Beyond peroxisome proliferator-activated receptor γ signaling: the multi-facets of the antitumor effect of thiazolidinediones

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Abstract

Certain members of the thiazolidinedione (TZD) family of the peroxisome proliferator-activated receptor γ (PPAR γ) agonists, such as troglitazone and ciglitazone, exhibit antitumor activities; however, the underlying mechanism remains inconclusive. Substantial evidence suggests that the antiproliferative effect of these TZD members in cancer cells is independent of PPAR γ activation. To discern the role of PPAR γ in the antitumor effects of TZDs, we have synthesized PPAR γ -inactive TZD analogs which, although devoid of PPAR γ activity, retain the ability to induce apoptosis with a potency equal to that of their parental TZDs in cancer cell lines with varying PPAR γ expression status. Mechanistic studies from this and other laboratories have further suggested that troglitazone and ciglitazone mediate antiproliferative effects through a complexity of PPAR γ -independent mechanisms. Evidence indicates that troglitazone and ciglitazone block BH3 domain-mediated interactions between the anti apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) members Bcl-2/Bcl-xL and proapoptotic Bcl-2 members. Moreover, these TZDs facilitate the degradation of cyclin D1 and caspase-8-related FADD-like IL-1-converting enzyme (FLICE)-inhibitory protein through proteasome-mediated proteolysis, and down-regulate the gene expression of prostate-specific antigen gene expression by inhibiting androgen activation of the androgen response elements in the promoter region. More importantly, dissociation of the effects of TZDs on apoptosis from their original pharmacological activity (i.e. PPAR γ activation) provides a molecular basis for the exploitation of these compounds to develop different types of molecularly targeted anticancer agents. These TZD-derived novel therapeutic agents, alone or in combination with other anticancer drugs, have translational relevance in fostering effective strategies for cancer treatment.

Endocrine-Related Cancer (2006) 13 401–413

Introduction

Thiazolidinediones (TZDs), including troglitazone (Rezulin), rosiglitazone (Avandia), pioglitazone (Actos), and ciglitazone, are synthetic ligands for the peroxisome proliferator-activated receptor γ (PPAR γ) (for review see Day 1999) (Fig. 1). This family of PPAR γ agonists improves insulin sensitivity by increasing transcription of certain insulin-sensitive genes involved in the metabolism and transport of lipids in adipocytes, such as lipoprotein lipase, adipocyte fatty acid-binding protein, acyl-CoA

synthase, and fatty acid transport protein (Koeffler 2003). In addition, TZDs have been demonstrated to promote the differentiation of preadipocytes by mimicking certain genomic effects of insulin on adipocytes (Tontonoz *et al.* 1994), and to regulate glucose homeostasis (Saltiel & Olefsky 1996). Consequently, the TZDs offer a new type of oral therapy in type II diabetes to reduce insulin resistance and to assist glycemic control.

Recent evidence indicates that certain TZD members, especially troglitazone and ciglitazone, exhibit moderate antiproliferative activities against

Figure 1 Chemical structures of troglitazone, ciglitazone, rosiglitazone, and pioglitazone, and their respective PPAR γ -inactive $\Delta 2$ derivatives ($\Delta 2$ -TG, $\Delta 2$ -CG, $\Delta 2$ -RG, and $\Delta 2$ -PG). The introduction of the double bond adjoining the terminal thiazolidinedione ring results in the abrogation of the PPAR γ ligand property.

many epithelial-derived human cancer cell lines including those of prostate (Kubota *et al.* 1998), breast (Yin *et al.* 2001), colon (Kato *et al.* 2004), thyroid (Ohta *et al.* 2001), lung (Tsubouchi *et al.* 2000), and pituitary carcinoma (Heaney *et al.* 2003). This growth inhibition was linked to the G1 phase cell cycle arrest through the up-expression of the cyclin-dependent kinase inhibitors p21 and p27 (Koga *et al.* 2001, 2003, Takeuchi *et al.* 2002, Yoshizawa *et al.* 2002, Bae *et al.* 2003) and/or repression of cyclin D1 expression (Wang *et al.* 2001, Yin *et al.* 2001, Lapillonne *et al.* 2003, Qin *et al.* 2003). Meanwhile, data from this and other laboratories have indicated that normal prostate epithelial cells (not shown) and T lymphocytes (Harris & Phipps 2002) are more resistant to apoptotic induction by these TZDs. In the light of this cancer-specific effect, the potential use of these PPAR γ agonists as chemopreventive agents has received much attention (for a review see Badawi & Badr 2002, Kopelovich *et al.* 2002, Smith & Kantoff 2002, Bull 2003, Koeffler 2003, Leibowitz & Kantoff 2003, Grommes *et al.* 2004, Jiang *et al.* 2004). Moreover, animal model studies have demonstrated the *in vivo* efficacy of troglitazone in colon, prostate, and liver cancers (Kubota *et al.* 1998, Sarraf *et al.* 1998, Yu *et al.* 2006) and that of rosiglitazone in pituitary tumors (Henry *et al.* 2000, Heaney *et al.* 2002). Despite these advances, the mechanism underlying the antitumor effects of TZDs remains unclear. As PPAR γ -mediated effects of TZDs promote the differentiation of preadipocytes, one school of thought attributes the

same mechanism to the terminal differentiation and cell cycle arrest of tumor cells (Tontonoz *et al.* 1997). However, the PPAR γ -target genes that mediate the antiproliferative effects remain elusive, as genomic responses to PPAR γ activation in cancer cells are highly complicated (Gupta *et al.* 2001). Reported causal mechanisms include attenuated expression of protein phosphatase 2A (Altioek *et al.* 1997), cyclins D1 and E, inflammatory cytokines and transcription factors (Koeffler 2003), and increased expression of an array of gene products linked to growth regulation and cell maturation (Gupta *et al.* 2001).

On the other hand, several lines of evidence have suggested that the inhibitory effect of TZDs on tumor cell proliferation is independent of PPAR γ activation. For example, sensitivity of cancer cells to TZD-induced growth inhibition does not correlate with the level of PPAR γ expression, and there exists a three orders of magnitude discrepancy between the concentration required to produce antitumor effects and that to modify insulin action (Day 1999). In addition, the *in vitro* antitumor effects appear to be structure specific irrespective of their potency in PPAR γ activation, i.e. troglitazone and ciglitazone are active while rosiglitazone and pioglitazone are not. To date, an array of non-PPAR γ targets has been implicated in the antitumor activities of troglitazone and/or ciglitazone in different cell systems, which include intracellular Ca²⁺ stores (Palakurthi *et al.* 2001), phosphorylating activation of extracellular signal-regulated kinases (Gouni-Berthold *et al.* 2001, Takeda *et al.* 2001), c-Jun N-terminal protein kinase, and p38 (Bae & Song 2003), up-regulation of early growth response-1 (Baek *et al.* 2003), the cyclin-dependent kinase (CDK) inhibitors p27^{kip1} (Motomura *et al.* 2000) and p21^{WAF/CIP1} (Sugimura *et al.* 1999), the tumor suppressor protein p53 and the p53-responsive stress protein Gadd45 (Okura *et al.* 2000), and altered expression of B-cell leukemia/lymphoma 2(Bcl-2) family members (Bae & Song 2003). However, some of these targets appear to be cell type specific due to differences in signaling pathways regulating cell growth and survival in different cell systems.

In considering the translational potential of using PPAR γ ligand in cancer prevention and therapy, our research has focused on elucidating the mechanisms underlying the antitumor effect of TZDs. In the following sections, evidence that dissociates the effect of TZDs on apoptosis from PPAR γ -activating activity is presented. This review also provides an

overview of recent findings from this and other laboratories concerning plausible PPAR γ -independent targets that underlie TZD-mediated apoptosis. Equally importantly, the use of TZDs as a molecular platform to develop novel mechanism-based therapeutic agents will be discussed.

Demonstration of PPAR-independent antiproliferative effects of TZDs

We hypothesized that if TZDs mediate antitumor effects independently of PPAR γ , one would be able to dissociate these two pharmacological activities via structural modifications of these molecules. This premise was corroborated by the development of a novel class of PPAR γ -inactive TZD analogs, which was accomplished by introducing a double bond adjoining the terminal thiazolidine-2,4-dione ring (Fig. 1) (Shiau *et al.* 2005). Two lines of evidence indicate that this structural modification abrogated the ligand-binding ability of PPAR γ . First, these $\Delta 2$ analogs (5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-2,4-thiazolidinedione ($\Delta 2$ -TG), 5-[4-[2-(methylpyridin-2-yl-amino)-ethoxy]-benzylidene]-2,4-thiazolidinedione ($\Delta 2$ -RG), 5-[4-[2-(5-ethyl-pyridin-2-yl)-ethoxy]-benzylidene]-2,4-thiazolidinedione ($\Delta 2$ -PG), and (5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione ($\Delta 2$ -CG)) were inactive in PPAR γ activation according to a PPAR γ transcription factor ELISA (Shiau *et al.* 2005). Secondly, DU-145 prostate cancer cells were transfected with a reporter construct that contains PPAR response element (PPRE) upstream of a luciferase gene. None of these $\Delta 2$ derivatives elicited any significant PPAR γ transactivation. The loss of PPAR γ activity was presumably attributed to the structural rigidity, as a result of the double bond introduction surrounding the heterocyclic system.

These PPAR γ -inactive TZD derivatives were examined in two prostate cancer cell lines with distinct PPAR γ expression status, androgen-independent PC-3 and androgen-dependent LNCaP. Between these two cell lines, PPAR γ was highly expressed in PC-3 cells, but was deficient in LNCaP cells (Fig. 2A). Nevertheless, despite a deficiency in PPAR γ , LNCaP cells exhibited a higher degree of susceptibility to troglitazone-mediated *in vitro* antitumor effects compared with the PPAR γ -rich PC-3 cells (Fig. 2B, left panel). In addition, $\Delta 2$ -TG, although devoid of PPAR γ -activating activity, was more potent than troglitazone in suppressing cell proliferation in both cell lines. This growth inhibition was attributable to apoptotic death, as

evidenced by mitochondrial cytochrome c release in PC-3 cell (Fig. 2B, right panel).

Similar results were obtained with ciglitazone and $\Delta 2$ -CG with respect to cytochrome c-dependent apoptotic death in PC-3 cells (Fig. 3), with relative potency similar to that of troglitazone and $\Delta 2$ -TG. In contrast, rosiglitazone, pioglitazone, and their $\Delta 2$ derivatives showed marginal effects, even at 50 μ M, on cell death in PC-3 cells (Fig. 3).

Together, these data suggest that TZDs mediated apoptosis induction in prostate cancer cell systems irrespective of PPAR γ activation. Mechanistic studies from this and other laboratories have further suggested that TZDs mediate proapoptotic effects through a complexity of PPAR γ -independent molecular targets, which include, but are not limited to, Bcl-2/Bcl-xL, cyclin D1, FADD-like IL-1-converting enzyme (FLICE)-inhibitory protein (FLIP), and prostate specific antigen (PSA), which are summarized as follows.

TZD-induced apoptosis involves the inhibition of Bcl-xL and Bcl-2 function

The functional relationship between antiapoptotic and proapoptotic Bcl-2 members in the regulation of mitochondrial integrity is well documented (Cory *et al.* 2003). Bcl-2 and Bcl-xL mediate their anti-apoptotic function through the sequestration of Bad, Bax, and other proapoptotic Bcl-2 members via BH3 domain-facilitated heterodimerization, thereby abrogating their ability to induce mitochondrial cytochrome c release (Diaz *et al.* 1997, Sattler *et al.* 1997, Otter *et al.* 1998, Nouraini *et al.* 2000, Finnegan *et al.* 2001). Thus, a balance between these two types of Bcl-2 members plays a crucial role in the regulation of the apoptotic machinery.

Effect of troglitazone on the expression of Bcl-2 family members

It has been reported that troglitazone at high doses repressed Bcl-2 expression in MCF-7 breast cancer (Elstner *et al.* 1998), and increased the expression level of the proapoptotic proteins Bad and Bax in HepG2 hepatoma cells (Bae & Song 2003). This phenomenon, however, appears to be cell line specific. In our study, troglitazone, even at 30 μ M, did not cause appreciable changes in the expression of any of the aforementioned Bcl-2 members in PC-3 cells with the exception of a slight decrease in Bad expression at 24-h exposure (Fig. 4) (Shiau *et al.* 2005).

Figure 2 Evidence that the effect of troglitazone on apoptosis in prostate cancer cells is dissociated from PPAR γ activation. (A) Western blot analysis of relative PPAR γ levels in PC-3 and LNCaP prostate cancer cells. (B, left panels) Dose-dependent effects of troglitazone and Δ 2-TG on the cell viability of PC-3 and LNCaP cells in serum-free RPMI 1640 medium for 24 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; (right panels) levels of cytochrome c release into cytoplasm induced by different doses of troglitazone and Δ 2-TG in PC-3 cells under the aforementioned treatment conditions. Values are means $\pm$ s.d. *, $P < 0.05$.

Figure 3 Differential effects of ciglitazone, rosiglitazone, pioglitazone, and their Δ 2 derivatives on apoptotic death in PC-3 cells in serum-free RPMI 1640 medium for 24 h. Values are means $\pm$ s.d.

Figure 4 Effect of troglitazone on the expression levels of Bcl-2 family members in PC-3 cells. PC-3 cells were exposed to 30 μ M troglitazone in serum-free RPMI 1640 medium for the indicated time, and equal amounts of proteins from cell lysates were electrophoresed and probed by western blotting with individual antibodies.

Inhibition of Bcl-xL and Bcl-2 function

Nevertheless, we obtained several lines of evidence that troglitazone and ciglitazone might inhibit the antiapoptotic function of Bcl-xL/Bcl-2 by blocking BH3 domain-mediated heterodimerization with proapoptotic Bcl-2 members (Shiau *et al.* 2005). First, data from the competitive fluorescence polarization analysis suggest that troglitazone, ciglitazone, and their $\Delta 2$ counterparts compete with a Bak BH3-domain peptide for binding to Bcl-xL and Bcl-2. Moreover, the potency of TZDs and their $\Delta 2$ analogs in inhibiting this peptide binding correlated with the respective effectiveness in inducing apoptotic death in LNCaP and PC-3 prostate cancer cells (Table 1).

For example, the inability of rosiglitazone and pioglitazone to trigger apoptotic death was reflected in their lack of potency in disrupting interactions between Bcl-xL/Bcl-2 and the Bak BH3 domain. Secondly, troglitazone and $\Delta 2$ -TG perturbed the dynamics of intracellular Bcl-2/Bak and Bcl-xL/

Bak interactions in PC-3 cells, i.e. the level of Bak associated with Bcl-2 and Bcl-xL was greatly reduced in troglitazone- and $\Delta 2$ -TG-treated cells as compared with DMSO control (Fig. 5A). As a result, liberation of proapoptotic Bcl-2 members induced apoptosis by facilitating cytochrome c release and consequent caspase-9 activation (Fig. 5B).

Thirdly, overexpression of Bcl-xL provided LNCaP cells conferred protection against troglitazone- and $\Delta 2$ -TG-induced apoptosis. As shown in Fig. 5C, ascending expression levels of Bcl-xL in three transfected LNCaP stable clones (B11, B1, and B3) diminished the susceptibility to troglitazone- and $\Delta 2$ -TG-induced apoptosis death as compared with that of parental LNCaP cells. The extent of cytoprotection conferred by ectopic Bcl-xL correlated with the Bcl-xL expression level. The excessive expression in B3 cells especially completely overcame the apoptotic effect of troglitazone and $\Delta 2$ -TG.

Antiproliferative mechanisms involve modulation of cell cycle effectors

Troglitazone and ciglitazone repress cyclin D1 expression via proteasome-facilitated proteolysis

PPAR γ agonists, including 15-deoxy- $\Delta 12,14$ -prostaglandin J₂ (PGJ₂), troglitazone, and ciglitazone, at high doses, have been shown to down-regulate cyclin D1 expression as part of their action to cause cell-cycle arrest and growth inhibition in breast cancer cells (Wang *et al.* 2001, Yin *et al.* 2001, Lapillonne *et al.* 2003, Qin *et al.* 2003, 2004, Huang *et al.* 2005). Cyclin D1 is a downstream effector of diverse proliferative and transforming signaling pathways, including those mediated by β -catenin (Shtutman *et al.* 1999), estrogen receptor

Table 1 Correlation between the IC₅₀ values of individual TZDs and $\Delta 2$ -TZDs for inhibiting BH3-mediated interactions of Bcl-xL and Bcl-2 with the Bak BH3 domain peptide and for inhibiting cell proliferation of PC-3 and LNCaP prostate cancer cells. Values are means $\pm$ s.d.

		TG	$\Delta 2$ -TG	CG	$\Delta 2$ -CG	RG	$\Delta 2$ -RG	PG	$\Delta 2$ -PG
IC ₅₀ in binding inhibition (μ M) ¹	Bcl-xL	22 $\pm$ 1	18 $\pm$ 1	26 $\pm$ 2	17 $\pm$ 2	>50	>50	>50	>50
	Bcl-2	22 $\pm$ 1	18 $\pm$ 1	24 $\pm$ 3	22 $\pm$ 3	>50	>50	>50	>50
IC ₅₀ in cell viability inhibition (M) ²	PC-3	30 $\pm$ 2	20 $\pm$ 2	22 $\pm$ 4	14 $\pm$ 1	>50	>50	>50	>50
	LN-CaP	22 $\pm$ 3	14 $\pm$ 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

¹ Determined by fluorescence polarization analysis of the ability of individual compounds to inhibit the binding of the Flu-BakBH3 peptide with Bcl-xL or Bcl-2.

² Determined by the MTT assay.
n.d., not determined.

Figure 5 Troglitazone (TG) and Δ 2-TG trigger caspase-dependent apoptotic death by inhibiting heterodimer formation of Bcl-2 and Bcl-xL with Bak. (A) Effect of TG and Δ 2-TG on the dynamics of Bcl-2/Bak (left) and Bcl-xL/Bax (right) interactions in PC-3 cells. PC-3 cells were treated with 50 μ M TG or Δ 2-TG for 12 h, and cell lysates were immunoprecipitated with anti-Bcl-2 or anti-Bcl-xL antibodies. The immunoprecipitates were probed with anti-Bak antibodies by western blot analysis (WB). The bar graphs indicate the relative Bak levels, normalized to IgG light chain levels, in the three treatments. Values are means $\pm$ s.d. ($n = 3$). * $P < 0.01$. (B) Dose-dependent effect of TG and Δ 2-TG on caspase-9 activation in PC-3 cells. PC-3 cells were treated with TG or Δ 2-TG at the indicated concentrations for 24 h. Caspase-9 antibodies recognize the large subunits (35 and 37 kDa). (C) Ectopic Bcl-xL protects LNCaP cells from TG- and Δ 2-TG-induced apoptosis by attenuating cytochrome c release in an expression level-dependent manner. (Left panel) Ascending expression levels of ectopic Bcl-xL in B11, B1, and B3 clones by western blot analysis. (Right panel) Dose-dependent effects of TG (left panel) and Δ 2-TG (right panel) on apoptosis in LNCaP, B11, B1, and B3 cells. Data are means $\pm$ s.d. ($n = 3$).

(ER) (Lukas *et al.* 1996, Wilcken *et al.* 1997, Prall *et al.* 1998), Her-2/Neu (Lee *et al.* 2000), nuclear factor- κ B (Joyce *et al.* 1999, Henry *et al.* 2000), Rac (Westwick *et al.* 1997), Ras (Albanese *et al.* 1995), Src (Lee *et al.* 1999), signal transducer and activator of transcription (Bromberg *et al.* 1999, Matsumura *et al.* 1999), and Wnt (D'Amico *et al.* 2000). In mammary cells, transcriptional activation of cyclin D1 in response to these mitogenic signals leads to G₁/S progression and increased proliferation. Cyclin D1 overexpression has been implicated in oncogene-induced mammary tumorigenesis; it has been noted that more than 50% of primary breast carcinomas correlated with poor prognosis

(McIntosh *et al.* 1995, Kenny *et al.* 1999). In addition to activating cyclin-dependent kinases (CDKs) and sequestering CDK inhibitors in the G₁/S transition, the function of cyclin D1 as a CDK-independent activator of ER α is especially noteworthy (Neuman *et al.* 1997, Zwijsen *et al.* 1997, McMahon *et al.* 1999, Lamb *et al.* 2000). Cyclin D1 overexpression confers resistance to antiestrogens in breast cancer cells (Musgrove *et al.* 2001, Hui *et al.* 2002), and represents a negative predictive factor for tamoxifen response (Stendahl *et al.* 2004). Together, these findings suggest that an anti-cyclin D1 therapy might be highly specific for treating human breast cancer (Yu *et al.* 2001). An urgent

need therefore exists to develop potent cyclin D1-ablative agents that are effective in the therapeutically attainable range ($\leq 5 \mu\text{M}$) for breast cancer prevention and/or therapy.

As troglitazone provides an attractive starting point for this drug discovery effort, the mechanism underlying its effect on cyclin D1 repression constituted the focus of our recent investigation (Huang *et al.* 2005). Several lines of evidence suggest that troglitazone mediates the repression of cyclin D1 expression via a PPAR γ -independent mechanism. First, $\Delta 2$ -TG and $\Delta 2$ -CG, although devoid of PPAR γ activity, were able to mediate cyclin D1 ablation with slightly higher potency than troglitazone and ciglitazone respectively in MCF-7 breast cancer cells (Fig. 6A), while the more potent PPAR γ agonists rosiglitazone and pioglitazone

lacked appreciable activity at comparable concentrations (not shown).

Secondly, troglitazone-mediated cyclin D1 down-regulation could not be rescued by the PPAR γ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662). Thirdly, despite significantly higher PPAR γ expression, MDA-MB-231 breast cancer cells were less susceptible to troglitazone-induced cyclin D1 ablation than MCF-7 cells. Although it has been reported that the PPAR γ ligands PGJ₂ and troglitazone inhibited cyclin D1 transcription by activating PPAR γ in MCF-7 cells (Wang *et al.* 2001) and mouse skin keratinocytes (He *et al.* 2004) respectively, data from this and other laboratories have indicated that troglitazone and ciglitazone mediate cyclin D1 ablation in MCF-7 cells by facilitating ubiquitination and proteasomal

Figure 6 Pharmacological evidence that the effect of troglitazone and ciglitazone on cyclin D1 down-regulation in MCF-7 cells is dissociated from PPAR γ activation. (A) Dose-dependent effect of troglitazone and $\Delta 2$ -TG on cyclin D1 expression for 24 h in MCF-7 cells by western blotting. (B) Dose-dependent effects of troglitazone on the expression of cyclins and CDKs in MCF-7 cells by western blot analysis.

proteolysis in a specific manner (Qin *et al.* 2003, Huang *et al.* 2005). For example, PCR analysis indicates that the mRNA level of cyclin D1 remained unaltered in drug-treated cells, indicating that the repression was mediated at the post-transcriptional level. Moreover, this drug-induced cyclin D1 repression could be rescued by proteasome inhibitors, and was preceded by increased ubiquitination.

It is noteworthy that the troglitazone-mediated cyclin D1 ablation was highly specific. Among all cyclins and CDKs examined (cyclins D2, D3, A, B, and E, and CDKs 2 and 4) as well as p53, only cyclin D2 and CDK4 showed a slight decrease in the expression level in drug-treated MCF-7 cells (Fig. 6B).

Troglitazone up-regulates the expression of the CDK inhibitor p21^{CIP1}

Moreover, western blotting analysis indicates that, in MCF-7 cells, troglitazone up-regulated the expression of p21^{CIP1}, while p27^{KIP1} exhibited a dose-dependent decrease (Fig. 6B) (Huang *et al.* 2005). However, the effect of troglitazone on the expression levels of these two CDK inhibitors varies among different cell lines. For example, troglitazone treatment led to increased expression of both p21^{CIP1} and p27^{KIP1} in several hepatocellular carcinoma cells examined (Elnemr *et al.* 2000, Koga *et al.* 2001), while in troglitazone-treated pancreatic cancer cells, only the expression level of p27^{KIP1}, but not that of p21^{CIP1}, increased (Motomura *et al.* 2000, Itami *et al.* 2001). These findings suggested that variations in responses exist among different cancer cell lines.

Other molecular mechanisms

In addition to the aforementioned effects, several other PPAR γ -independent mechanisms have been reported to account for the antiproliferative action of TZDs in different cancer cells. Among these proposed mechanisms, the effects on proteasome-mediated degradation of FLIP (Kim *et al.* 2002) and down-regulation of PSA expression (Kubota *et al.* 1998, Hisatake *et al.* 2000) are especially noteworthy in light of their clinical implications in cancer therapy.

FLIP inhibits apoptosis induced by tumor necrosis factor (TNF) family death receptor by blocking caspase 8 activation (Wajant 2003). As demonstrated by antisense-mediated down-regulation, decreased expression of FLIP confers sensitivity

against death receptor-induced apoptosis. Inhibition of FLIP expression in tumor cells is of particular importance to TNF-related apoptosis inducing ligand (TRAIL)-based cancer therapy (Wajant 2003). TRAIL is the ligand of death receptors, and has attracted substantial clinical interests in light of its ability to induce apoptosis preferentially in tumor cells. However, not all tumor cells respond to TRAIL, in part because of intracellular resistance mechanisms (Ashkenazi 2002). Recent reports indicate that PPAR γ agonists including troglitazone could sensitize tumor cells to TRAIL-induced apoptosis (Goke *et al.* 2000, Kim *et al.* 2002) as a result of the proteasome-dependent proteolysis of FLIP (Kim *et al.* 2002). As the mode of FLIP down-regulation is reminiscent of that of cyclin D1, there may exist cross-reactivity in troglitazone-mediated proteasomal degradation of both proteins.

PSA is used as a serum biomarker for diagnosis and progression of prostate cancer (Polascik *et al.* 1999). It has been shown that troglitazone at concentrations ($\leq 10 \mu\text{M}$) lower than that required for growth inhibition ($\geq 20 \mu\text{M}$) was able to down-regulate PSA expression in LNCaP prostate cancer cells through the inhibition of androgen activation of the androgen response elements in the regulatory region of the PSA gene (Hisatake *et al.* 2000). Moreover, the same report indicates that troglitazone at 600–800 mg/day stabilized PSA levels in a prostate cancer patient with a radical prostatectomy throughout a 14-month period.

Pharmacological exploitation of troglitazone to develop novel mechanism-based anticancer agents

The above discussion clearly demonstrates that the antiproliferative activity of troglitazone is mediated through multiple molecular targets independently of PPAR γ . From a mechanistic perspective, dissociation of the aforementioned pharmacological activities from PPAR γ activation provide a molecular rationale to use troglitazone as a starting point to develop novel mechanism-based therapeutic agents. The proof of the principle of this premise is 5-[4-(6-allyloxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-2,4-thiazolidinedione ($\Delta 2$ -TG-6) (Huang *et al.* 2005), a close structural analog that exhibited an order of magnitude higher potency than troglitazone and $\Delta 2$ -TG in repressing cyclin D1 expression and inhibiting MCF-7 cell proliferation (Fig. 7).

Figure 7 $\Delta 2$ -TG-6, a structurally optimized cyclin D1-ablative agent. (A) Structure of $\Delta 2$ -TG-6. (B) Dose-dependent effect of $\Delta 2$ -TG-6 on cyclin D1 down-regulation in MCF-7 cells. (C) Dose-dependent effects of $\Delta 2$ -TG-6 versus TG and $\Delta 2$ -TG on MCF-7 cell viability as analyzed by MTT assay. (D) Dose-dependent effects of $\Delta 2$ -TG-6 versus TG on PARP cleavage. PARP proteolysis to the apoptosis-specific 85 kDa fragment was monitored by western blotting.

As shown, $\Delta 2$ -TG-6 reduced cyclin D1 levels at concentrations as low as 2.5 μ M compared with ≥ 20 μ M for $\Delta 2$ -TG (Fig. 7B), and exhibited IC_{50} of 8 μ M versus 55 μ M for $\Delta 2$ -TG in inhibiting MCF-7 cell viability (Fig. 7C). This antiproliferative effect was attributable to apoptosis induction, as evidenced by poly(ADP-ribose)polymerase (PARP) cleavage (Fig. 7D). Lead optimization of $\Delta 2$ -TG-6 to generate more potent cyclin D1-ablative agents is currently undergoing, the clinical implication of which is multifold. First, cyclin D1 ablation provides specific protection against breast carcinogenesis (Yu *et al.* 2001). Secondly, in light of the role of cyclin D1 overexpression in antiestrogen resistance, cyclin D1 ablation may help overcome the resistance. Thirdly, the synergistic interaction between flavopiridol and trastuzumab in inhibiting breast cancer cell proliferation is attributable, in part, to the reduction of cyclin D1 expression (Wu *et al.* 2002). These agents may sensitize cells to the antiproliferative action of either CDK inhibition or Her-2/Akt inhibition.

A similar approach has been taken to develop a potent Bcl-2/Bcl-xL inhibitor (TG-88) with an IC_{50} of 1.8 μ M based on fluorescence polarization analysis. Oral TG-88 at a daily dose of 100 or 200 mg/kg was effective in suppressing PC-3 xenograft tumor growth without causing weight loss or apparent toxicity, indicating its oral bioavailability and potential clinical use (Shiau *et al.* 2005). It is also

noteworthy that the structural requirement for Bcl-2/Bcl-xL inhibition differed from that of cyclin D1 ablation.

Conclusion

Data from this and other laboratories have clearly demonstrated that the effect of TZDs on triggering apoptosis in cancer cells is dissociated from that of PPAR γ activation. To date, a number of important signaling mechanisms have been identified to underlie TZD-mediated antitumor activities, including, but not limited to, inhibition of Bcl-2/Bcl-xL function and repression of the expression of cyclin D1, FLIP, and PSA. These findings provide molecular underpinnings for the molecular exploitation of troglitazone and ciglitazone to develop different types of molecularly targeted anticancer agents. Our data indicate that structural preference for Bcl-2/Bcl-xL inhibition differs from that of cyclin D1 ablation, while that between cyclin D1 and FLIP is currently being investigated. Because of the heterogeneity in the cancer cell population, various genetic lesions/molecular defects allow different subsets of tumor cells to exhibit differential sensitivity to apoptotic signals generated by different chemotherapeutic agents. Thus, these troglitazone-derived agents in combination with other therapeutic agents have translational relevance in fostering effective strategies for cancer treatment.

Acknowledgements

This work was supported by Public Health Service Grants CA-94829 and CA-112250 (C-SC), and K08CA101875 (JJP) from National Cancer Institute, NIH, Department of Health and Human Service, and by Army Grant W81XWH-05-1-0089 from Department of Defense Prostate Cancer Research Program (C-SC). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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