Oestrogen-related receptor alpha inverse agonist XCT-790 arrests A549 lung cancer cell population growth by inducing mitochondrial reactive oxygen species production

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Abstract

Objective: Although oestrogen-related receptor α (ERR α) is primarily thought to regulate energy homeostasis, it also serves as a prognostic marker for cancer. The aim of this study was to investigate any connection between ERR α activity and cell population growth.

Materials and methods: XCT-790, an ERR α specific inverse agonist, was employed to suppress ERR α activity in human non-small cell lung cancer cells (NSCLC) A549. Gene expressions were detected using quantitative real-time PCR and Western blot analysis. Mitochondrial mass, membrane potential and reactive oxygen species (ROS) production were measured by staining with Mitotracker green, JC-1 and CM-H₂DCFDA dyes respectively. Rate of progression through the tricarboxylic acid (TCA) cycle was analysed by measuring activities of citrate synthase and succinate dehydrogenase. Cell cycle analysis was performed by using flow cytometry.

Results: We found that XCT-790 treatment reduced mitochondrial mass but enhanced mitochondrial ROS production by increasing rate through the TCA cycle, elevating mitochondrial membrane potential ($\Delta\Psi_m$) and down-regulating expression of superoxide dismutase. It was further demonstrated that XCT-790-induced ROS modulated p53 and Rb signalling pathways and suppressed cell replication.

Conclusions: ERR α affects cell cycle mechanisms through modulating mitochondrial mass and function. Dysregulation of this essential pathway leads to

elevation in mitochondrial ROS production, which in turn modulates activities of tumour suppressors, resulting in cell cycle arrest.

Introduction

Oestrogen-related receptor alpha (ERR α) is a member of the nuclear hormone receptor superfamily that has been identified due to its sequence similarity to the oestrogen receptor (ER) (1). Initial studies demonstrated that ERR α does not bind to ER agonist 17 β -oestradiol; thus, ERR α is recognized as an orphan nuclear receptor with constitutive activity. However, several studies have suggested that ERR α and its close family members ERR β and ERR γ can nonetheless, bind to selective synthetic ligands. Specifically, synthetic compounds XCT-790 and compound A have been shown to be ERR α -specific inverse agonists, while synthetic oestrogen diethylstilboestrol acts as an inverse agonist for all ERRs (2–5). Recently, we also identified that phytoestrogen kaempferol is an ERR α and ERR γ inverse agonist (6).

ERR α regulates energy homeostasis by interacting with peroxisome proliferator-activated receptor γ coactivator-1 α and 1β (PGC-1 α and PGC-1 β). In a coordinated manner, they control transcription of genes of the oxidative phosphorylation pathway (7–10). ERR α has also been implicated as a prognostic marker for breast, ovarian, colon and prostate cancers (11–15); that is, ERR α immunoreactivity or mRNA overexpression is significantly associated with increased risk of cancer development and adverse clinical outcome (11) suggesting a role of ERR α in regulating cell proliferation. Indeed, suppressing expression of ERR α by RNA interference inhibits population growth of oestrogen receptor-negative breast cancer cells (16). ERR α inverse agonist XCT-790 synergizes with the oestrogen receptor antagonist to inhibit proliferation of oestrogen receptor-positive breast cancer cells

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(4,17). Another $ERR\alpha$ inverse agonist, compound A, inhibits proliferation of both oestrogen receptor-positive and -negative breast tumour xenografts in mouse (18). However, it still leaves unexplained how $ERR\alpha$ mechanistically regulates cell division. There is no direct evidence that links $ERR\alpha$ to expression of cell cycle regulatory genes in these cancer cell types. Besides, a comprehensive study of $ERR\alpha$ regulated genes in cardiac muscle, by chromatin immunoprecipitation followed by microarray analysis, reveals that cell cycle regulatory genes are unlikely to be direct transcriptional targets of $ERR\alpha$ (19). Therefore, it remains to be established how dysregulation of $ERR\alpha$ activity impacts cell population growth and whether $ERR\alpha$ influences cell cycle machinery through modulating mitochondrial function and energy metabolism.

In the present study, using $ERR\alpha$ specific inverse agonist XCT-790 as a probe, we found that suppressing $ERR\alpha$ activity resulted in suppression of mitochondrial mass. Our results also showed that XCT-790 increased mitochondrial reactive oxygen species (ROS) production by elevating mitochondrial membrane potential ($\Delta\Psi_m$) and down-regulating expression of superoxide dismutase (SOD). Furthermore, we demonstrated that XCT-790 modulated p53 and Rb pathways and suppressed cell replication by increasing ROS production. These pieces of evidence collectively provide a mechanistic basis for the role of $ERR\alpha$ as a cancer prognostic marker and suggest that pharmacological suppression of $ERR\alpha$ activity may represent a novel strategy to treat cancer.

Materials and methods

Cell cultures and treatments

Human lung cancer A549 cells were purchased from the American Type Culture Collection (ATCC) and cultured according to ATCC instructions. For cell proliferation assays, cells were seeded in 24-well plates at a density of 40 000 cells/well with or without compounds added. Cell number was counted after time periods, as indicated. Assays were performed in triplicate and were repeated at least three times.

Plasmids and transfections

Expression vectors in pCMV-Gal4-DBD were generated by subcloning ligand binding domains (LBDs) of the corresponding nuclear hormone receptors ($ERR\alpha$: amino acids 144–423; $ERR\gamma$: amino acids 168–350) into the pCMV-BD vectors. All plasmids were sequence verified. Transient transfection was performed as we have reported previously with pFRLacZeo vectors used as internal control (6).

siRNA transfection

$ERR\alpha$ and control siRNA were purchased from Ribo Bio (Guangzhou, China). Cells were transfected with siRNA using tremegene (Roche, Mannheim, Germany) according to the manufacturer's instructions. After 24 h, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was generated using a reverse transcription kit (Invitrogen). Gene expression was detected by real-time PCR. Total protein was extracted with RIPA buffer and protein expression was detected by Western blotting.

Mitochondrial mass assay

After cells were treated for 24 h with different doses of XCT-790, Mitotracker green (Invitrogen) diluted in serum-free 1640 medium (pre-warmed to 37 °C) was added to XCT-790-treated or -untreated cells in fresh medium. Adherent cells were washed twice in cold phosphate-buffered saline (PBS) and suspended in 1 ml PBS for analysis using a Becton Dickinson (FAC-SCalibur; BD Biosciences, CA, USA) flow cytometer. Mitotracker green was excited at 488 nm and fluorescence was detected at 525 nm.

Succinate dehydrogenase and citrate synthase activity assay

Cells were plated at 3000 cells/well in 96-well plates and incubated for 24 h. Then XCT-790 was added and cells were incubated for an additional period of 24 h. Succinate dehydrogenase activity (SDH) was quantified by dissolving MTT-formazan in dimethyl sulphoxide (DMSO) and reading absorbance of the resulting solution at 570 nm. Citrate synthase (CS) activity was detected as described previously (20). SDH and CS activity were normalized to protein concentrations from extracted cell samples.

Measurement of ROS accumulation

Cells were plated at 70 000 cells/well in 24-well plates and incubated for 24 h. Then XCT-790 was added and cells were incubated for an additional period of 24 h. They were then incubated with phenol red-free medium (Opti-MEM) (Gibco, Grand Island, NY, USA) containing 1 μ M 2',7'-dichlorofluorescein diacetate (CM-H₂DCFDA) (Beyotime, Jiangsu, China) in the dark for 30 min at 37 °C. Cells were then harvested and ROS levels were analysed using a flow cytometer (FAC-SCalibur; BD Biosciences, CA, USA) and data were processed using the CellQuest program (BD Biosciences).

Western blot analysis and quantitative real-time PCR

Cell extracts were prepared in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF and 10 μ g/ml Aprotinin) and whole cell extracts (50–75 μ g of protein) were treated with SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blot analysis using an ECL Western Blotting System (RPN2108, Amersham Pharmacia, UK). p53 and ERR α antibodies were purchased from BD PharmingenTM (San Jose, CA, USA) and Upstate (Temecula, MA, USA), respectively, while total Rb, phospho-Rb (Ser795), phospho-Rb (Ser807/811) and β -actin antibodies were purchased from Cell Signaling (Danvers, MA, USA). Quantitative real-time PCR was performed as previously reported (6). Primers and PCR conditions can be provided upon request.

Assessment of apoptosis after Hoechst 33342 staining

Cells were seeded into six-well plates at 2×10^5 /well. The following day, XCT-790 was added to cells and they were cultured for 48 h. Cells were washed twice in PBS and fixed in 4% formaldehyde for 10 min. They were then washed again in PBS and stained with 10 μ g/ml of Hoechst 33342 for 10 min. Cells were examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Determination of mitochondrial membrane potential

Cells were plated at 70 000 cells/well in 24-well plates and incubated overnight. XCT-790 was added and they were incubated for further 24 h. Cells were harvested and incubated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride (JC-1) or rhodamine 123 (Sigma, St. Louis, MO, USA) and washed twice in PBS before being analysed by flow cytometry.

Cell cycle analysis

Cells treated for 48 h with different doses of XCT-790 were fixed in 70% ethanol, treated with 50 μ g/ml RNase A (Sigma), stained with 50 μ g/ml propidium iodide (Sigma), and analysed by flow cytometry for DNA synthesis and cell cycle status (FACSCalibur instrument and CELL-QUEST software, Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Hypothesis-testing methods included one-way analysis of variance (ANOVA) followed by least significant difference test. Results represent mean $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$. Significance was considered at $P < 0.05$.

Results

XCT-790 specifically down-regulated ERR α activity and arrested A549 cell population growth

To explore any connection between ERR α and cell cycle progression, we first investigated whether ERR α inverse agonist XCT-790 was sufficient to arrest cell population growth in oestrogen-independent human non-small cell lung cancer cells (NSCLC) A549. Using a Gal4-DBD-ERR α LBD system in which we stimulated the ERR α activity with cotransfection of coactivator PGC1 α expression plasmids (21), we showed that XCT-790 dose dependently suppressed ERR α but not ERR γ activities (Fig. 1a,b). We then examined the effect of XCT-790 on cell population growth and found that XCT-790 dose-dependently inhibited cell division with IC₅₀ at around 2.5 μ M (Fig. 1c) that closely matches IC₅₀ of the Gal4-DBD-ERR α -LBD assay. In addition to functioning as an ERR α inverse agonist, XCT-790 weakly induced activity of peroxisome proliferator-activated receptor γ (PPAR γ) (4). As PPAR γ agonists such as rosiglitazone and troglitazone at high concentrations have been shown to suppress cancer cell replication (9), it is possible that XCT-790-mediated proliferation suppression may be dependent on PPAR γ . We then tested whether GW9662, an antagonist of PPAR γ , was sufficient to block the proliferation suppressive effect of XCT-790. Although GW9662 was sufficient to block the induction of PPAR γ activity mediated by rosiglitazone (data not shown), our results showed that GW9662 could not overcome the suppressive effect of XCT-790 in our lung cancer cells (Fig. 1d), suggesting that the proliferation suppressive effect of XCT-790 is not mediated by PPAR γ but rather correlates with suppressing activity of ERR α .

Additionally, we used RNA interference to suppress expression of ERR α to confirm our hypothesis that down-modulating ERR α would be sufficient to suppress cancer cell population growth. Initially, we designed and synthesized an siRNA that specifically targeted ERR α . We established that ERR α siRNA down-modulated expression of ERR α at both protein and mRNA levels (Fig. 1e). We then transfected ERR α siRNA into A549 cells and measured its effect on cell proliferation. Compared to control siRNA, ERR α -specific siRNA dose-dependently reduced cancer cell division (Fig. 1f). These data collectively suggest that down-modulating activity or expression of ERR α is sufficient to reduce cancer cell population growth.

XCT-790 suppressed mitochondrial mass and altered mitochondrial function

ERR α partially regulates expression of PGC-1s and coordinates with PGC-1s to mediate mitochondrial mass

Figure 1. XCT-790 specifically down-regulates ERR α activity and arrests A549 cell growth. (a) and (b) Effects of XCT-790 on ERR α and ERR γ ligand binding domain activities. A549 cells were transfected with expression plasmids of Gal4-DBD-ERR α LBD or -ERR γ LBD (20 ng per well), pcDNA-PGC-1 α (10 ng/well) together with reporter pFR-Luc (20 ng per well) and pFRlaczeo control vectors (5 ng/well). Cells were treated with different doses of XCT-790 for 24 h before luciferase and β -galactosidase assays. Relative luciferase activity indicates the ratio of luciferase versus β -galactosidase activities. Data shown was from one representative experiment done in triplicate (mean $\pm$ SD) of three independent experiments. (c) Effects of XCT-790 on cell growth. A549 cells were seeded in 24-well plates at 4×10^4 /well. The following day, different doses of DMSO (0 μ M XCT-790) as control was set to 100%. (d) Effects of GW9662 on XCT-790-induced growth suppression. Cells were pre-treated with GW9662 for 2 h and then incubated with XCT-790 for another 48 h before cell counting. (e) Effects of ERR α siRNA on ERR α expression. Cells were transfected with 100 nmol control or ERR α -specific siRNA for 24 h. ERR α mRNA levels were detected using real-time PCR with 18S rRNA as an internal control. ERR α protein expression levels were detected using Western blot analysis with β -actin as an internal control. (f) Effects of ERR α siRNA on cell growth. Cell number was counted after cells were transfected with siRNA for 48 h. The number of cells treated with control siRNA as a control was set to 100%. Results represent mean $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 2. XCT-790 suppresses mitochondrial biogenesis and alters mitochondrial function. (a) Effects of XCT-790 on *PGC-1 α* and *PGC-1 β* gene expression. Gene expressions were detected using quantitative real-time PCR and shown as % of control. Cells treated with DMSO (0 μ M XCT-790) as control was set to 100%. 18S rRNA was used as an internal control. (b) Effects of XCT-790 on mitochondrial mass. Mitochondrial mass was detected by Mitotracker™ Green staining, which was performed as described in Materials and methods. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. (c) Effects of XCT-790 on PDK2 and PDK4 gene expression. Gene expressions were detected using quantitative real-time PCR and shown as % of control. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. 18S rRNA was used as an internal control. (d) Effects of XCT-790 on CS and SDH activities. Citrate synthase and SDH activities were measured as described in Materials and methods. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. Results represent mean $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$.

(7,22). We then checked mRNA expression levels of PGC-1s upon suppressing ERR α activity and found that XCT-790 dose-dependently reduced expression of PGC-1 α , while marginally suppressing expression of PGC-1 β (Fig. 2a). We then asked whether reduced levels of PGC-1s would lead to reduction of mitochondrial mass. We treated cells with Mitotracker Green that stains mitochondria independent of mitochondrial membrane potential. Using flow cytometric analysis and confocal imaging, we found that XCT-790 dose-dependently decreased mitochondrial mass (Fig. 2b and data not shown).

In addition to suppressing mitochondrial mass, we wondered whether XCT-790 would also alter mitochondrial function. Pyruvate dehydrogenase complex (PDC) catalyses conversion of pyruvate to acetyl-CoA. Phosphorylation of PDC by pyruvate dehydrogenase kinases 2 and 4 (PDK2 and PDK4) inhibits PDC activity and reduces rates of glucose utilization and tricarboxylic acid cycle (TCA cycle) (23). PDK2 and PDK4 are controlled by ERR α at the transcriptional level (23,24). We found that XCT-790 decreased mRNA expression levels of PDK2 and PDK4 (Fig. 2c). Reduction in PDK2 and PDK4 expression levels would be expected to enhance PDC activity and hence rate of passage through the TCA cycle. Indeed, we found that XCT-790 increased enzymatic activities of two key enzymes in the TCA cycle,

citrate synthase (CS) and succinate dehydrogenase (SDH) (Fig. 2d), suggesting that XCT-790 hyperactivated the TCA cycle.

XCT-790 increased mitochondrial reactive oxygen species production

The mitochondrial electron transport chain is one of the main sources of ROS. PGC-1 α can modulate ROS production by regulating expression of key ROS detoxifying enzyme SODs and uncoupling proteins (UCPs) (25,26). As XCT-790 specifically reduced expression of PGC-1 α , we explored whether XCT-790 would affect mRNA expression levels of SODs and UCPs. As expected, XCT-790 dose-dependently decreased expression of SOD1, SOD2, UCP2 and UCP3 (Fig. 3a,b). Reduced SOD expression may elevate mitochondrial ROS production. We found that XCT-790 dose- and time-dependently increased ROS levels (Fig. 3c). In addition, down-modulating expression of ERR α by siRNA also induced ROS production (Fig. 3d). UCPs are inner mitochondrial membrane proton carriers that modulate mitochondrial membrane potential ($\Delta\Psi_m$) (27). Reduced expression levels of UCP2 and UCP3 together with the hyperactivated TCA cycle, which generates more NADH and FADH $_2$ to enter into the electron transport chain, would be expected to

Figure 3. XCT-790 increases mitochondrial reactive oxygen species production. (a) Effects of XCT-790 on SOD1 and SOD2 gene expression. Gene expressions were detected using quantitative real-time PCR and shown as % of control. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. 18S rRNA was used as an internal control. (b) Effects of XCT-790 on UCP1/2/3 gene expression. Gene expressions were detected as in (a). (c) Effects of XCT-790 on ROS production. ROS level was measured as described in materials and methods. Results were expressed as mean DCF fluorescence. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. (d) Effects of ERR α siRNA on ROS production. Cells were transfected with 100 nM control or ERR α siRNA and incubated for 24 h, and ROS level was analysed as in (c). (e) Effects of XCT-790 on mitochondrial membrane potential. Mitochondrial membrane potential was assayed by JC-1 staining and quantified by Flow Cytometry. (f) Effects of rotenone on XCT-790-reduced mitochondrial mass. Cells were pre-treated with 200 nM rotenone for 2 h and then incubated with DMSO or 10 μ M XCT-790 for another 24 h before mitochondrial mass was measured by Mitotracker Green staining as in material and methods. (g) Effects of rotenone on XCT-790-induced ROS production. Cells were treated as in (f) before ROS level was measured as in (c). Experiments were performed as described in Materials and methods. Results represent mean $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$.

elevate $\Delta\Psi_m$. Using a mitochondrial dye JC-1 that provides green fluorescence when $\Delta\Psi_m$ is low and a red fluorescence when $\Delta\Psi_m$ is high, we confirmed that XCT-790 elevated $\Delta\Psi_m$ (Fig. 3e). Similar results were obtained using rhodamine123 as an alternative dye (data not shown). Therefore, reduced expression of anti-oxidative enzyme SODs and elevated $\Delta\Psi_m$ may be dually responsible for increase in mitochondrial ROS production.

To confirm further whether the increase in ROS originated from mitochondria, we used an electron transport chain complex I inhibitor, rotenone, to block mitochondrial activity and asked if this inhibitor would selectively affect increase in ROS production. While rotenone had no significant effect on the ability of XCT-790 to reduce mitochondrial mass (Fig. 3f), it effectively reduced amounts of ROS induced by XCT-790 (Fig. 3g), strongly suggesting that increase in ROS was derived from mitochondria.

XCT-790 suppressed cell growth by increasing ROS production

To establish whether the increase in ROS was primarily responsible for the cell population growth suppressive effect of down-modulating ERR α activity, we used an anti-oxidative compound Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTBAP) to block induction of ROS by XCT-790 (Fig. 4a). We found that MnTBAP could reverse the replication suppressive effect of XCT-790 (Fig. 4b). To confirm further that ROS induced by XCT-790 arrested cell division and cell cycle progression, we employed propidium iodide staining to analyse cell cycles of these cancer cells treated with XCT-790 and found that the proportion of cells at G₀/G₁ phase was higher, while those at S- and G₂-phases were lower in a dose-dependent manner (Fig. 4c). Ability of XCT-790 to arrest cell cycle progression through G₀/G₁ phases was also blocked by MnTBAP (Fig. 4d). However, we did not find that XCT-790 substantially changed proportions of cells in the sub-G₁ phase. Hoechst 33342 staining results also showed that XCT-790 did not induced apoptosis

when cells were cultured in medium supplemented with 10% foetal bovine serum (FBS); however, XCT-790 induced significant apoptosis in cells cultured in medium supplemented with 0.1% FBS (Fig. 4e). Thus, these data collectively suggest that cell population growth suppression and cell cycle arrest induced by XCT-790 was dependent on increase in mitochondrial ROS production.

XCT-790 modulates p53 and Rb pathways by increasing mitochondrial ROS production

As ROS is critical to cell replication suppressive effect of XCT-790 and that ROS has been shown to induce tumour suppressor p53 expression (28,29), we examined the effect of XCT-790 on p53 protein level and the role of ROS in this process. Using Western blot analysis, we found that XCT-790 dose-dependently induced p53 at the protein level and anti-oxidative compound MnTBAP blocked this induction (Fig. 5a,c). To discover whether XCT-790 increased p53 transcriptional activity, we checked to see if p53-regulated gene cell cycle inhibitor p21^{WAF1} and DNA repair enzyme GADD45 α were induced by XCT-790. We found that p21^{WAF1} and GADD45 α mRNA levels were induced by XCT-790 and MnTBAP blocked these inductions (Fig. 5b,d).

As cell cycle inhibitor p21^{WAF1} functions to block the activity of G₁ phase cyclin-dependent kinases (CDK2 and CDK4), which phosphorylate tumour suppressor Rb at serine 795 to promote cell division, we additionally examined whether XCT-790 would affect the Rb tumour suppressor pathway. We found that XCT-790 dose-dependently reduced total Rb protein level (Fig. 5a,c). Intriguingly, the phosphorylation status of Rb was differentially affected by XCT-790. XCT-790 selectively reduced Rb phosphorylation at serine 795 while leaving phosphorylation at serine 807 intact (Fig. 5a,c), consistent with the idea that reduction of CDK2 and CDK4 activities by XCT-790 treatment was associated with induction of p21^{WAF1}. The shift in phosphorylation status of Rb may promote function of Rb as a repressor of transcription factor E2F (30). We indeed found that XCT-790 reduced

Figure 4. XCT-790 suppresses cell growth by increasing ROS production. (a) Effects of anti-oxidative compound MnTBAP on XCT-790-induced ROS production. Cells were pre-treated with different concentrations of MnTBAP for 2 h and then incubated with DMSO or 10 μM XCT-790 for another 24 h before ROS level was measured. (b) Effects of MnTBAP on XCT-790-mediated growth inhibition. Cells were seeded in 24-well plates at 4×10^4 /well. The following day, different concentrations of MnTBAP were added to cells 2 h prior to the addition of DMSO or 10 μM XCT-790. Cell number was counted after 48 h. (c) Effects of XCT-790 on the cell cycle. A549 cells were treated with different doses of XCT-790 for 48 h. Cell cycle was analysed by propidium iodide (PI) staining as described in materials and methods. (d) Effects of MnTBAP on XCT-790 induced cell cycle arrest. Cells were treated with or without 200 μM MnTBAP before either DMSO or 10 μM XCT-790 treatment for 48 h. Cell cycle was analysed as in (c). (e) Cells were seeded into six-well plates at 2×10^5 /well and cultured overnight. XCT-790 was added to the cells in 10% FBS and incubated for another 48 h. Alternatively, cells were seeded into six-well plates at 4×10^5 /well, cultured overnight and then starved in medium with 0.1% FBS for 24 h. XCT-790 was added to the cells and incubated for another 24 h in 0.1% FBS. Apoptosis was detected by staining with Hoechst 33342 dye. Arrow points to apoptotic cells.

expression of E2F target genes such as cyclin E and p18^{INK4c} (31–33) (Fig. 5b and data not shown). Furthermore, MnTBAP blocked effects of XCT-790 on the Rb tumour suppressor pathway (Fig. 5b,d). These data suggested that XCT-790 modulates p53 and Rb pathways by increasing mitochondrial ROS production, and both pathways may be involved in XCT-790-mediated cell cycle arrest. Finally, as cyclin D1 is a target of the oestrogen receptor in breast cancer cells and that ERRα had been previously implicated in affecting ER-regulated gene expression (34), we also found that expression of cyclin D1 was suppressed by XCT-790 treatment in a ROS-dependent manner (Fig. 5b,d).

Discussion

With the retrovirally transduced dominant negative mutant version of thyroid hormone receptor v-erb A serving as an example, several members of the nuclear hormone receptor superfamily have been linked directly or indirectly to

oncogenesis (35–37). The oestrogen receptor and the androgen receptor play important roles in stimulating breast and prostate cancer development, respectively. Importantly, oestrogen receptor antagonists such as 4-hydroxytamoxifen and androgen receptor antagonists such as bicalutamide are currently used clinically to suppress hormone-dependent cancers (38,39). On the other hand, agonists for peroxisome proliferator-activated receptor γ (PPARγ) such as troglitazone and rosiglitazone have been demonstrated to suppress cancer cell population growth *in vitro* (40,41). However, the therapeutic values of PPARγ agonists in treating cancer remain controversial, because genetic manipulation of PPARγ expression in animal models of cancer has yielded contradictory results and many developing PPARγ agonists increased tumour incidence in rodents (42).

Although not classically considered to be an oncogene, ERRα has been suggested as a cancer prognostic marker; that is, higher level of ERRα expression is positively related to adverse clinical outcome (11–15). ERRα

Figure 5. XCT-790 modulates p53 and Rb pathways by increasing mitochondrial ROS production. (a) Effects of XCT-790 on p53 and Rb protein expressions. Total p53, total Rb, Rb Ser 795 phosphorylation and Rb Ser 807 phosphorylation levels were assayed by Western blots with β -actin as an internal control as in materials and methods. (b) Effects of XCT-790 on cyclin D, cyclin E, p21 and GADD45 α gene expression. Gene expression was detected using quantitative real-time PCR and shown as % of control. Cells treated with DMSO (0 μ M XCT-790) as a control was set to 100%. 18S rRNA was used as an internal control. (c) Effects of MnTBAP on p53 and Rb protein levels regulated by XCT-790. Cells were treated with or without 200 μ M MnTBAP before either DMSO or 10 μ M XCT-790 treatment for 48 h. Western blots were performed as in (a). (d) Effects of MnTBAP on cyclin D, cyclin E, p21 and GADD45 α gene expression regulated by XCT-790. Cells were treated with or without 200 μ M MnTBAP before either DMSO or 10 μ M XCT-790 treatment for 24 h. Gene expressions were detected as in (b).

can bind to oestrogen-related receptor responsive element (ERRE) and oestrogen-responsive element (ERE) and cross-talks to ER α in breast cancer cells to regulate expression of an oestrogen responsive gene *pS2* (43), suggesting that ER α and ERR α may dually play important roles in breast cancer development. However, how ERR α contributes to development of other non-oestrogen-dependent tumours is not well understood.

Previously, in differentiated 3T3-L1 adipocytes, we have established that suppressing activity of ERR α through XCT-790 leads to reduced mitochondrial mass and increased ROS production (44). In the present study, we further explored whether ERR α regulated cell population growth through this mechanism, in the context of a cancer cell. We found that down-regulating ERR α activity in A549 lung cancer cells reduced mitochondrial mass with compensatory increase in $\Delta\Psi_m$ caused by suppression of PDK2/4 and UCP2/3 expression. The increase in $\Delta\Psi_m$ and the reduced expression of anti-oxidative enzymes, SOD1 and SOD2, in turn induced ROS production.

Importantly, there are differences between differentiated adipocytes and cancer cells; that is, XCT-790 preferentially reduced mRNA expression level of PGC-1 α in cancer cells in contrast to PGC-1 β in 3T3-L1 adipocytes. Consistent with the role of PGC-1 α in modulating the ROS detoxification system (25,26), expression levels of SOD1, SOD2, UCP2 and UCP3 levels were strongly reduced in A549 (Fig. 3). Coincidentally, the fold induction of ROS by XCT-790 was higher in A549 than in 3T3-L1 cells. These differences may be cell type specific. They may also indicate mitochondrial regulation is different between cell cycle arrested/differentiated cells and actively growing cancer cells.

The highly induced mitochondrial ROS production activates both p53 and Rb tumour suppressor pathways and arrests cell growth. Although ROS have been suggested to promote tumour formation or cell population growth by inducing DNA damage and gene activation, recent studies have revealed a more complicated picture, in which ROS could also be utilized to suppress cancer

cell population growth and even induce apoptosis (45,46). Our study thus provides a plausible mechanistic explanation for the impact of $ERR\alpha$ on cell cycle mechanics in a ROS-dependent manner, through modulating mitochondrial mass and function.

The roles of $ERR\alpha$ and $PGC-1\alpha$ in regulating oxidative phosphorylation have been well established. In particular, reduced expression of $PGC-1\alpha$ in muscle tissue is thought to contribute to insulin resistance (47). Thus, a number of pharmaceutical companies have been interested in screening for agonists of $ERR\alpha$ to restore oxidative phosphorylation capacity through enhancing $PGC-1\alpha$ expression, as a novel treatment strategy for diabetic patients. Our results and those of Chisamore *et al.* raise intriguing possibilities that $ERR\alpha$ inverse agonists would potentially have therapeutic value as novel chemotherapeutics for treating cancers (18). In addition to suppressing lung cancer cell population growth, we also demonstrated that XCT-790 functioned in suppressing breast, prostate and colon cancer cell population growth (data not shown). As the activity of $ERR\alpha$ has also been shown to be enhanced by receptor tyrosine kinase activation, it would be interesting to test whether cancer cells with hyperactive receptor tyrosine kinase activation, like that of HER2-overexpressing breast cancer cells, would have higher sensitivity to $ERR\alpha$ inverse agonists. In conclusion, our study provides another possible link between obesity, diabetes and cancer, and that alterations in metabolic pathways may be potential strategies for cancer treatment.

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