Rosiglitazone sensitizes MDA-MB-231 breast cancer cells to anti-tumour effects of tumour necrosis factor- α , CH11 and CYC202

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Abstract

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a member of the nuclear hormone superfamily and has multiple endogenous and pharmacological ligands, including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ and two thiazolidinediones (TZD), rosiglitazone and pioglitazone, which are used clinically to treat type-2 diabetes mellitus. PPAR γ agonists regulate development, cellular growth and metabolism in various tissues and have been documented to decrease cellular proliferation and to induce apoptosis of various tumour phenotypes, including breast cancer. However, the full spectrum of anti-tumour effects occurs only at suprapharmacological doses. In this study, we investigated the mechanism of rosiglitazone-induced anti-tumour effects of MDA-MB-231 human breast cancer cells, and used that information to predict rosiglitazone-induced sensitization of breast cancer cells to the effects of other compounds. We first confirmed that 100 μ M rosiglitazone, but not lower doses, decreases MDA-MB-231 cell viability *in vitro*. We then used microarray gene expression analysis to determine early rosiglitazone-induced gene expression changes after 4-h exposure, which included 1298 genes that we grouped into functional categories. We selectively confirmed rosiglitazone-mediated effects on expression of key regulators of breast cancer proliferation and apoptosis, including p53, p21 and Bax. Finally, we used this information to predict that rosiglitazone would sensitize MDA-MB-231 cells to the anti-tumour effects of CH11, which trimerizes Fas, as well as tumour necrosis factor- α . Moreover, we used the confirmed array data to predict cooperative activity of rosiglitazone and R-roscovitine (CYC202), an inhibitor of multiple cyclin-dependent kinases. We conclude that microarray analysis can determine early TZD-modulated changes in gene expression that help to predict effective *in vitro* drug combinations.

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Introduction

Peroxisome proliferator-activated receptor- γ (PPAR γ), a member of the nuclear hormone superfamily, is expressed in many tissues. PPAR γ has endogenous and pharmacological ligands including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ and two thiazolidinediones (TZD), rosiglitazone and pioglitazone, which enhance insulin sensitivity and are

used clinically to treat type-2 diabetes mellitus (Theocharis *et al.* 2004). Ligand binding induces heterodimerization with retinoid X receptor, and the resultant complex transcriptionally regulates many genes (Theocharis *et al.* 2004). However, recent evidence suggests that non-receptor effects of PPAR γ agonists may be biologically important (Clay *et al.* 2002, Shiau *et al.* 2005).

PPAR γ agonists regulate development and cellular growth and metabolism in various tissues. In particular, they mediate adipocyte differentiation, which has led to an examination of their potential anti-tumour effects. PPAR γ agonists have been documented to decrease cellular proliferation and to induce apoptosis of various tumour phenotypes, including breast cancer (Nahle 2004, Theocharis *et al.* 2004). However, anti-tumour effects occur only at suprapharmacological doses; this has been a source of criticism, even though doses up to 1500 times those used clinically in humans were well tolerated in mouse tumour models (Heaney *et al.* 2002, 2003). Regardless, investigators are reluctant to use very high doses in clinical trials. Therefore, it was our aim to examine, *in vitro*, a short-course of high-dose rosiglitazone treatment, and to develop a rational approach to predict how rosiglitazone might enhance the activity of other agents, and/or how rosiglitazone might be used for short periods of time in combination therapy strategies.

We investigated the mechanism of rosiglitazone-induced anti-tumour effects on ER-negative MDA-MB-231 human breast cancer cells, and used that information to determine rational combination therapies that include rosiglitazone and other agents. We first confirmed a rosiglitazone dose that inhibited viability of MDA-MB-231 cells *in vitro*. We then used microarray gene expression analysis to determine very early rosiglitazone-induced primary gene expression changes. We confirmed expression changes of key regulators of breast cancer proliferation and apoptosis. Finally, we used this information to predict rosiglitazone-mediated sensitization of MDA-MB-231 cells to apoptosis by CH11, which trimerizes Fas, and tumour necrosis factor- α (TNF α). Moreover, we used the confirmed array data to predict synergistic activity of rosiglitazone and R-roscovitine (CYC202), an inhibitor of multiple cyclin-dependent kinases (CDK).

Materials and methods

Reagents

A stock solution of 14.3 mM rosiglitazone (Cayman Chemicals, Ann Arbor, MI, USA) in DMSO was stored at -20°C and a working solution was prepared in the culture medium just before use. CH11 was obtained from Cell Signaling (Beverly, MA, USA). TNF α was obtained from Sigma-Aldrich, CYC202 was obtained from Cyclacel Ltd (Dundee, Scotland, UK) and GW9662 was obtained from EMD Chemicals, Inc. (Gibbstown, NJ, USA). PPRE-x3-TK-LUC reporter vector that contains three copies of a consensus PPAR-response element (PPRE) upstream of the thymidine

kinase promoter-luciferase fusion gene, was kindly provided by Dr Bruce Spiegelman (Harvard University, Cambridge, MA, USA).

Cell culture

MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified incubator containing 5% CO_2 . Cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

Assays of cellular viability and DNA synthesis

Cellular viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MDA-MB-231 cells were seeded in triplicate ($n=6$) at 8×10^3 cells per well of a 96-well plate in DMEM. After overnight incubation, cells were replenished with fresh media containing 0.1, 1, 10, and 100 μM rosiglitazone and incubated for 4 or 6 days. Media containing drugs or vehicle were changed on alternate days. The effect of rosiglitazone on DNA synthesis was carried out using a BrdU Cell Proliferation ELISA kit purchased from Roche following the manufacturer's instruction.

The effect of rosiglitazone on sensitizing MDA-MB-231 cells to CH11, TNF α or CYC202 was assessed using the MTT assay. MDA-MB-231 cells were seeded in triplicate at 1×10^4 cells per well of a 24-well plate in DMEM. After overnight incubation, cells were replenished with fresh media containing 1.4 or 100 μM rosiglitazone and incubated for the indicated time periods. A dose of 1.4 μM is analogous to the peak plasma concentration of an 8 mg dose of rosiglitazone given to treat diabetes; i.e. $C_{\text{max}} = 598 \pm 117 \text{ ng/ml}$. The molecular weight of rosiglitazone equals 357.44; so the molar concentration in serum equals 1.4 μM .

Cells were pretreated with 100 μM rosiglitazone for 24 h followed by exposure to 50 ng/ml CH11 or 20 ng/ml TNF α for an additional 48 h. Control cells received vehicle (DMSO) at a concentration equal to that of the drug-treated cells. Media containing drug or vehicle was changed on alternate days. For readings at each time point, cells were incubated with fresh media containing 1 mg/ml MTT (Sigma-Aldrich). Following 2-h incubation at 37°C , media containing MTT was removed and 200 μl of vehicle was added per well. Cells were incubated at room temperature for 20 min with rotation and then transferred to a 96-well plate.

The absorbance was determined at 570 nm with a microtiter plate reader.

MDA-MB-231 cells were also subjected to simultaneous treatment with 100 μ M rosiglitazone plus vehicle (DMSO), or vehicle (DMSO) plus 20 nM CYC202, or 100 μ M rosiglitazone plus 20 nM CYC202 for 96 h. Media containing drug or vehicle was changed on alternate days. At 96 h, MTT assays were performed as previously described.

Western blot analysis

MDA-MB-231 cells were seeded at 1×10^6 cells per well in a six-well plate in DMEM. After treatment with 100 μ M rosiglitazone for the indicated time periods, cells were washed twice in cold $1 \times$ PBS, then scraped and collected in chilled lysis buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.2 mM Na_3VO_4 , 1 mM dithiothreitol and protease inhibitor cocktail). Cells were lysed by gently vortexing for 5 s every 5 min for a total of 20 min; cells were kept cold on ice in between vortexing. After centrifugation at 10 000 *g* for 5 min at 4 °C, the supernatant (lysate) was transferred to a fresh tube. Protein concentration was then determined using the Bradford Assay (Bio-Rad). Total extract (50 μ g) was mixed with $2 \times$ Laemmli sample buffer (Bio-Rad) and boiled for 5 min. Samples were run on 4–20% Tris-HCl precast gel (Bio-Rad) along with Precision Plus Kaleidoscope Protein Standards (Bio-Rad). After electrophoresis at 30 mA, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 100 V for 1 h at 4 °C. The membrane was blocked with PBS/0.1% Tween-20 (PBST) containing 5% non-fat dry milk for 1 h at room temperature and washed three times for 5 min each in PBST. The membrane was then incubated with the appropriate primary antibody overnight at 4 °C. Bax rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) was diluted in the ratio of 1:1000 in PBST containing 5% non-fat dry milk; and p53 mouse monoclonal antibody (Cell Signaling) was diluted in the ratio of 1:2000 in PBST containing 5% BSA. The membrane was washed three times for 5 min each in PBST and then incubated with HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) diluted in the ratio of 1:2000 or HRP-conjugated goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in the ratio of 1:10 000 in PBST containing 5% non-fat dry milk for 1 h at room temperature. The immunoblot was visualized by chemiluminescence using SuperSignal West Dura

Extended Duration Substrate (Pierce) on a Kodak 2000MM Multimodal Imaging System.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Total RNA (1 μ g) was digested using DNase I (Invitrogen). The DNase-treated RNA was then reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad) which contains a blend of oligo (dT) and random hexamer primers under the following conditions: 25 °C for 5 min; 42 °C for 30 min; and 85 °C for 5 min. Triplicate samples of cDNA were amplified by PCR using the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification reaction mixture (25 μ l total volume) contained 1 μ l cDNA, 20 pmol forward primer (*p21* F primer CCT AAT CCG CCC ACA GGA A, *Bax* F primer ATG GAC GGG TCC GGG GAG CAG CCC, and *18S* F primer GCC GCT AGA GGT GAA ATT CTT G), 20 pmol reverse primer (*p21* R primer AAG ATG TAG AGC GGG CCT TTG, *Bax* R primer GGT GAG CAC TCC CGC CAC AAA GAT, and *18S* R primer CAT TCT TGG CAA ATG CTT TCG), 12.5 μ l iTaq SYBR Green Supermix (Bio-Rad), and 9.5 μ l DEPC- H_2O . RRNA (18S) was used as an endogenous control to normalize the quantification of the target transcripts in each sample.

Luciferase assay

To test for transcriptional activity of PPAR γ , cells were seeded at a density of 2.5×10^5 cells per well of a six-well plate in triplicate 1 day prior to transfection. Cells were transiently co-transfected with PPRE-x3-TK-LUC reporter (2 μ g) and β -galactosidase vector (1 μ g) using FuGENE6 Transfection Reagent (Roche Diagnostics) according to the manufacturer's instructions. After 12 h, the FuGENE6-DNA mix-containing media was replaced with fresh media and incubated for another 24 h. This was followed by treatment with rosiglitazone or vehicle for the indicated time periods and concentrations. Cells were then harvested and lysed with $1 \times$ Reporter Lysis Buffer (Promega). Luciferase and β -galactosidase activity were measured using the Luciferase Assay System and β -Galactosidase Enzyme Assay System respectively (Promega). Luciferase activities were normalized against β -galactosidase levels to adjust for transfection efficiency.

Microarray analysis

Sample preparation and processing

MDA-MB-231 cells were seeded at 4.0×10^5 cells per 25 cm^2 flask in three replicates each for vehicle and rosiglitazone treatment. The following day, cells were treated with $100 \mu\text{M}$ rosiglitazone or vehicle for 4 h. Total RNA was isolated using Trizol reagent (Invitrogen) and subsequently purified with RNeasy Mini Kit (Qiagen). Sample quality was assessed on RNA 6000 Nano LabChip using the Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). Only samples that yielded high quality RNA with minimal degradation and sharp, clear 18S/28S ribosomal peaks were chosen for microarray analysis, which was performed in triplicate for vehicle and rosiglitazone treated samples. *In vitro* transcription (IVT), microarray hybridization and scanning were performed according to Affymetrix protocols. Briefly, total RNA from each sample was synthesized into double-stranded cDNA by reverse transcription. IVT reactions were performed on cDNA to generate biotinylated cRNA targets. These were then chemically fragmented and hybridized overnight at 45°C to Affymetrix Human Genome Focus Array chips, which represent over 8500 well-described human genes. Hybridized chips were washed and stained with streptavidin–phycoerythrin using the Affymetrix Fluidics Station 400 and scanned with an Affymetrix GeneChip 3000 confocal scanner. Fluorescent images from scanned chips were processed using Affymetrix GeneChip Operating System.

Microarray data analysis

Comparative analysis between expression profiles for vehicle- and rosiglitazone-treated samples was performed using GeneSpring software v7.0 (Agilent Technologies). Gene expression data were normalized, by selecting ‘per chip normalization’. In this case, all expression data on a chip were normalized to the 50th percentile of all values on that chip. The ‘cross gene error model for deviation from 1.0’ was inactive.

There were three replicates per group (vehicle versus rosiglitazone). The genes were filtered on the basis of flag value ‘present’ in all six samples. This criterion allowed for selection of genes based on the data quality flags in the original files. The filtered gene list was subsequently used to identify genes that were differentially expressed between vehicle and rosiglitazone-treated cells using a one-way ANOVA parametric test with a false discovery rate of 0.05. The expression of each gene was reported as the ratio of the value obtained after treatment relative to the control after normalization of the data.

Results

Rosiglitazone-mediated inhibition of cell viability and DNA synthesis

In order to determine the rosiglitazone dose that inhibited MDA-MB-231 cell viability, cells were treated with 0.1, 1, 10 or $100 \mu\text{M}$ rosiglitazone and MTT assay was carried out after 4 and 6 days. A significant decrease in viability was observed only when cells were treated with a $100 \mu\text{M}$ rosiglitazone (Fig. 1a).

Other groups noted decreases in cell proliferation as measured by thymidine H^3 incorporation with $10 \mu\text{M}$ rosiglitazone; we, therefore, evaluated the effects of 0.1, 1, 10 and $100 \mu\text{M}$ rosiglitazone on DNA synthesis as measured by BrdU incorporation. We showed that the lower doses decreased DNA synthesis, but the effect was not dose-dependent and was much smaller than the effect of $100 \mu\text{M}$ rosiglitazone (Fig. 1b). Moreover, these effects were seen only after 4 and 6 days of treatment and not at earlier time points.

Transcriptional activation of a PPAR γ response element by rosiglitazone

PPAR γ is expressed in MDA-MB-231 cells. To assess the functional activity of PPAR γ , MDA-MB-231 cells were transfected with a reporter construct (PPRE-x3-TK-LUC) that contains three copies of PPRE upstream of the firefly luciferase gene (Hortobagyi 1998, Lu *et al.* 2005). In Fig. 2a cells were exposed to vehicle, $100 \mu\text{M}$ rosiglitazone, $100 \mu\text{M}$ GW9662, or $100 \mu\text{M}$ rosiglitazone plus $100 \mu\text{M}$ GW9662. Vehicle-treated cells exhibited only basal luminescence; however, PPAR γ promoter activity was significantly induced after 4-h treatment with $100 \mu\text{M}$ rosiglitazone but not with GW9662. Furthermore, GW9662 abrogated the rosiglitazone-mediated transactivation indicating the effect was specific. Moreover, $1.4 \mu\text{M}$ rosiglitazone was unable to significantly enhance PPRE activity (Fig. 2b).

Rosiglitazone-induced differential gene expression

The Affymetrix Human Genome Focus Array, which represents over 8500 verified human sequences, was used to identify genes induced or repressed in MDA-MB-231 cells treated with $100 \mu\text{M}$ rosiglitazone, the dose that attenuated cell viability. A short treatment time (4 h) was used with the intent of identifying direct PPAR γ target genes and to avoid detecting the later, secondary effects of protein changes induced by rosiglitazone. By filtering the data based on flag value ‘present’ in all of the replicates analysed, 4002

Figure 1 Effect of rosiglitazone on cellular viability and DNA synthesis. (a) MDA-MB-231 cells were treated with vehicle, 0.1, 1, 10 or 100 μM rosiglitazone and cell viability was measured by MTT assay at 4 and 6 days. Data are expressed as the mean of three independent experiments and compared using Student's *t*-test. * $P < 0.05$, 100 μM Rosiglitazone vs DMSO control. ** $P < 0.01$, 100 μM Rosiglitazone vs DMSO control. (b) MDA-MB-231 cells were treated with vehicle, 0.1, 1, 10 or 100 μM rosiglitazone and DNA synthesis was measured by BrdU ELISA assay at 4 and 6 days. Data are expressed as the mean of three independent experiments and compared using Student's *t*-test. * $P < 0.01$, 100 μM Rosiglitazone vs DMSO control.

out of 8793 genes passed the filter in MDA-MB-231 cells. After performing one-way ANOVA with a false discovery rate of 0.05 on the filtered data set, a total of 1298 genes were identified as being differentially expressed between vehicle and rosiglitazone treatment in MDA-MB-231 cells.

To assess the biological process or function of the genes that showed a significant alteration of expression level, the expression analysis systematic explorer (EASE) classification system database

Figure 2 Rosiglitazone-mediated PPRE transactivation. (a) Cells were transiently transfected with a PPRE-luciferase reporter construct and treated with vehicle, 100 μM rosiglitazone, 100 μM GW9662, or both for the indicated time periods before assaying for promoter activity. Promoter activity is represented as relative luciferase activity after normalization with β-galactosidase. Data are expressed as the mean of three determinants from two independent experiments. Data were compared using Student's *t*-test. (b) Cells were transiently transfected with a PPRE-luciferase reporter construct and treated with vehicle, 1.4 μM rosiglitazone, 1.4 μM GW9662, or both for the indicated time periods before assaying for promoter activity. Promoter activity is represented as relative luciferase activity after normalization with β-galactosidase. Data are expressed as the mean of three determinants from two independent experiments. Data were compared using Student's *t*-test. * $P < 0.01$ vs DMSO control.

(<http://david.niaid.nih.gov/david/ease.htm>) was queried using the gene list derived from our microarray analysis. We focused our attention on categories that were relevant to this study (i.e. cell cycle, cell growth/maintenance, cell communication, regulation of transcription, apoptosis and cell proliferation). We reasoned that this approach would allow us to determine an association between the altered gene set and the functional pathways that might be involved in rosiglitazone-mediated apoptosis/growth inhibition. Based on this analysis, Table 1 lists genes that are overexpressed (≥ 1.25 -fold, $P \leq 0.05$) and underexpressed (≤ 0.75 -fold, $P \leq 0.05$) after rosiglitazone treatment relative to the vehicle. A comprehensive list of statistically significant gene changes can be found in the supplemental data.

Table 1 List of upregulated and downregulated genes in rosiglitazone-treated MDA-MB-231 cells

	P value	Fold change	Common	Genbank	Description
a. Upregulated genes					
Induction of apoptosis/apoptotic program	0.015	2.39	BAD	U66879	BCL2-antagonist of cell death
	0.020	1.57	DAP	NM_004394	Death-associated protein
	0.013	2.89	BAK1	NM_001188	BCL2-antagonist/killer 1
	0.046	1.47	BAX	NM_004324	BCL2-associated X protein
	0.038	3.91	BCL2L1	AL117381	BCL2-like 1 (bcl-xL)
	0.0002	1.55	DEDD	AK022531	Death effector domain containing
	0.006	2.26	DDX41	NM_016222	DEAD (Asp–Glu–Ala–Asp) box polypeptide 41
	0.0187	2.26	TP53	NM_000546	Tumour protein p53
Programmed cell death	0.015	3.01	LTBR	NM_002342	Lymphotoxin β receptor (TNFR superfamily, member 3)
Cell cycle regulation	0.014	1.93	NBL1	D28124	Neuroblastoma, suppression of tumourigenicity 1
	0.034	1.54	G0S2	NM_015714	Putative lymphocyte G0/G1 switch
	0.014	1.93	NBL1	D28124	Neuroblastoma, suppression of tumourigenicity 1
	8.39×10^{-6}	4.16	PPP2R1A	NM_014225	Protein phosphatase 2 (formerly 2A), regulatory subunit A α
	0.032	1.82	PPM1G	NM_002707	Protein phosphatase 1G (formerly 2C), magnesium-dependent, γ
	0.019	1.82	CDK4	NM_000075	Cyclin-dependent kinase 4
	0.048	1.61	CDKN1A	NM_000389	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	0.00948	6.64	TGFB1	BC000125	Transforming growth factor, β 1
	0.013	7.79	RAB1B	NM_030981	RAB1B, member RAS oncogene family
	0.018	2.32	PPP1R15A	U83981	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
b. Downregulated genes					
Induction of apoptosis/apoptotic program	0.00263	0.66	CASP3	NM_004346	Caspase 3
	0.00778	0.67	CASP4	U25804	Caspase 4
	0.018	0.68	DAP3	U18321	Death-associated protein 3
	0.00141	0.59	APAF1	NM_013229	Apoptotic protease activating factor
Cell cycle regulation	0.014	0.69	RAN	AF054183	RAN, member RAS oncogene family
	0.033	0.61	CCNL1	NM_020307	Cyclin L1
	0.018	0.65	CCNC	AL137784	Cyclin C
	0.019	0.65	ANAPC10	NM_014885	Anaphase promoting complex, subunit 10
	0.044	0.61	CETN3	BC005383	Centrin
	0.00285	0.65	CDK7	L20320	Cyclin-dependent kinase 7
Cell proliferation	0.00563	0.68	STAMBP	NM_006463	STAM-binding protein
	0.047	0.73	FGFR1OP	NM_007045	FGFR1 oncogene partner
	0.00228	0.50	NRP1	BE620457	Neuropilin 1

Validation of selected targets by western blot and quantitative RT-PCR

Microarray analysis of MDA-MB-231 cells established p53, p21 and Bax as potential target genes that were upregulated by rosiglitazone that are involved in tumour suppression and/or apoptosis. Therefore, we focused on these three genes for validation studies. Protein levels were assessed by western blot for later time points than were used for the microarray screening in order to allow enough time for translation to occur. MDA-MB-231 cells treated with 100 μ M rosiglitazone for 24 and 48 h demonstrated an increase in p53 and p21 protein levels when compared with vehicle-treated cells (Fig. 3a). However, no change in the Bax protein level was detected. Bax protein levels were then analysed 4- and 8-h post-treatment, which also failed to show a change in expression (Fig. 3b). Therefore, Bax mRNA expression was analysed by real-time RT-PCR at 4- and 24-h post-treatment. Results indicate an increase in expression by 1.43-fold at 4 h, closely correlating with the array data; however, no change in mRNA levels was detected at 24 h (data not shown).

Figure 3 Western blot analysis to validate genes modulated by rosiglitazone (a) MDA-MB-231 cells were treated with 100 μ M rosiglitazone or vehicle and harvested at indicated times. Cell extracts were separated on SDS-PAGE and protein levels were detected with antibodies against Bax, p21 and p53. (b) MDA-MB-231 cells were treated with 100 μ M rosiglitazone or vehicle for the indicated time periods and then harvested. Cell extracts were separated on SDS-PAGE and protein levels were detected with antibody against Bax.

Rosiglitazone sensitizes MDA-MB-231 cells to CH11 and TNF α

Since rosiglitazone-treated cells demonstrated higher levels of p53 and p21 protein, we hypothesized that rosiglitazone could sensitize MDA-MB-231 cells to agents that also act on these apoptosis/cell cycle pathways. MDA-MB-231 cells were pretreated with 100 μ M rosiglitazone for 24 h followed by the treatment with either CH11 or TNF α for an additional 48 h at doses that are known to induce apoptosis (Lejeune *et al.* 2006, Wajant 2006). This resulted in enhanced growth inhibition when compared with treatment with any of the agents alone. Rosiglitazone followed by CH11 treatment resulted in 42% cell death, whereas vehicle followed by CH11 treatment inhibited cell growth by only 1% (Fig. 4). Rosiglitazone followed by TNF α treatment resulted in a 45% cell death, while vehicle followed by TNF α inhibited cell growth by only 5%. Rosiglitazone followed by vehicle treatment inhibited growth by only 17% (Fig. 5a). Since the ultimate goal of these studies is to determine whether a short exposure and/or lower doses of TZD can ultimately be used clinically, we evaluated whether 1.4 μ M rosiglitazone was able to enhance the effects of TNF α on DNA synthesis. As seen in Fig. 5b, 1.4 μ M rosiglitazone was unable to augment the effect of TNF α .

Rosiglitazone enhances CYC202 effects

MDA-MB-231 cells co-treated with 100 μ M rosiglitazone and 20 nM CYC202 demonstrated reduced viability when compared with either agent alone. Rosiglitazone plus CYC202 demonstrated a 91% inhibition of growth by 96 h when compared with 45 and 21% for CYC202 plus vehicle and rosiglitazone plus vehicle respectively (Fig. 6a). Moreover, 1.4 μ M

Figure 4 Effect of rosiglitazone plus CH11 on cellular viability. Cells were pretreated with 100 μ M rosiglitazone for 24 h, then treated with 50 ng/ml CH11 for an additional 48 h. Cell viability was measured by MTT assay. Data are expressed as the mean of three independent experiments. * P <0.01 vs DMSO; * P <0.01 vs Rosiglitazone; † P <0.01 vs CH11, Student's *t*-test.

Figure 5 Effect of rosiglitazone plus TNF α on cellular viability. (a) Cells were pretreated with 100 μ M rosiglitazone for 24 h, then treated with 20 ng/ml TNF α for an additional 4 h. Cell viability was measured by MTT assay. Data are expressed as the mean of three independent experiments. (b) Cells were pretreated with 1.4 μ M rosiglitazone for 24 h, then treated with 20 ng/ml TNF α for an additional 4 h. Cell viability was measured by MTT assay. Data are expressed as the mean of three independent experiments. * P <0.01 vs DMSO; ** P <0.01 vs Rosiglitazone, £ P <0.05 vs TNF, Student's t -test.

rosiglitazone was not able to enhance the effects of CYC202 on DNA synthesis (Fig. 6b).

Discussion

PPAR γ agonists facilitate adipocyte differentiation when combined with other agents. Several lines of evidence indicate that fibroblasts differentiate into adipocytes when exposed to glucocorticoid plus either insulin or insulin-like growth factor I plus a PPAR γ agonist (Kletzien *et al.* 1992, Tontonoz *et al.* 1995). Hu *et al.* (1995) were able to transdifferentiate myoblasts into adipocytes using dexamethasone plus 5,8,11,14-eicosatraynoic acid (an endogenous PPAR γ

Figure 6 Effect of rosiglitazone plus CYC202 on cellular viability. (a) Cells were treated with vehicle, 100 μ M rosiglitazone plus vehicle, CYC202 plus vehicle, or CYC202 plus 100 μ M rosiglitazone for 96 h. Cell viability was measured by MTT assay. Results represent mean $\pm$ s.d. of three experiments and were compared by Student's t -test. (b) Cells were treated with vehicle, 1.4 μ M rosiglitazone plus vehicle, CYC202 plus vehicle, or CYC202 plus rosiglitazone for 96 h. Cell viability was measured by MTT assay. Results represent mean $\pm$ s.d. of three experiments and were compared by Student's t -test. * P <0.05 vs DMSO. ** P <0.01 vs Rosiglitazone. † P <0.01 vs CYC202.

ligand) plus insulin. Several pathways regulate adipogenesis, including PPAR γ -induced degradation of β -catenin during preadipocyte differentiation, and CCAAT/enhancer binding proteins (C/EBP β), which induces C/EBP α , enhances PPAR γ production, and is required for PPAR γ -associated adiponectin expression during the terminal stages of adipogenesis (Farmer 2005). In agreement, Wnt-1, an upstream activator of β -catenin, blocks adipogenesis *in vitro* (Ross *et al.* 2000). These data indicate a complex, tightly regulated pathway where multiple agents are needed to fully induce differentiation. Therefore, we surmised that

TZDs might be more effective anti-tumour agents in combination with other compounds.

Heaney *et al.* (2002, 2003) investigated rosiglitazone's anti-tumour effects using a pituitary tumour model. They demonstrated that PPAR γ expression is restricted to normal pituitary corticotroph cells in rodents and humans, but is overexpressed in all pituitary tumour phenotypes. Furthermore, suprapharmacological doses of rosiglitazone significantly inhibited growth of incipient and established gonadotroph, somatotroph and corticotroph tumours in mice. In each case, rosiglitazone decreased the tumour-mediated hormone oversecretion (Heaney *et al.* 2002, 2003). Because suprapharmacological doses of rosiglitazone were required, this suggests that 1) anti-tumour effects might occur only after saturation of endogenous PPAR γ receptors, 2) co-treatment with other agents might potentiate TZD effects and 3) rosiglitazone doses up to 1500 times those used in humans are well tolerated by mice.

In this study, we confirmed that a high dose of rosiglitazone was required to inhibit MDA-MB-231 cell viability, but that lower doses could inhibit DNA synthesis over the course of 4 or 6 days. However, only 100 μ M rosiglitazone was able to transactivate a PPRE reporter construct at 1 and 4 h, which are consistent with findings of other *in vitro* and *in vivo* studies (Heaney *et al.* 2002, 2003, Sheu *et al.* 2006). One possibility for the requirement of very high doses to inhibit cell viability is that the endogenous PPAR γ receptors, which are often overexpressed in cancer, may need to be saturated prior to intracellular levels of TZD rising sufficiently to mediate anti-tumour effects. Indeed, Seargent *et al.* (2004) demonstrated that the PPAR γ antagonist GW9662 selectively occupied PPAR γ receptors, yet enhanced rosiglitazone's anti-tumour effects in breast cancer cells, supporting the concept that by binding to PPAR γ , GW9662 permitted a higher intracellular level of rosiglitazone (Seargent *et al.* 2004). Alternatively, the amount of TZD that can enter a tumour cell may be limited. Another possibility is that the molecular machinery required for growth inhibitory/apoptotic effects may be incomplete or less functional in breast cancer cells. Finally, the potency of TZDs as activators of PPAR γ and their PPAR γ -independent anti-tumour effects may not correlate. For example, rosiglitazone and troglitazone are both potent PPAR γ activators; however, rosiglitazone's anti-tumour effects are seen only at suprapharmacological doses, whereas troglitazone demonstrates some anti-tumour activity at pharmacologic doses (Sharma *et al.* 2004). We attempted to simulate a clinically relevant dose (an 8 mg dose of rosiglitazone leads to a serum concentration of $\sim$ 1.4 μ M) and to increase the dose to

70 times of that level (100 μ M). In contrast, Heaney *et al.* used 1500 times the maximal clinical dose in *in vivo* murine studies. Of course, direct exposure of cells in culture to rosiglitazone is not necessarily analogous to *in vivo* exposure, regardless of the dose.

In an effort to evaluate only primary direct PPAR γ target gene changes, we performed a microarray analysis only after 4-h rosiglitazone treatment in order to avoid detecting secondary effects of protein changes induced by rosiglitazone, which then might further alter gene expression. A similar approach was taken by Gerhold *et al.* (2002) who investigated the gene expression profile of adipocyte differentiation by performing microarray analysis only after 6-h treatment with a PPAR γ agonist. They used the Affymetrix platform to identify 579 out of 6347 genes, which were either up- or downregulated. Their findings support the notion that early genomic changes are diverse and robust. Our array analysis demonstrated that rosiglitazone altered expression of 1298 genes only after 4 h. These changes affected key pathways involved in growth and apoptosis. Based on EASE analysis, genes were grouped into functional categories. We focused on categories most relevant to anti-cancer effects, including cell cycle, growth/maintenance, communication, transcriptional regulation, apoptosis and proliferation.

We hypothesized that rosiglitazone would alter expression of genes that regulate cell death, thereby sensitizing cancer cells to known apoptosis-inducing drugs. Since several pro-apoptotic genes were upregulated in our microarray screen, we predicted cooperative interactions with agents that also act in the apoptotic pathway. We chose to focus on members of the TNF death-receptor family including TNF α and Fas, which induce apoptosis through pathways involving ligand-mediated receptor activation. TNF α and CH11 (which trimerizes the Fas receptor) have been shown to promote apoptosis in tumour cells (Toillon *et al.* 2002, Butt *et al.* 2005). As seen in Fig. 4a and b, co-treatment with rosiglitazone plus CH11 or rosiglitazone plus TNF α resulted in greater cell death than treatment with either of the agents alone, but only at suprapharmacological doses.

Since rosiglitazone increased p53 and p21 protein levels (Fig. 3a) and has previously been shown to induce G1/S phase arrest in a variety of cell lines, we aimed to determine whether co-treatment with a drug that arrested cells at the G2/M phase would potentiate the anti-tumour effects of rosiglitazone in breast cancer cells (Artwohl *et al.* 2005, Gruszka *et al.* 2005, Sheu *et al.* 2006). As can be seen in Fig. 5, co-treatment of MDA-MB-231 cells with rosiglitazone and CYC202 reduced proliferation greater than treatment with either

drug alone, but again only at suprapharmacological doses. CYC202 is an ATP analogue that inhibits several cellular CDKs at various levels of sensitivities but has greatest effect against CDK2/cyclin E (Gray *et al.* 1999, McClue *et al.* 2002). CYC202 has been shown to inhibit the growth of a variety of tumour cell lines, including B cell lymphomas, and breast, lung and colon cancer cell lines (Mgbonyebi *et al.* 1999, McClue *et al.* 2002, Wojciechowski *et al.* 2003, Alvi *et al.* 2005). Treatment of tumour cell lines with CYC202 induced p53 expression resulting in growth arrest and apoptosis (Mgbonyebi *et al.* 1999, Wojciechowski *et al.* 2003, Alvi *et al.* 2005). These results suggest that targeting two different cell cycle checkpoints simultaneously might be an effective therapeutic strategy in breast cancer. Moreover, CYC202 may be advantageous in co-treatment as it has proven effective against p53-null and p53-positive tumour cell lines (Alvi *et al.* 2005). Furthermore, CYC202 had little toxicity in phase I clinical trials (Fischer & Gianella-Borradori 2003).

In summary, we conclude that rosiglitazone induces diverse early genomic changes that include key regulators of apoptosis and cell cycle progression. In addition, these early genomic changes can be used to guide investigators to rationally combine rosiglitazone with other agents. Finally, early rosiglitazone-induced genomic changes have functional relevance and can predict some early protein expression changes.

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