo

To determine if 15d-PGJ2 and ABT510 cooperatively blocked angiogenesis in vivo, the mice with corneal implants containing bFGF received systemic treatment with ABT510 ± 15d-PGJ2. 15d-PGJ2 alone and ABT510alone blocked bFGF-induced angiogenesis by 34% and 44%, respectively. When combined, ABT510 and 15d-PGJ2 inhibited corneal

FIGURE 1. PPARγ ligands increased CD36 expression by HMVECs. A. The cells treated for 48 hours as indicated were stained with anti-CD36 FITC or isotype control. CD36-positive and propidium iodide – positive cells were detected by flow cytometry. Note increase in CD36-positive EC and apoptosis in 15d-PGJ2-treated population. B. HMVECs were treated for 48 hours with increasing 15d-PGJ2 concentrations and CD36 presentation was measured by flow cytometry. C and D. HMVECs were treated for 48 hours with GW9662 (1 μM) and/or TZD (1 μM), rosiglitazone (C), or troglitazone (D). ○, control vehicle; ●, TZD alone; □, GW9662; ■, TZD + GW9662. Note a decreased response to TZD in the presence of the inhibitor. *, P < 0.002, significantly different from control. **, P < 0.008, significantly different from rosiglitazone. E. RNA from cells treated for 72 hours with indicated doses of rosiglitazone (Rsg) were analyzed by semiquantitative reverse transcription-PCR for the expression of CD36 and PPARγ, with actin as a loading control.
angiogenesis by 41%. 15d-PGJ2 showed similar cooperative effect when combined with ABT510 in Matrigel plug assay (Fig. 4). When nude mice carrying Matrigel plugs containing bFGF were treated with rosiglitazone and/or ABT510, computer-assisted microvessel density (MVD) measurements showed inhibition of neovascularization by ABT510 (1 mg/kg/d) and rosiglitazone (50 mg/kg/d) by 39% and 45%, respectively. Combined inhibition by ABT510 and rosiglitazone was 80% (Fig. 5A and B).

Remarkably, EC apoptosis induced by ABT510 in vivo was increased by rosiglitazone from 22% to 60%, exceeding additive effect, which would have been nearing 40% (Fig. 5A and C). To determine possible contribution of immune cells to the cooperative antiangiogenic effect of ABT510 and PPARγ ligands, experiments were carried out in the wild-type C57/BL6 mice with similar results (data not shown).

**FIGURE 2.** PPARγ DNA binding increased after 15d-PGJ2 treatment. Nuclear extracts were incubated with [32P]-labeled oligonucleotide containing PPRE from a human CD36 promoter, 10-fold molar excess of cold PPRE (specific competitor), or AP2 (nonspecific competitor) oligonucleotides. Lane 1, negative control; lanes 2 to 4, control nuclear extracts; lanes 5 to 7, 15d-PGJ2-treated nuclear extracts. Note increased intensity of the shifted band in lanes 5 and 7.

Troglitazone and 15d-PGJ2 Improved the Antitumor Activity of ABT510

To study the combined effect of TSP1 peptide and PPARγ ligands on tumorigenesis, we used TSP1-negative bladder carcinoma cell 253J B-v that responds well to ABT510 (7). Systematic treatment of tumor-bearing mice with ABT510 (1 mg/kg/d) or troglitazone (50 mg/kg/d) caused 32% and 31% decrease in tumor volume, respectively, whereas decrease by combined treatment was 74% (Fig. 6A). Lower dose of troglitazone (10 mg/kg/d) by itself reduced tumor volume by 35% and by 48% when combined with ABT510 (Fig. 6A). 15d-PGJ2 (2.5 mg/kg/d) also augmented ABT510 effect on tumor growth (Fig. 6B). Treatment with either ABT510 or Tro50 lowered tumor MVD by 20%, whereas combination of ABT510 and Tro50 resulted in 45% MVD reduction (Fig. 6C). As shown previously (7), ABT510 triggered apoptosis in the tumor-associated endothelium (~2.8%). Troglitazone by itself at low and high doses also induced apoptosis, albeit at low levels (~1.3%; Fig. 6D). When ABT510 was combined with high-dose troglitazone, apoptosis was synergistically increased to ~11% (Fig. 6D).

The checkerboard analysis with the fractional product of Webb yielded the following values: for the combination of ABT510 (1 mg/kg) and Tro10, E refuses was 0.56%, whereas E obeys was 0.475%, indicating no interaction. However, when troglitazone was increased to 50 mg/kg, E remain low (0.531), whereas E obeys increased to 0.739, pointing to a possibility of synergy. The results of analysis with Chou and Talalay values were similar (E refuses = 0.425 versus E obeys = 0.485 for Tro10 and 0.698 versus 0.739 for Tro50). To perform limited isolation analysis, we used the values from the previous studies. ABT510 blocked tumor growth by ~70% at 30 mg/kg (20, 22), whereas 70% to 80% inhibition of tumor volume required 100 mg/kg TZD (20, 22). In combination, the same agents produced 70% inhibition at 1 and 50 mg/kg, respectively. These values yielded interaction index I = 0.83 (<1.0), which also indicated synergy.

CD36 expression throughout the tumor vasculature was nonuniform with a trend toward lower expression in larger capillaries. Staining of the tumor sections for the endothelial markers CD31 and CD36 revealed significantly higher number of vascular structures positive for CD36 in tumors treated with Tro50 compared with control vehicle (82% versus 50%; Fig. 6E). Tro10 had no discernible effect on CD36 expression. Among larger capillaries (diameter >10 μm) chosen at random in control and ABT510-treated tumor sections, only 8 of 23 were CD36 positive (35%). Conversely, in tumors treated with Tro50 with or without ABT510, 20 of 22 randomly chosen vessels were CD36 positive (90%). Apoptotic ECs could be easily found in larger endothelial structures of the tumors treated with Tro50/ABT510 combination (5 of 10 vessels, all highly positive for CD36; Fig. 6F). However, in all other treatment groups, only 1 of 35 vessels contains apoptotic ECs. Our data indicate that PPARγ ligands cooperate with ABT510 in blocking tumor growth by increasing CD36 expression and inducing EC apoptosis in tumor vasculature.

**Discussion**

Blocking angiogenesis is a promising way to control tumor growth without serious toxic side effects. However, the efficacy of the method is limited by the potency of available agents. Limited knowledge of specific receptors and mechanisms of angiogenesis inhibitors hampers the efforts to design supplementary strategies to improve their efficacy or to design small nonpeptide mimics. TSP1 is unique among inhibitors in that its antiangiogenic signaling receptor CD36 is known and the downstream signaling events are defined in considerable detail (10, 11, 23). Short peptides derived from TSP1 (15), some active at low nanomolar concentrations, hold great promise as antiangiogenic/antitumor agents (7). However, the inhibitory effect of nascent TSP1 and derivatives is applied predominantly to CD36-positive ECs (10, 11), although non-CD36-dependent...
pathways were also shown (14, 24). The expression of CD36 in vivo is quite heterogeneous. It is absent on the large vessel ECs, with the exception of some arteries, and is expressed at varying levels by the microvascular endothelium (16). The response of the cultured EC population to TSP1 is proportional to the percentage of CD36-positive cells (10).

CD36 is expressed by non-ECs, predominantly monocytes, where it participates in adipogenesis and glucose metabolism (17), but in monocytes, TSP1/CD36 binding fails to induce apoptosis (25). CD36 promoter contains PPRE of direct repeat sequences (DR-1; ref. 17), although the PPRE is imperfect and the promoter does not contain an important adjacent sequence (26). Recently, CD36 promoter was found to contain two independent proximal and distal PPREs. Unexpectedly, neither of them can be induced by PPARγ ligands alone, suggesting the existence of signaling intermediates responsible for the tissue specificity of CD36 regulation (27). ECs express PPARγ, but its role in CD36 regulation remained unexplored. We found that PPARγ is critical for CD36 up-regulation as it was abolished by GW9662, a PPARγ specific inhibitor.

TZD directly inhibits proliferation and induces apoptosis in multiple cancer cell lines, including liposarcoma, breast adenocarcinoma, carcinomas of the prostate and colon, non-small cell lung cancer, and the pancreatic, renal cell, and gastric carcinomas (17). Troglitazone blocks the growth of human colon cancer xenografts, and PPARγ mutations that disrupt ligand binding are frequent in colon carcinoma (28).

PPARγ ligands have been recently discovered as antiangiogenic agents. 15d-PGJ2 blocks angiogenesis by VEGF and leptin (18, 29), troglitazone relieves choroidal neovascularization (19), and rosiglitazone reduces primary tumor growth and metastases by blocking angiogenesis (20). However, the mechanism(s) of their action is largely unexplored, with the exception of a few studies demonstrating PPARγ-dependent dephosphorylation/inactivation of survival kinase Akt via phosphatase tensin homologue (29) and up-regulation of tissue inhibitor of metalloproteinase activity by rosiglitazone (20). 15d-PGJ2 has been earlier shown to induce EC apoptosis via an unspecified mechanism (30).

PPARγ-dependent increase of CD36 on the capillary endothelium improved antiangiogenic efficacy of TSP1 and related peptides. TZD increased the PPARγ message in both HMVECs and 253J B-v cells. In the late-passage CD36-deficient HMVECs, PPARγ ligands drove CD36 expression to the ambient or higher levels and thus restored the ability to respond to TSP1. Combinations of mildly inhibitory doses of ABT510 with 15d-PGJ2, troglitazone, or rosiglitazone produced profound antiproliferative effect in ECs but not in tumor cells. Similar combination treatments had cooperative effect in the neovascularization and tumorigenicity assays.

Our study provides another mechanism behind the angioinhibitory activity of PPARγ ligands and offers the way to use them as components of complex antiangiogenic treatment. We showed that CD36 was critical for the cooperative antiangiogenic effect by PPARγ ligand rosiglitazone and ABT510. When used as a single agent, PPARγ ligand 15d-PGJ2 slowed tumor progression but had no effect on the MVD. 253J B-v completely lacks secreted TSP1 and thus requires exogenous TSP1 to induce CD36-dependent apoptosis. This is consistent with reports where 15d-PGJ2 antitumor effects on breast cancer cells stemmed from PPARγ-independent apoptosis (31). When antiangiogenic TSP1 mimic was reintroduced into the system, even low doses of ABT510 combined with 15d-PGJ2 caused visible MVD reduction, far exceeding the decrease achieved by the single ABT510 treatment, and synergistically improved the antitumor effect of 15d-PGJ2. Both low and high doses of troglitazone alone showed similar antitumor activity; however, only at high dose did troglitazone induce CD36 expression on tumor endothelium and cooperatively with ABT510 induce endothelial apoptosis, thus blocking angiogenesis and tumor growth. Endogenous endothelial-derived and tumor-derived TSP1 is an important factor in the antiangiogenic effect of metronomic chemotherapy (9). However, no significant changes in TSP1 mRNA were observed in HMVECs treated with 15d-PPARγ ligands (data not shown). Although increased availability of CD36 undoubtedly improved sensitivity to the endogenous TSP1, the concentrations of exogenous ABT510 in this case far exceed endogenous TSP1 levels, thus masking its contribution.

In addition to their effect on CD36 presentation, PPARγ ligands up-regulate VEGF expression by cancer cells, macrophages, and vascular smooth muscle cells (32, 33), conflicting the report by Panigrahy et al., which shows ~60% reduction of VEGF expression by several tumor types (20). VEGF increase may in fact lead to an increased susceptibility of the tumor-associated endothelium to TSP1 (23) due to increased presentation of Fas, a secondary signaling intermediate. However, further increase of inducer levels may activate prosurvival pathways and protect ECs from apoptosis by inhibitors, thus outbalancing inhibitor-induced apoptosis. A dramatic increase

FIGURE 3. PPAR ligands improved the response of late-passage HMVECs to TSP1 and ABT510. A, HMVECs (passages 8-10) pretreated for 36 hours with 15d-PGJ2 (3 μmol), LDL (10 μg/mL), or oxLDL (10 μg/mL) as indicated were starved for 12 hours and used in the chemotaxis assays in parallel with non-pretreated cells. TSP1 alone (3 nmol; ○) or with bFGF (20 ng/mL; ●) were added where indicated. *, P < 0.01, significantly different from untreated and LDL-treated HMVECs. B, Untreated HMVECs (○, no bFGF; ●, 20 ng/mL bFGF) or pretreated with 15d-PGJ2 (3 μmol/L), as in A (○, no bFGF; ●, 20 ng/mL bFGF), were tested in chemotaxis assay in the presence of increasing concentrations of ABT510. Note that ABT510 blocks bFGF-induced migration of HMVECs pretreated with 15d-PGJ2 at low nanomolar concentrations compared with micromolar concentrations in untreated EC. C, CD36 neutralizing antibody (FA6-162) blocked the inhibitory effect of ABT510 on HMVECs treated with PPARγ ligands. HMVECs, untreated (○) or treated with 15d-PGJ2 (3 μmol), pioglitazone (2 nmol; ●), troglitazone (1 nmol; □), and rosiglitazone (1 nmol; ■), were tested in chemotaxis assay. ABT510 (1 nmol), bFGF (20 ng/mL), anti-CD36 neutralizing antibody FA6-152 (2 μg/mL), and control IgG (2 μg/mL) were added as indicated. *, P < 0.01, significantly different from untreated HMVECs. **, P < 0.03, significantly different from ABT510 + bFGF + IgG. D and E, Rosiglitazone (Rosg) and ABT510 cooperatively inhibited HMVEC proliferation. Late-passage HMVECs (D) or bladder carcinoma cells (E) were treated for 72 hours with rosiglitazone and/or ABT510 concentrations and proliferation was assessed with WST-1 kit. Rosiglitazone at increasing doses blocked EC but not bladder carcinoma proliferation (D and E, left). Increasing ABT510 concentrations caused a mild decrease in HMVEC proliferation, which was cooperatively increased by rosiglitazone (1 μmol, D, right). 253J B-v remained unaffected (E, right). Proliferation levels of untreated cells are shown on days 0 and 3 (dotted lines).
in multiple angiogenic stimuli, including VEGF, was noted in tumor cells, which bypassed one round of antiangiogenic treatment, causing resistance to antiangiogenic agent (34). Besides increased production of angiogenic stimuli, there are multiple pathways by which tumors become resistant to angiogenesis inhibitors/hypoxia including the loss of p53 (35). Thus, it is of particular importance to maximize the efficacy of the initial round of antiangiogenic therapies—the major goal of our study.

In keeping with our Matrigel experiments, oral administration of rosiglitazone (50 mg/kg/d) reduces corneal vascularization by ~40% (20). Cooperatively with ABT510, used at a dose that produced similar effect, angiogenesis was decreased >80%. 15d-PGJ2 (10 μg/mL) applied locally completely blocked angiogenesis in the cornea (18).

Because PPARγ ligands increase CD36 expression and thus oxLDL uptake by macrophages (21, 36), its use as a anticancer agent may lead to atherosclerosis due to increased macrophage-foam cell conversion. However, troglitazone blocks formation of early atherosclerotic lesions possibly as a result of the downregulated expression of vascular cell adhesion molecule-1 on the vascular cells, lowering monocyte adherence to the lumen,

**FIGURE 4.** 15d-PGJ2 and ABT510 cooperatively inhibited neovascularization. Matrigel plugs containing bFGF (100 ng/mL) and heparin (64 units/mL) were implanted in the median abdominal area of C57/BL6 mice (five mice per group). The mice were treated for 6 days with 15d-PGJ2 (1.25 mg/kg/d), ABT510 (1.0 mg/kg/d), or both, and the plugs were then recovered, sectioned, and stained for the endothelial marker CD31. The sections were examined under Olympus fluorescent microscope (Melville, NY). Scale bar, 20 μm.

**FIGURE 5.** The cooperative effect of ABT510 and rosiglitazone in vivo. A. Athymic nu/nu mice (five mice per group) received Matrigel implants containing bFGF (100 ng/mL) and heparin (64 units/mL). Mice were treated for 6 days with vehicle control, rosiglitazone (50 mg/kg/d; Rosi), ABT510 (1 mg/kg/d), or both, as indicated. The plugs were sectioned and stained for the endothelial marker CD31 (red) using Rhodamine X–labeled secondary antibodies and for apoptosis with ApopTag kit (green). Merged images are shown. Yellow, apoptotic ECs. Scale bar, 50 μm. A minimum of 10 high-powered fields at the hotspots of vascularization have been analyzed for CD31-positive structures (MVD; B) and apoptosis (C). Control; ABT510; Rosi; ABT510 + rosiglitazone. Statistical significance of the differences between treatment groups was determined using two-tailed t test. *P < 0.01, for the control vehicle and single agent treatments. Note strong cooperative activity of rosiglitazone and ABT510. Bars, SEM.
FIGURE 6. Inhibition of tumor growth by ABT510 and PPARγ ligands. A. 253J B-v cells were injected s.c. in the hindquarters of nu/nu mice (five mice per group), and 7 days were allowed for the tumors to establish. The animals were then started (day 1) on the regimen of vehicle PBS (○), ABT510 (■), Tro10 (□), Tro50 (△), Tro10 + ABT510 (▲), and Tro50 + ABT510 (●). Single agents inhibited tumor growth (ABT510, \( P < 0.05 \); Tro10, \( P < 0.02 \); Tro50, \( P < 0.03 \); Tro50 + ABT510 versus single treatments \( P < 0.005 \)). B. Single agent treatments were allowed to establish for 14 days and then treated (day 1) with vehicle PBS (○), ABT510 (●), 15d-PGJ2 (▲), or both (●). Note the divergence in growth rates between the tumors in the combined treatment group and the groups on single ABT510 or 15d-PGJ2 treatment \( (P < 0.05) \). C. Tro50 + ABT510 significantly reduced MVD compared with control treatment. ○, vehicle; ■ ABT510; □, Tro10; △ Tro50; ▲, Tro10 + ABT510; ●, Tro50 + ABT510. * \( P < 0.04 \), ** \( P < 0.001 \), versus single treatments. D. ABT510 alone \( (P < 0.02) \) or in combination with Tro50 (versus single treatments, \( P < 0.001 \)) caused significant apoptosis in tumor ECs. ○, vehicle; ■ ABT510; □, Tro10; △, Tro50; ●, Tro10 + ABT510; ▲, Tro50 + ABT510. E. Staining of tumor sections for CD36 (green), CD31 (red), and apoptosis (blue). Note visible differences in MVD reduction between single and combined treatments. CD36-positive ECs (bright green arrows) can be seen in all tumor samples. 1, control; 2, ABT510; 3, Tro50; 4, Tro50 + ABT510. CD36-negative ECs (white arrows) were present in tumors from control and ABT510-treated mice. Significant number of CD36-positive ECs underwent apoptosis in the combined treatment group (4, white on merged images, blue arrows). F. Tro50 increased CD36 expression/apoptosis in larger tumor vessels. Larger vessels from control and ABT510-treated tumors showed low level of CD36, whereas those from Tro50-treated tumors had significantly higher levels of CD36 and EC apoptosis. Panels are arranged as in E. Scale bar, 50 μm.
decreased inducible nitric oxide synthase, gelatinase B, and scavenger receptor A (37). Due to liver and other toxicities of troglitazone, it has been removed from the market, but rosiglitazone and other PPARγ ligands can be used to enhance the efficacy of ABT510 and other TSP1 peptides. In conclusion, PPARγ ligands at doses lower than those required for complete reversal of angiogenesis improved the efficacy of TSP1 derivatives in causing endothelial apoptosis, blocking EC chemotaxis in vitro, and angiogenesis in several in vivo systems. Our results combined with the data in ref. 20 may lead to the more effective use of these potentially synergistic antiangiogenic, antimetastatic therapies, possibly in conjunction with other antiangiogenic or cytotoxic therapies.

Materials and Methods

Cells and Reagents

HMVECs (Clonetics, San Diego, CA) were grown in EC basal medium, 5% fetal bovine serum, human epidermal growth factor (0.1 μg/mL), hydrocortisone (0.01 μg/mL), bovine brain extract (120 μg/mL), amphotericin (50 μg/mL), and gentamicin sulfate (50 μg/mL) and were used at passages 5 to 10. The following antibodies were used for immunostaining: rat anti-CD31 (BD PharMingen, San Diego, CA), rabbit anti-collagen IV (Santa Cruz Biotechnology, Santa Cruz, CA), and Rhodamine-X–conjugated donkey anti-rat and FITC-conjugated mouse CD36 (Santa Cruz Biotechnology, Santa Cruz, CA), and were used at 1:100 dilutions. For immunohistochemistry, Thy-1, CD36, and the primary antibody, FITC-labeled mouse anti-human CD36 (Santa Cruz Biotechnology, Santa Cruz, CA), and Rhodamine-X–conjugated donkey anti-rat antibody SM-ACTGGAGAGGCAAAGG, 5′-GCTACGAGCTGCCTGACGG, 174 bp ref. 39). The template was equalized using β-actin as a marker and the number of cycles was chosen to keep product amount in a linear range (34 cycles for CD36 and PPARγ, 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute; 23 cycles for β-actin, 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute).

Flow Cytometry Analysis

Flow cytometry analysis was done using Epics XL-MCL cytometer (Beckman Coulter, Miami, FL) and WinMDI 2.8 software. HMVECs were stained, stained for 45 minutes with the primary antibody, FITC-labeled mouse anti-human CD36 antibody SMΦ, or isotype control antibody (Ancell, St. Paul, MN), and counterstained with propidium iodide (0.5% carboxymethylcellulose (Sigma Chemical)). The animals were treated for 6 days with saline, rosiglitazone, or ABT510, or both. Rosiglitazone in 10% DMSO, rosiglitazone, ABT510, or both. Rosiglitazone in 10% DMSO–hydron/sucralfate pellets containing bFGF (50 ng) were implanted into the cornea of C57BL/6 mice (National Cancer Institute, Bethesda, MD) and the animals started on the indicated regimen of 15d-PGJ2 (1.25 mg/kg/d), ABT510 (1.25 mg/kg/d), or saline. The eyes were examined by slit-lamp microscopy on day 6. Clearly visible capillary growth from the limbus was scored as a positive response (+) and no visible vascularization was considered negative. Statistical significance was evaluated with Pearson χ² test.

For Matrigel plug assay, 4- to 6-week-old C57BL6 or nu/nu mice (five animals per group, National Cancer Institute) were injected s.c. in the median abdominal area with 0.5 mL growth factor–depleted Matrigel (BD Labware, Bedford, MA) containing bFGF (100 ng/mL) and heparin (64 units/mL, Sigma Chemical). The animals were treated for 6 days with saline, rosiglitazone, ABT510, or both. Rosiglitazone in 10% DMSO-0.5% carboxymethylcellulose (Sigma Chemical) was given by gavage at 50 mg/kg/d, and ABT510 (1 mg/kg/d) was injected intraperitoneal injection 24 hours after implantation.
i.p. Plugs were extracted, snap frozen, sectioned, and stained for the endothelial marker CD31. Apoptosis was visualized by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (Apoptag kit, Intergen, Purchase, NY). The sections were examined with an LSM510 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) and MVD was determined in 10 high-powered fields per sample. For the 15d-PGJ2 experiments, mice carrying Matrigel plugs were treated with 15d-PGJ2 (2.5 mg/kg/d), ABT510 (1 mg/kg/d), or both for 6 days and sections were stained for CD31 and examined by fluorescent microscopy.

**Electrophoretic Mobility Shift Assays**

Nuclear extracts were prepared as described (42) and analyzed with a commercial kit (Promega, Madison, WI). HMVECs (5 x 10^6), untreated or treated for 48 hours with 15d-PGJ2 (3 µmol), were swelled for 10 minutes in 400 µL ice-cold buffer A [10 mmol HEPES-KOH (pH 7.9), 1.5 mmol MgCl₂, 10 mmol KCl, 0.5 mmol DTT, 0.2 mmol phenylmethylsulfonyl fluoride]. The extent of the lysis was assessed by light microscopy. The nuclei were precipitated (13,000 rpm), rinsed with 400 µL cold buffer A, resuspended in 30 µL buffer C [20 mmol HEPES-KOH (pH 7.9), 25% glycerol, 420 mmol NaCl, 1.5 mmol MgCl₂, 0.2 mmol EDTA, 0.5 mmol DTT, 0.2 mmol phenylmethylsulfonyl fluoride], incubated for 20 minutes at 4°C, and cleared by centrifugation (2,000 rpm, 13,000 rpm).

Double-stranded oligonucleotide containing PPARγ binding site of the human CD36 gene promoter with 4-bp 5’ overhangs corresponding to BamHI and EcoRI sites (5'-AAATCCGGGG-TCTGTAAGTCAGGGCGAAGGG-3’) was end labeled using Klenow fragment in the presence of α-32P-dATP. Binding reactions (2-5 µg nuclear extract per reaction) were done following the manufacturer’s recommendations.

**Tumorigenicity Assay**

253J B-v bladder tumor cells (10^6) were injected s.c. in the hindquarters of 4- to 6-week-old nu/nu female mice (5 mice per group, National Cancer Institute). The tumors were allowed to establish for 1 to 2 weeks (palpable tumors detectable) and the animals were randomly distributed into groups and treated systemically with i.p. injections of saline, 15d-PGJ2 (2.5 mg/kg/d), and ABT510 (1 mg/kg/d), Troglitazone (10 or 50 mg/kg/d) in 10% DMSO-0.5% carboxymethylcellulose was given by gavage. Tumors were measured every 2 days and the volumes determined in 10 high-powered fields per sample. For the 15d-PGJ2 experiments, mice carrying Matrigel plugs were treated with 15d-PGJ2 (2.5 mg/kg/d), ABT510 (1 mg/kg/d), or both and the tumors were harvested and snap frozen. Cryosections were stained for CD31 and examined by fluorescent microscopy.

**Mathematical Analysis**

The analysis was done at Northwestern University Bioinformatics Core. For the checkerboard analysis (43), fractional product of Webb was calculated according to the equation EEXP = E1 + E2 - E1 x E2. Limited isobolar analysis (43) was done, with the interaction index calculated as follows: I = 2 E1 E2 (i = 1, 2, 3...), where d_i is the dose of a compound i used in combined treatment and D_i is the dose producing the same effect as a single agent. If the interaction is synergistic, the following ratios are expected: EEXP < E0BS and I < 1.0 (E0BS is the observed effect of combined treatment). Finally, the equation by Chou and Talalay (44) was used to determine the expected concentration for the individual compounds: D = D0 x (E / E0BS - 1)^1/m, which was subsequently used to calculate the fractional product of Webb.

**References**


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