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The Estrogen-Related Receptor α Inverse Agonist XCT 790 Is a Nanomolar Mitochondrial Uncoupler

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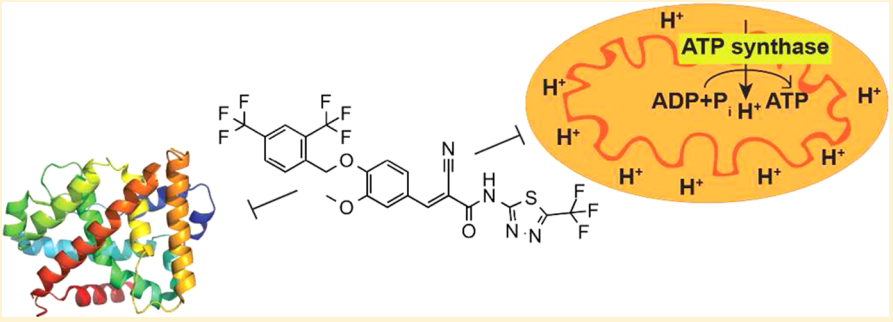

ABSTRACT: XCT 790 is widely used to inhibit estrogen-related receptor α (ERRα) activity as an inverse agonist. Here, we report that XCT 790 potently activates AMP kinase (AMPK) in a dose-dependent and ERRα-independent manner, with active concentrations more than 25-fold below those typically used to perturb ERRα. AMPK activation is secondary to inhibition of energy production as XCT 790 rapidly depletes the pool of cellular ATP. A concomitant increase in oxygen consumption rates suggests uncoupling of the mitochondrial electron transport chain. Consistent with this, XCT 790 decreased mitochondrial membrane potential without affecting mitochondrial mass. Therefore, XCT 790 is a potent, fast-acting, mitochondrial uncoupler independent of its inhibition of ERRα. The biological activity together with structural features in common with the chemical uncouplers FCCP and CCCP indicates likely mode of action as a proton ionophore.

The development of cell-permeable synthetic chemical compounds that directly and specifically antagonize or agonize a biological entity of interest has been hugely beneficial as they have become tools for probing the mechanism of action of small molecules, proteins, and organelles. These chemical probes (also known as tool compounds) are commonly employed in dynamic perturbation contexts that cannot be mimicked genetically, to modulate both the timing and the extent of target inhibition and/or activation.[1,2] Such contexts are usually essential for unraveling the most proximal biochemical mechanisms of action for a target of interest within cells and tissues.[3] The specificity of the chemical−target interaction thus becomes a defining feature for productive tool compounds. The presence of multiple cellular targets for a chemical probe, especially if they are unrecognized and mechanistically diverse, can obviously severely confound interpretation of biological observations associated with that probe. As such, careful consideration of alternate mechanisms of action of tool compounds is essential for the constructive application of chemical probes to biological investigation.[2,4]

The thiadiazoleacrylamide XCT 790 is the first potent and selective inhibitor of estrogen-related receptor α (ERRα).[5,6] Its development was motivated by the recognition of ERRα as a key participant in the regulation of cell differentiation, energy homeostasis, and metabolic syndromes. The mechanism of inhibition appears to be the disruption of ERRα−coactivator complexes upon binding of XCT 790 to the inferred ligand-binding domain of ERRα.[5−7] As such, it has since been extensively employed as a tool compound to directly evaluate the participation of ERRα in numerous cell biological processes.

From an investigation employing XCT 790 to help elaborate the contribution of ERRα to oncogenic transformation, we unexpectedly discovered abrupt and potent activity against mitochondrial energy production. This activity occurred within minutes in all cell lines tested, and at doses >25-fold lower than those commonly employed to inhibit ERRα. Notably, this activity was completely independent of the expression of ERRα, the only known target of XCT 790. Measurements of mitochondrial membrane potential, oxygen consumption, and extracellular acidification rates indicated acute and dose-dependent uncoupling of oxidation from phosphorylation in mitochondria by XCT 790, most likely through direct action as a proton ionophore.

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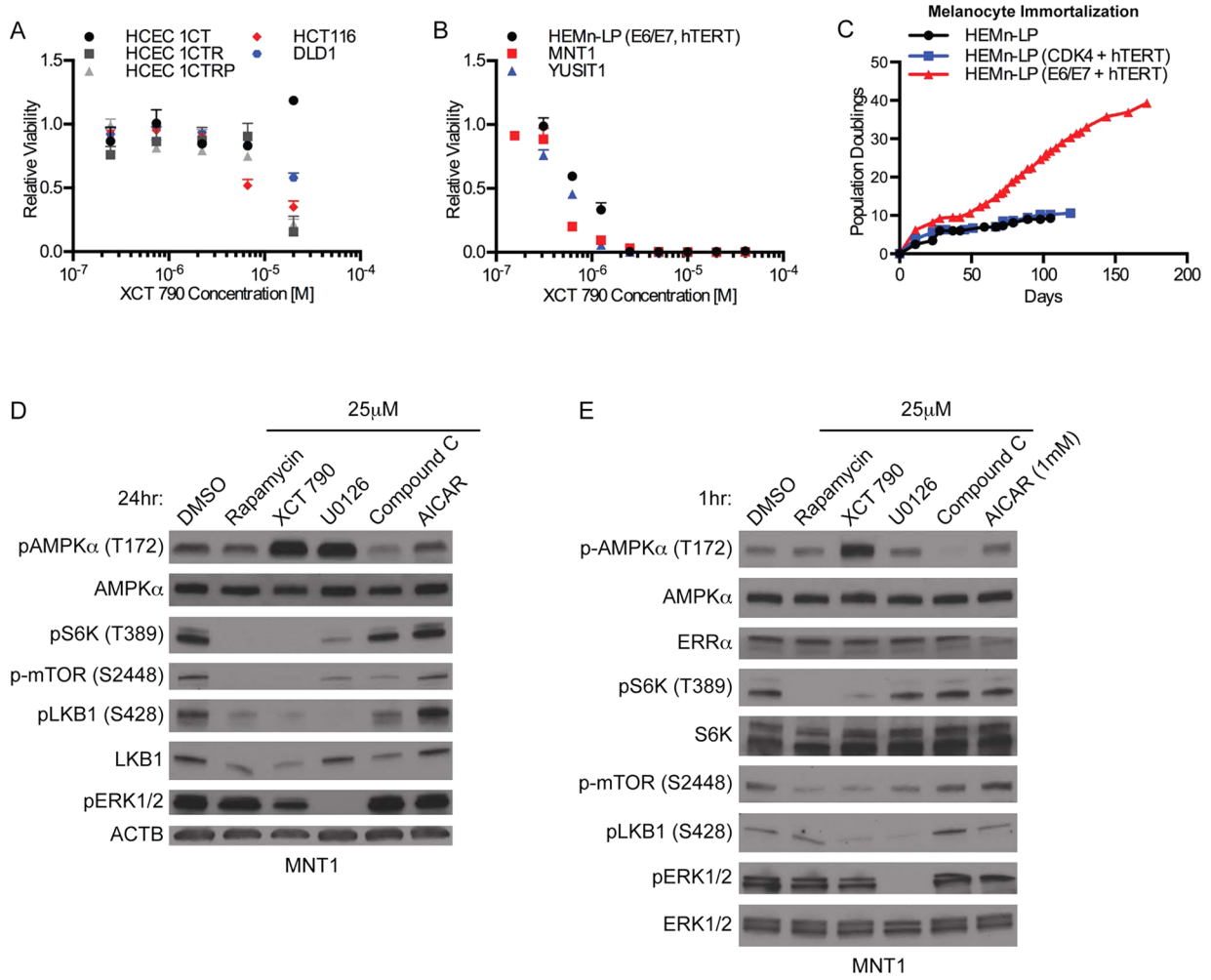

Figure 1. Selective viability and signaling perturbations by XCT 790. (A) Normalized cell viability of immortalized human colonic epithelial cells and colon cancer cell lines or (B) immortalized human melanocytes and melanoma cell lines 72 h after exposure to XCT 790 at the indicated doses. Bars indicate means ± the standard deviation ($N = 3$). (C) Doubling of the populations of HEMn-LP, HEMn-LP (CDK4/hTERT), and HEMn-LP (E6/E7/hTERT) cells for the indicated times. (D and E) Whole cell lysates of MNT1 cells, exposed to XCT 790 for 24 (D) or 1 h (E), were assessed for the accumulation of indicated phosphorylated proteins.

MATERIALS AND METHODS

Materials. XCT 790, rapamycin, and Compound C were purchased from Sigma-Aldrich. U0126 was purchased from Cell Signaling Technology. AICAR was purchased from Toronto Research Chemicals. The following antibodies were used: phospho-AMPKα (Thr172), AMPKα, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-mTOR (Ser2448), phospho-LKB1 (Ser428), LKB1, phospho-Raptor (Ser792), phospho-ACC (Ser79), ACC, phospho-ULK1 (Ser555), COX IV, phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), and p44/42 MAPK (ERK1/2) from Cell Signaling Technology; ERRα (V19), VDAC1/2/3 (FL-283), cytochrome c (7H8), BCL2 (C2), and TOM20 (FL-145) from Santa Cruz Biotechnology; and ACTB from Sigma-Aldrich.

Cell Culture. Human colonic epithelial cells, HCEC 1CT, were immortalized by ectopically expressing cyclin-dependent kinase 4 (CDK4) and the catalytic component of human telomerase (hTERT).[8] HCEC 1CTR and HCEC 1CTRP cells were generated by ectopically expressing $KRAS^{V12}$ and $KRAS^{V12}$ together with shRNA against P53, respectively.[9] HCEC growth medium and culture conditions have been described previously.[8] HCT116 and DLD1 colon cancer cell lines were maintained in DMEM supplemented with 10% FBS and assayed in HCEC medium. Neonatal human epidermal melanocytes from a lightly pigmented donor (HEMn-LP) were purchased from Invitrogen and immortalized by ectopically expressing hTERT and human papilloma virus, HPV16 E6/E7 protein. HEMn-LP (E6/E7, hTERT) cells were cultured in Medium 254 supplemented with human melanocyte growth supplement (HMGS) purchased from Invitrogen with penicillin (100 units/mL) and streptomycin (0.1 mg/mL) from Invitrogen. MNT1, YUSIT1, A375, YUMAC, YUSIT1, and C8161 human melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (catalog no. 12430) with 10% FBS from Atlanta Biologicals and with penicillin (100 units/mL) and streptomycin (0.1 mg/mL). Human bronchial epithelial cells, HBEC3, -30, and -34, were isolated from three patients and were immortalized by ectopically expressing CDK4 and hTERT.[10] HBECs were cultured in Keratinocyte-SFM medium and supplements from Invitrogen and with penicillin (100 units/mL) and streptomycin (0.1 mg/mL). U2OS GFP-LC3 cells were cultured in DMEM, 10% FBS, penicillin (100 units/mL), streptomycin (0.1 mg/mL), 1 mg/mL G418, and 5 μg/mL blasticidin.

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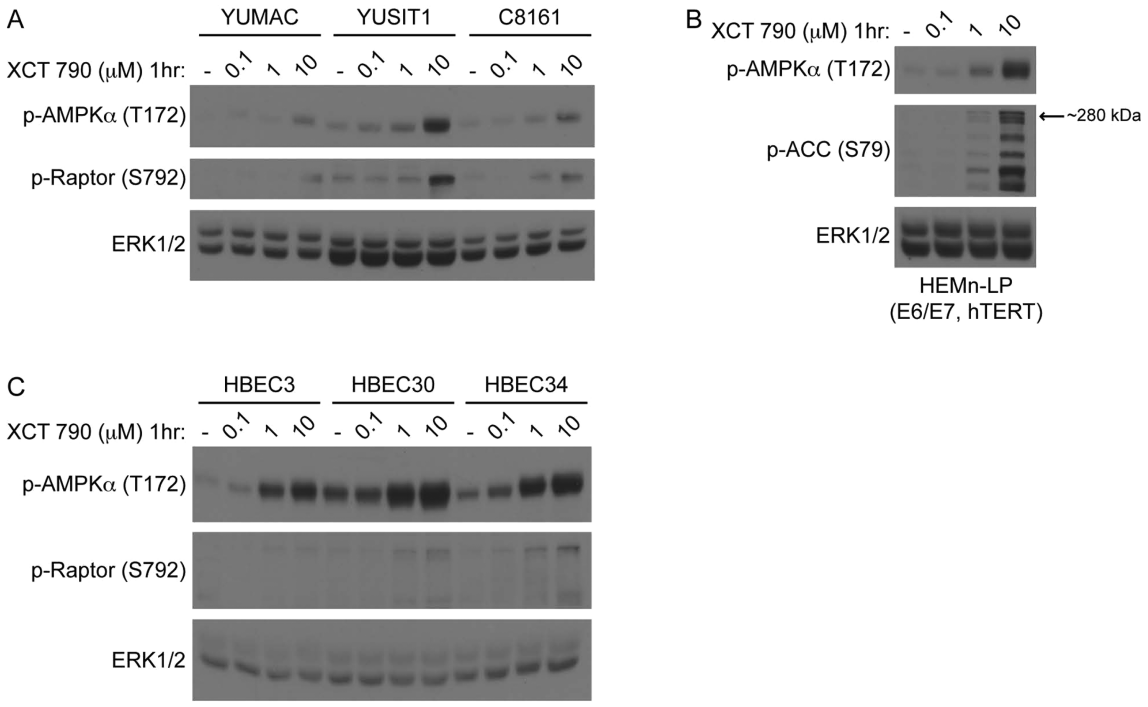

Figure 2. Abrupt AMPK activation is a common response to XCT 790 in both normal and cancer cell lines. (A–C) Whole cell lysates from melanoma cells (A), immortalized human melanocytes (B), and human bronchial epithelial cells (C) were assessed for the accumulation of phosphorylated AMPK and AMPK substrates (phosphorylated Raptor at Ser792[11] and ACC at Ser79[13]) following exposure to the indicated concentrations of XCT 790.

Cell Viability Assays. Cells were plated in 96-well plates and treated with either DMSO (0.5%) or XCT 790 for 72 h. CellTiter-Glo reagent (Promega) was added and mixed for 2 min on a plate shaker followed by incubation for 10 min at room temperature. Luminescence was determined using an EnVision multilabel plate reader.

Immunoblotting and Pathway Analysis. Cells were lysed in 50 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 10% glycerol. Samples were separated on SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. AMP kinase (AMPK) activation was assessed by the accumulation of phosphorylated AMPK at Thr170 and AMPK substrates: phosphorylated Raptor (S792),[11] phosphorylated ULK1 (S555),[12] and phosphorylated ACC (S79).[13] Additionally, Raptor phosphorylation by AMPK inhibits mTOR activation, leading to decreased mTOR and p70 S6 kinase phosphorylation (Figures 1D,E and 3A).

siRNA Transfections. Cells were reverse transfected using siRNAs targeting LONRF1 (negative control) and ERRα (Dharmacon, GE Life Sciences). siRNAs were used in pools, each pool containing four different oligos targeting a specific gene. Seventy-two hours after being transfected, cells were treated with XCT 790 for the indicated periods of time followed by immunoblotting or mitochondrial membrane potential or respiration assays.

Mitochondrial Respiration and Glycolysis. Oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were measured with an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were plated at 30K per well density and cultured for 48 h and then assayed as described previously.[14]

Mitochondrial Membrane Potential and Mass Analyses. Cells were treated with either DMSO (0.5%) or XCT 790 (10, 25, or 40 μM) for 15 min, and then the growth medium was removed and replaced with growth medium containing either DMSO (0.5%) or XCT 790 (10, 25, or 40 μM) with MitoTracker CMXRos and MitoTracker Green FM probes from Invitrogen and incubated for an additional 15 min. Cells were then washed with warm growth medium, trypsinized, spun down, washed with cold PBS, and resuspended in either FACS buffer [0.1% BSA, 10 mM HEPES (pH 7.4), penicillin (100 units/mL), and streptomycin (0.1 mg/mL) in Leibowitz's L15 medium] or PI/RNASE staining buffer from Invitrogen and immediately processed for fluorescence-activated cell sorting (FACS) using FACSCalibur (BD Biosciences) and analyzed with FlowJo.

RESULTS

Estrogen-related receptor α (ERRα) has recently been reported to be necessary for enhanced metabolic capacity and anchorage-independent growth of oncogenic Ras transformed mouse embryonic fibroblasts.[15] To begin to investigate the commonality of ERRα addiction in RAS- and RAF-driven human tumors, we tested the relative toxicity of the ERRα inhibitor XCT 790 in a small panel of colon cancer cell lines, melanoma cell lines, telomerase-immortalized colonocytes,[8,9] and melanocytes (Figure 1A–C). Interestingly, we identified a significant sensitivity window between tumor-derived cell lines and colonocytes and also found oncogenic KRAS expression was sufficient to sensitize colonocytes to XCT 790 (Figure 1A). However, melanocytes and melanoma cells were indiscriminately sensitive to doses of XCT 790 10-fold lower than those required to affect the colorectal cancer cell lines (Figure 1B). A distributive analysis of baseline oncogenic pathway activity upon exposure of MNT1 cells to XCT 790 revealed potent inactivation of the mechanistic target of rapamycin complex 1

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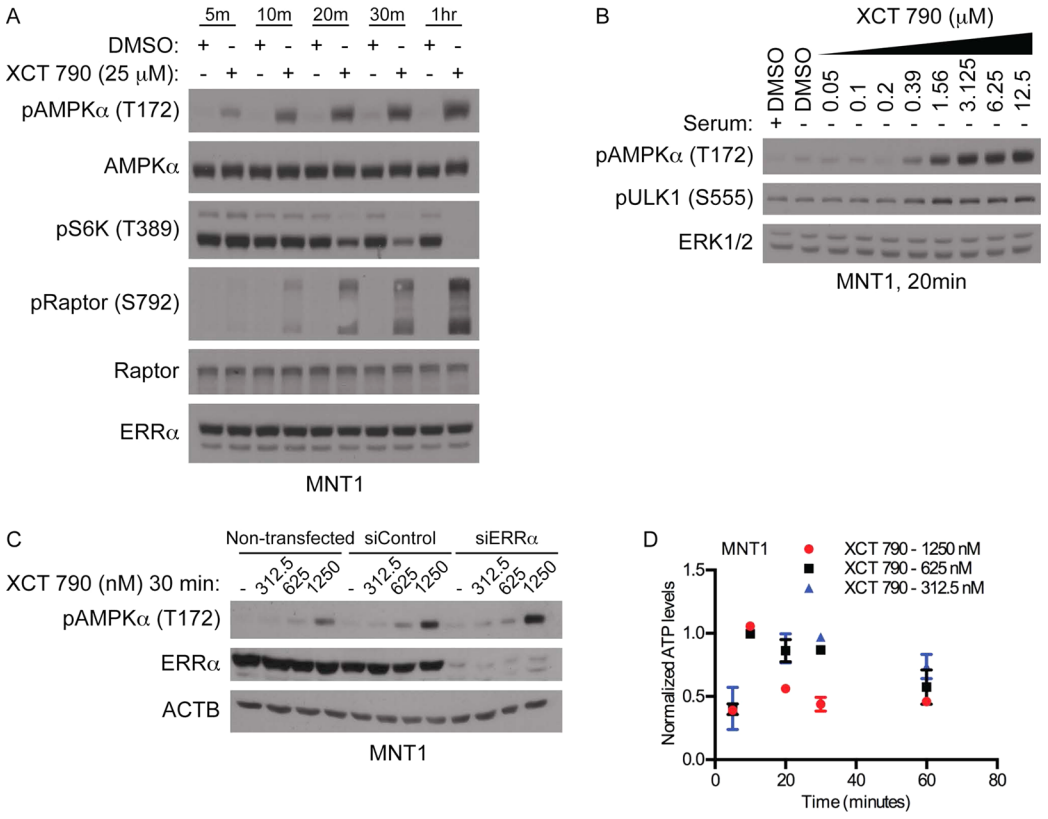

Figure 3. XCT 790 activates AMPK in a dose-dependent and ERRα-independent manner. (A) Whole cell lysates from MNT1 cells, exposed to XCT 790 for the indicated periods of time, were assessed for the accumulation of the indicated phosphorylated proteins by immunoblotting. (B) AMPK activation was visualized as in panel A following exposure to the indicated concentrations of XCT 790. (C) The AMPK response to XCT 790 was assessed 72 h after transfection with siRNAs targeting LONRF1 (negative control) or ERRα. (D) Normalized ATP levels in MNT1 cells, exposed to XCT 790 for the indicated periods of time, as measured by ATP-coupled luciferase assays. Bars indicate means ± the standard deviation (N = 3).

(mTORC1) signaling together with the accumulation of active AMP kinase (AMPK) as indicated by T172 phosphorylation status (Figure 1D). These alterations occurred within 1 h and were uncoupled from the accumulation of ERRα (Figure 1E). AMPK activation by a low micromolar concentration of XCT 790 was observed in all cell lines tested, including multiple melanoma lines (Figure 2A), telomerase-immortalized human melanocytes (Figure 2B), and bronchial epithelial cells from three different patients[10] (Figure 2C).

Time and dose response analyses indicated AMPK pathway activation occurred within 5 min of XCT 790 exposure (Figure 3A) at concentrations as low as 390 nM (Figure 3B). Importantly, siRNA-mediated ablation of ERRα had no effect on this response, indicating the participation of an alternate target of XCT 790 (Figure 3C). A decrease in the ATP concentration, as measured by a luciferin/luciferase coupled assay, occurred within 20 min of XCT 790 exposure (Figure 3D), suggesting that the observed AMPK activation may be secondary to deregulation of energy production. To examine this directly, we tested the consequence of XCT 790 exposure on mitochondrial respiratory potential through measurements of oxygen consumption and extracellular acidification. We observed a substantial increase in oxygen consumption rates (OCRs) as soon as 8 min after XCT 790 exposure (Figure 4A), concomitant with an increase in the level of extracellular acidification (ECAR) (Figure 4B). An elevated OCR was persistent at the smallest doses tested but eroded over time in a dose-dependent fashion (Figure 4A). The XCT 790-induced OCR was insensitive to the ATP synthase inhibitor oligomycin or to the proton ionophore FCCP but collapsed in the presence of the complex 1 inhibitor rotenone (Figure 4C). These mitochondrial electron transport chain inhibitors also did not further increase the ECAR above levels observed with XCT 790 alone (Figure 4D), suggesting that XCT 790 had induced the maximal adaptive lactate production downstream of mitochondrial respiratory defects. Taken together, these observations suggest XCT 790 rapidly uncouples oxygen consumption from ATP production in intact mitochondria (Figure 4A,C). Importantly, these effects were completely independent of ERRα (Figure 4E,F) expression or overt alterations in the concentration of mitochondrial proteins over the time course of the experiment (Figure 4G).

These observations, together with the capacity of FCCP to mimic XCT 790 activation of AMPK (Figure 5A) and to decrease ATP levels (Figure 5B) with similar timing and potency, strongly suggest XCT 790 is a chemical uncoupler of mitochondrial membrane potential that is independent of the interaction with ERRα. Direct measurements of $\Delta\psi$, using MitoTracker CMXRos, demonstrated a dose-dependent inhibition of mitochondrial membrane potential (Figure 5C) by XCT 790 in the absence of effects on mitochondrial mass (Figure 5D). The consequent defect in cellular ATP production was evident as a dramatic inhibition of autophagolysosomal maturation, which requires a high rate of ATP consumption to fuel lysosomal acidification (Figure 5E).

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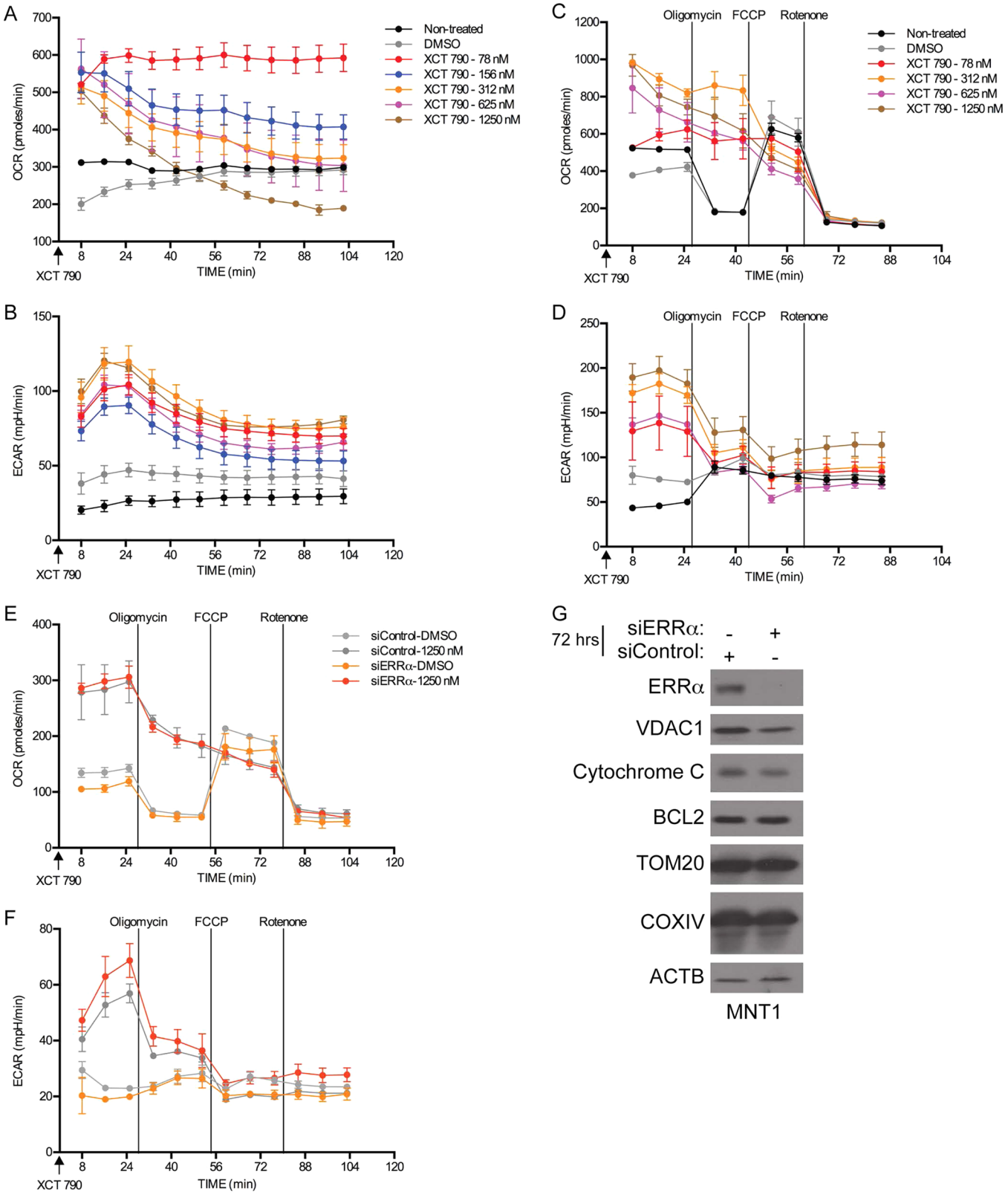

Figure 4. ERRα-independent perturbation of mitochondrial respiration by XCT 790. (A–D) OCR (A and C) and ECAR (B and D) were measured in MNT1 cells, exposed to XCT 790 at the indicated concentrations 8 min before the assay, in the absence (A and B) or presence (C and D) of mitochondrial electron transport chain inhibitors. (E and F) OCR (E) and ECAR (F) were measured starting 8 min after XCT 790 exposure at the indicated doses. Cells were assayed 72 h after transfection with the indicated siRNAs. Bars indicate means ± the standard error of the mean ($N = 3$). (G) Whole cell lysates from cells treated as described for panels E and F were assessed for ERRα and representative mitochondrial protein expression by immunoblotting.

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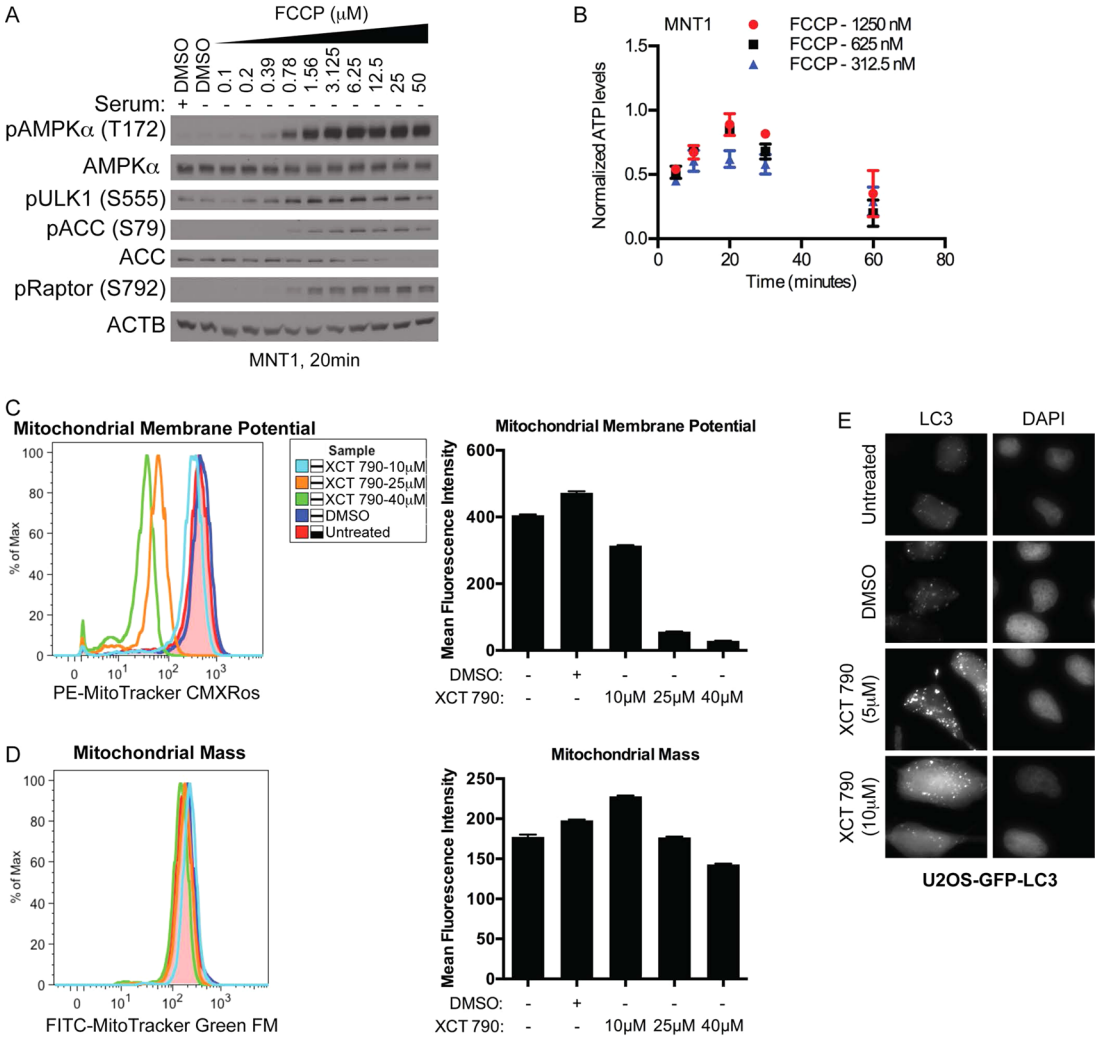

Figure 5. XCT 790 is a mitochondrial uncoupler. (A) Result of FCCP exposure on AMPK pathway activation measured as described for panels A and B of Figure 1. (B) Normalized ATP levels in MNT1 cells, exposed to FCCP for the indicated periods of time, as measured by ATP-coupled luciferase assays. Bars indicate means ± the standard deviation ($N = 3$). (C and D) Mitochondrial membrane potential (C) and mass (D) measured by FACS in MNT1 cells, treated with XCT 790 for 30 min, using mitochondrial dyes MitoTracker CMXRos and MitoTracker Green FM, respectively. Bar graphs indicate the means ± the standard deviation of triplicate measurements. (E) Representative micrographs of U2OS-GFP-LC3 cells treated with XCT 790 or a carrier at the indicated concentrations for 24 h.

DISCUSSION

Here we find that the thiadiazoleacrylamide XCT 790, developed as an ERRα inverse agonist, is a potent mitochondrial uncoupler. This activity leads to the rapid depletion of cellular ATP pools, which in turn activates AMPK, a master regulator of metabolic homeostasis. Importantly, these effects are completely independent of ERRα activity. ERRα depletion neither mimicked nor rescued the effects of XCT 790 measured here. XCT 790 is commonly used at concentrations of 1−20 μM for 8−24 h to examine the biological significance of ERRα activity in cells.[15−36] Rapid XCT 790-induced perturbations of energy production and energy sensing pathways occurred at much smaller doses. Thus, most, if not all, reported biological responses to XCT 790 are in the context of combinatorial inhibition of ERRα and mitochondrial respiratory activity and should be interpreted accordingly.

The biochemical phenotype, timing, and dose sensitivity of the cellular response to XCT 790 are identical to those of the well-studied proton ionophore, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP).[37] This molecule and the closely related carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) disrupt mitochondrial transmembrane electrochemical gradients by directly facilitating proton transport across the lipid membrane barrier.[37,38] All three chemicals are amphipathic nitriles, which in the case of FCCP and CCCP is a structural feature that facilitates discharge of the mitochondrial pH gradient. Thus, it is highly likely that XCT 790 is also a proton ionophore with a consequent direct mechanism of action against mitochondrial respiration.

There is considerable genetic, molecular, and biochemical evidence that ERRα is a bona fide regulator of energy homeostasis in cells and tissue. Through interactions with PGC-1α and PGC-1β, ERRα can directly induce the expression of genes that support oxidative phosphorylation and mitochondrial biogenesis in response to changes in energy demand.[39−43] Furthermore, XCT 790 can clearly interfere with this activity by directly dissociating ERRα from its protein cofactors. Thus, the potent and rapid destruction of mitochondrial membrane potential by XCT 790, in a manner independent of ERRα

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activity, is a particularly confounding phenotype associated with use of this tool compound for the exploration of ERRα biology.

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Notes

The authors declare no competing financial interest.

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