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Aldose reductase inhibitor, fidarestat regulates mitochondrial biogenesis via Nrf2/HO1/AMPK pathway in colon cancer cells

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Abstract

Although we have shown earlier that aldose reductase (AR) inhibitors prevent colorectal cancer cell (CRC) growth in culture as well as in nude mice xenografts, the mechanism(s) is not well understood. In this study, we have investigated how AR inhibition prevents CRC growth by regulating the mitochondrial biogenesis via Nrf2/HO-1 pathway. Incubation of CRC cells such as SW-480, HT29, and HCT116 with AR inhibitor, fidarestat that non-covalently binds to the enzyme, increases the expression of Nrf2. Further, fidarestat augmented the EGF-induced expression of Nrf-2 in CRC cells. Fidarestat also increased the Nrf2 -DNA binding activity as well as expression of HO-1 and NQO1 and activation of SOD and catalase in SW480 cells. Similarly, in nude mice xenograft tumor tissues, Nrf2 and HO-1 levels were significantly higher in fidarestat-treated mice compared to controls. Further, stimulation of CRC cells with EGF in the presence of fidarestat increased the mRNA levels of PGC-1 α , Nrf1 and TFAM and protein levels of PGC-1 α , TFAM and COX-IV and decreased the mitochondrial DNA damage as measured by 8-hydroxy-2'-deoxyguanosine levels. AR inhibitor also modulated the phosphorylations of AMPK and mTOR and expression of p53 in EGF-treated cells. Collectively, our results indicate that AR inhibitor prevents CRC growth by increasing mitochondrial biogenesis via increasing the expression of Nrf2/HO-1/AMPK/p53 and decreasing the mitochondrial DNA damage.

Graphical abstract

Aldose reductase inhibitor, fidarestat prevents cancer cell growth by upregulating AMPK/Nrf2/HO-1/PGC-1 α signals.

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Keywords

Aldose reductase; colon cancer; fidarestat; mitochondria; Nrf2; Heme oxygenase-1; oxidative stress; growth factors

1. Introduction

Colorectal cancer (CRC) is one of the major cause of tumor-related morbidity and mortality worldwide. Poor prognosis and consequences of its metastatic spread make CRC the second most common cause of cancer-related deaths in western countries [1]. Apart from other factors such as environmental and genetic, it has been shown that increased reactive oxygen species (ROS) produced by cytokines, chemokines, and growth factors contribute to colon cancer growth [2]. Normally ROS is reduced directly by glutathione (GSH) and/or through various anti-oxidative enzymes. However, excessive ROS cause lipid peroxidation and lipid aldehydes formation. Lipid aldehydes such as hydroxy-*trans*-2-nonenal (HNE) are reduced by aldose reductase (AR; AKR1B1) to corresponding alcohol, 1, 4-dihydroxynonene (DHN) or conjugated by GSH to form GS-lipid aldehydes such as GS-HNE which is also reduced by AR to GS-DHN [3]. We have demonstrated earlier that GS-DHN activates PKC, PI3K, and PLC which in turn activate transcription factors NF- κ B and AP-1[4,5]. Inhibition of AR by fidarestat, a specific inhibitor of AR, prevents the bFGF- and PDGF-induced activation of NF- κ B, expression of cyclooxygenase-2 (Cox-2), and production of prostaglandin E₂ (PGE₂) which promote colon cancer cells growth[6,7]. Although it is well known that

deregulation of mitochondrial biogenesis plays a significant role in the progression of cancer growth [8,9], the role of AR in the regulation of mitochondrial biogenesis is not known.

Recent studies show that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is activated under oxidative stress and binds to the antioxidant response element (ARE) in the 5'-promoter region of cytoprotective genes and induces the expression of a number of antioxidant proteins such as superoxide dismutase (SOD), glutathione reductase, NAD(P)H:quinone oxidoreductase 1 (NQO1), and hemeoxygenase-1 (HO-1) which prepare the cells to withstand oxidative stress[10,11]. Also, Nrf2 regulates mitochondrial biogenesis by interacting with various mitochondrial proteins and maintains the levels of peroxisome proliferator-activated receptor gamma coactivator (PGC-1 α) and Nrf-1[12]. It has also been shown that PGC-1 α co-activates Nrf-1 which further activates the mitochondrial transcription factor A (TFAM) and transcribes proteins involved in mitochondrial DNA repair [13,14]. Further, AMP-activated protein kinase (AMPK) is an energy sensor of the cells and works as a key regulator of mitochondrial biogenesis by upregulating PGC1 α [13,14]. TFAM could also modulate mitochondrial DNA and upregulate p53 (a tumor suppressor) transcriptional activity and play a crucial role in mitochondrial biogenesis.

In this study, we have investigated how AR inhibition prevents the CRC growth by regulating the mitochondrial biogenesis via Nrf2 and HO-1 pathway. Our results demonstrate that AR inhibitor increases the expression of Nrf2 and related antioxidant enzymes in CRC cells, which in turn regulate mitochondrial biogenesis by increasing the expression of PGC-1 α , Nrf1, and TFAM and decreasing the mitochondrial DNA damage.

2. Materials and methods

2.1 Materials

McCoy's 5A medium, RPMI-1640, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. Antibodies against Nrf2 were purchased from Santa Cruz. Antibodies against KEAP1, HO-1, NQO1, PGC1 α , TFAM, COX-IV, Phospho- and total-AMPK α 1, phospho- and total-mTOR p53, GAPDH, β -actin, human epidermal growth factor (hEGF) and anti-rabbit IgG-F(ab)2 (Alexa Fluor 488) were purchased from Cell Signal Inc. Fidarestat was a gift from Livwel Therapeutics Inc, USA. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), Catalase Enzymatic Assay kit and other reagents used in Western blot analysis were obtained from Sigma. FlexiTube Nrf2-siRNA and control-siRNA were purchased from Qiagen. Nrf2 transcription factor assay kit, SOD assay kit and 8-hydroxy-2'-deoxyguanosine assay kit were purchased from Cayman chemicals. HO-1 assay kit was purchased from Abcam.

Cell culture and Treatment

Human CRC cells (SW480, HT29, and HCT116) were obtained from the American Type Culture Collection (ATCC). SW480 cells were cultured in RPMI 1640 while HCT116 and HT29 cells were grown in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Prior to treatment, cells were serum-starved in the respective medium containing 0.1% FBS in the

absence or presence of fidarestat (10 μ M) for 24 h and further stimulated with EGF (10ng/mL) for different time periods. Cell viability was measured by MTT assay by recording the absorbance at 560 nm spectrophotometrically. SOD activity was determined by a kit from Cayman Chemical according to manufacturer's instructions, and catalase activity was determined by enzymatic assay kit obtained from Sigma.

2.2. Isolation of mitochondria

Mitochondria from the EGF $\pm$ fidarestat-treated colon cancer cells were isolated by the method described earlier[15]. Briefly, cells were suspended in ice-cold hypotonic buffer containing NaCl, MgCl₂, and Tris-HCl for 15 min on ice. The swollen cells were homogenized in a buffer containing mannitol, sucrose, Tris-HCl, and EDTA. Homogenates were centrifuged at 2000g for 5 min to remove nuclei and large membrane fragments. Mitochondrial pellets were obtained by centrifuging the supernatant at 17,000g for 15 min. Mitochondrial DNA was isolated by using Trizol reagent (Life Technologies).

2.3. Immunoblot Analysis

Equal amounts of nuclear and cytoplasmic proteins were subjected to Western blot analysis using specific antibodies. The antigen-antibody complexes were detected by enhanced Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Membranes were stripped with RestoreTM PLUS stripping buffer (Thermo Scientific) and used for reprobing with other antibodies or loading control. Densitometric analysis was performed using Image J software (NIH) and fold changes were calculated by normalizing to either loading control GAPDH or respective total proteins.

2.4. Ablation of Nrf2 by siRNA

SW480 cells were cultured in RPMI 1640 medium containing 10% FBS. The cells were then incubated with Nrf2-siRNA(150nM) or siRNA-negative control with HiPerfect transfection reagent and processed as per the supplier's protocol (Qiagen, USA). The cells were incubated for 48 h at 37°C in a humidified CO₂ incubator. Silencing of Nrf2 was confirmed by Western blot analysis.

2.5. Immunohistochemical Analysis

SW480 cells were cultured in RPMI-1640 medium on culture slides and sub-confluent cancer cells were growth-arrested in 0.1% FBS $\pm$ fidarestat (10 μ M). After 24 h, cells were further treated with EGF (10 ng/ml) $\pm$ fidarestat and incubated for 30, 60 and 120 min. Subsequently, the cells were washed and fixed in 4% formaldehyde. Expression of Nrf2 was visualized using monoclonal antibody followed by the addition of FITC-labeled secondary antibodies (green). DAPI (blue) staining was also performed to visualize the nuclei. Further, the expression of Nrf2 and HO-1 in tumor sections from control and Fidarestat treated HT29 nude mice xenografts were determined by immunohistochemistry [16].

2.7. Quantitative RT-PCR

Q-RT-PCR was performed in the mitochondrial DNA isolated from CRC cells. Synthesis of cDNA was performed with 1.0ug of total RNA in 20 μ l using the TaqMan reverse

transcription reagents kit from Life Technologies (N8080234). Q-PCR amplifications were performed by using 1µl of cDNA in a total volume of 20µl using the iTaq Universal SYBR Green Supermix (Bio-Rad 1725125). The final concentration of the primers was 300nM. Relative RT-PCR assays were performed with GAPDH housekeeping gene as a normalizer. The relative expression of mRNA was calculated using the 2^{-C_t} method, and the results were graphically presented as fold change [17]. The absolute analysis was performed by using a known amount of a synthetic transcript of the gene of interest. All PCR assays were run on the ABI Prism 7500 Sequence Detection System. The following primers were used: HO-1 forward: 5'-CGGGCCAGCAACAAAGTG-3'; reverse: 5'-CCAGAAAGCTGAGTGTAAGGACC-3'. NRF1 forward 5'-CTGATGGCACTGTCTCACTTATCC-3' reverse 5'-CCACGGCAGAATAATTCATTG-3'. PPARGC1a forward 5'-CACACACAGTCGCAGTCACAAC-3', reverse 5'-CACACTTAAGGTGCGTTCAATAGTC-3'. TFAM forward 5'-GGTCTGGAGCAGAGCTGTGC-3' reverse 5'-TGGACAACCTGCCAAGACAGAT-3'.

2.8. Statistical Analysis

Data presented as mean $\pm$ SD (n=5) and unpaired Student's *t*-test was used to analyze the *p* values (Microsoft Office Excel 2013). *p* < 0.05 was considered as statistically significant.

3. Results

3.1 Effect of AR inhibition on Nrf2 expression in CRC cells

To examine how AR inhibitor prevents colon cancer cell viability by upregulating the expression of Nrf2, we treated colon cancer cells (SW480, HT29, and HCT116) with EGF $\pm$ fidarestat. Incubation of cancer cells with fidarestat alone or EGF alone induced the Nrf2 expression in the nucleus. However, pre-incubation with fidarestat followed by EGF augmented the Nrf2 expression as compared to EGF or fidarestat alone (Fig 1A). Immunocytochemical analysis also indicated the nuclear accumulation of Nrf2 in the SW-480 cells treated with fidarestat alone or in EGF alone treated cells (Fig 1B). We next examined the effect of fidarestat on EGF-induced Nrf2 DNA binding activity in SW-480 cancer cells. The Nrf-2 DNA binding activity increased in fidarestat-treated cells as compared to untreated cells. Pre-incubation with fidarestat followed by EGF enhanced the Nrf2 transcriptional activity in the colon cancer cells (Fig 1C). Furthermore, AR inhibitor or EGF also decreased the expression of Keap-1, a negative regulator of Nrf-2, in the colon cancer cells and pre-incubation with fidarestat followed by EGF also reduced the expression of the Keap1 protein in the cytoplasm of colon cancer cells (Fig 1D).

3.2. Effect of AR inhibition on anti-oxidative protein expressions in CRC cells

The results described above demonstrated that pre-incubation with AR inhibitor enhances the Nrf2 activity by EGF stimulation which may help the cells to adopt to oxidative stress through up-regulation of anti-oxidative enzymes. Therefore, we studied the effect of EGF $\pm$ AR inhibitor on HO-1 and NQO1 expression in CRC cells. Fidarestat alone or EGF alone increased the protein levels of HO-1 and NQO1 (Fig 2A). Further, EGF $\pm$ fidarestat-treated cells showed an increase in the HO-1 and NQO1 protein expression as compared to cells

treated with EGF or fidarestat alone (Fig 2A). Similar results were observed when we measured the levels of HO-1 in cancer cell lysates (Fig 2B). Furthermore, mRNA expression of HO-1 increased significantly in cells stimulated with EGF in the presence of fidarestat as compared to EGF or fidarestat alone (Fig 2C). SOD and catalase activities were also increased significantly in SW480 cells treated with EGF+fidarestat as compared to EGF or fidarestat alone (Fig 2D and 2E). Thus, our results indicate that inhibition of AR could enrich and prepare the antioxidant environment inside of the cells to fight against oxidative stress.

3.3. Inhibition of AR increases Nrf2 and HO-1 expression in nude mice xenografts

We determined the expression of Nrf2 and HO-1 in HT29 colon cancer xenografts obtained from the nude mice treated without or with fidarestat [16]. Immunostaining of tumor sections with Nrf2 and HO-1 antibodies demonstrated that fidarestat increased the expression of Nrf2 and HO-1 in nude mice xenograft tissues (Fig 3).

3.4. Effect of AR inhibitor on Nrf2-mediated colon cancer cells growth

We have shown earlier that fidarestat prevents colon cancer growth [4–7]. To examine if Nrf2 activation is required for the prevention of CRC cells growth by fidarestat, we examined the viability of CRC cells grown in the Nrf2-transfected cells $\pm$ fidarestat. EGF caused an increase in the CRC cells viability in both Nrf2-siRNA- and control-siRNA-transfected cells (Fig 4). However, fidarestat prevented the EGF-induced CRC cells growth in control-siRNA-transfected but not in the Nrf2-siRNA-transfected cells suggesting that Nrf2 activation is required for the effects of fidarestat.

3.5. Effect of AR inhibition on EGF-stimulated mitochondrial biogenesis

Since Nrf2 has been shown to regulate mitochondrial biogenesis that controls cell survival, differentiation, and growth, we next determined the protein and mRNA expression of the major regulators of mitochondrial biogenesis, i.e., PGC-1 α , Nrf1, and TFAM in CRC cells treated with EGF $\pm$ fidarestat. The results suggest that stimulation with EGF in the presence of AR inhibitor augmented the mRNA expression of Nrf1, PGC-1 α , and TFAM as compared to fidarestat or EGF alone (Fig 5A). Similar to mRNA levels, the protein expression of PGC-1 α , TFAM, and COX-IV increased in the cells stimulated with EGF $\pm$ fidarestat as compared to EGF-treated cells (Fig 5B). These results suggest that pretreatment with fidarestat enhances the mitochondrial biogenesis under oxidative stress. We next determined the mitochondrial DNA damage by determining 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in CRC cells. Cancer cells treated with EGF alone showed significantly increased DNA damage as compared to control ($p < 0.05$), whereas cancer cells treated with EGF + fidarestat showed significantly ($p < 0.05$) less mitochondrial DNA damage (Fig 5C) suggesting that pretreatment of CRC cells with fidarestat increases mitochondrial biogenesis and protects DNA damage under stress conditions.

3.6. Effect of AR inhibition on EGF-induced expression of AMPK, mTOR and p53 in CRC cells

Since the activation of AMP-protein kinase induces the mitochondrial biogenesis, we next examined the effect of AR inhibitor on EGF-induced AMPK-phosphorylation. The results shown in the Fig. 5D indicate that fidarestat increases the phosphorylation of AMPK- α 1 in EGF+fidarestat-treated cells as compared to EGF alone. Further, fidarestat also increased the expression of tumor suppressor protein p53 in EGF-treated SW-480 cells (Fig 5D). Since mTOR activation is known to be regulated by AMPK, we next examined the effect of fidarestat on EGF-induced mTOR phosphorylation. Our results shown in the Fig 5E indicate that the treatment of SW480 cells with EGF caused a significant phosphorylation of mTOR while pre-treatment of SW480 cells with fidarestat attenuated EGF-induced phosphorylation of mTOR.

4. Discussion

We have shown earlier that AR inhibitors prevent CRC growth and metastasis in vitro and in vivo models by inhibiting the NF- κ B-mediated pro-inflammatory pathways [4,5]. In this study, we show that AR inhibitor, fidarestat increases the activity of Nrf2-mediated anti-oxidative pathways and treatment of CRC with fidarestat before the addition of EGF augmented it. Further, AR inhibitor-induced EGF-stimulated mitochondrial biogenesis and decreased mitochondrial DNA damage. These results suggest a novel bifunctional role of AR inhibition; i.e., by preventing the NF- κ B-mediated pro-inflammatory pathways and increasing the Nrf2-mediated anti-oxidative/anti-inflammatory pathways, AR inhibitors could act as chemopreventive drugs. Further, Nrf2 is an important transcription factor that controls cellular redox equilibrium by regulating the expression of antioxidant and anti-inflammatory mediators such as GST, NQO1, CAT, SOD, and HO-1 by binding to respective antioxidant response elements and plays a major role in protecting the cells under oxidative stress conditions [18]. However, Nrf2 has been shown to play protective as well as harmful effects in various forms of cancers [19]. Some studies indicate that expression of Nrf2 causes chemoresistance while several other studies suggest that Nrf2 expression decreases tumor burden [20,21]. Specifically, Nrf2 null mice showed increased tumor growth in azoxymethane- and dextran sodium sulfate-induced colon cancer models as compared to control mice [22,23]. Similarly, Nrf2 null mice have been shown to aggressively develop tumors in benzo(a)pyrene- and N-nitrosobutyl(4-hydroxybutyl)amine –induced gastric and bladder cancers [24,25]. These studies indicate that Nrf2 protects against oxidative stress-induced tumor growth initiated by oxidants such as chemical carcinogens, cytokines, and growth factors. In the current study, we have shown that fidarestat increases the expression, nuclear translocation, and DNA binding activity of Nrf2 in CRC cells. Consistently, fidarestat pretreatment further activated EGF-induced Nrf2 expression and transcriptional activity. Our studies also suggest that fidarestat increases the expression of Nrf2-mediated downstream anti-oxidative proteins such as HO1, NQO1, and SOD and it augments the EGF-induced anti-oxidative proteins. These studies indicate that pre-incubation with fidarestat prepares the cells against oxidative stress by inducing Nrf2/HO1 pathway which contributes to its anti-oxidative/anti-inflammatory properties. Consistent with our data, several studies have also shown that natural chemopreventive agents such as curcumin,

cinnamaldehyde, sulforaphane, and resveratrol exert their effects by increasing the expression of Nrf2 pathway-mediated defense responses such as expression of phase-II detoxification enzymes and transporters which protect cells from xenobiotic and carcinogenic stresses [23,26–29]. Similarly, some synthetic Nrf2 activators such as Oltipraz and dimethyl fumarate have also been shown to ameliorate various cancer forms including colon cancer [30–32].

To further demonstrate the significance of Nrf2 activation in the prevention of EGF-induced CRC growth by AR inhibitor, we examined the effect of fidarestat on EGF-induced cell viability in Nrf2-ablated cells. Our results suggest that AR inhibitor prevented the EGF-induced cell viability in control cells but not in Nrf2-ablated cells suggesting that Nrf2-mediated anti-oxidative pathways are required for AR-regulated cell viability. Since Nrf2 also transcribes genes responsible for mitochondrial biogenesis [12], we examined the effect of AR inhibitor on mitochondrial biogenesis in CRC cells. Our results demonstrate that AR inhibitor increased the expression of PGC-1 α , a master regulator of the transcriptional network which regulates mitochondrial biogenesis in CRC cells. It is also suggested that PGC-1 α causes apoptosis and acts as a tumor suppressor in some cancers [33]. Furthermore, expression of another transcription factor, NRF-1, activated by PGC-1 α , acts on nuclear genes coding for proteins necessary for the mitochondrial biogenesis or for mtDNA transcription and replication [13–15], significantly increased in the cells pretreated with fidarestat. Both PGC-1 α and NRFs co-activate the expression of TFAM, which is also important for the regulation and maintenance of mtDNA integrity [13]. Our results show that the protein expression of PGC-1 α and TFAM associated with mitochondrial biogenesis increased in CRC cells treated with EGF+fidarestat. Further, AR inhibitor prevented the EGF-induced mitochondrial DNA damage. These observations indicate that AR inhibition could prevent CRC cell growth by increasing the mitochondrial biogenesis and expression of tumor suppressor genes such as PGC-1 α and p53 and decreasing the DNA damage [34,35]. Our results also suggest that AMPK plays a significant role in the maintenance of intracellular energy balance, phosphorylates Nrf2 at Ser550, promotes nuclear accumulation of Nrf2, and upregulates PGC-1 α and mitochondrial biogenesis [36]. Similarly, tumor suppressor p53 also plays a major role in cellular response to diverse intracellular and extracellular stresses including DNA damage and oncogene expression [37].

In summary, our studies reveal that AR inhibitor, fidarestat besides inhibiting the NF- κ B – mediated pro-inflammatory pathway, prevents CRC growth by activating Nrf2/HO1-mediated anti-oxidative and anti-inflammatory pathways. Fidarestat also upregulates mitochondrial biogenesis and decreases mitochondrial DNA damage in growth factor-treated CRC cells. These findings suggest a novel mechanism(s) through which inhibitors of AR prevent growth factor-induced cancer cell growth. Q

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Abbreviations

8-OHdG 8-hydroxy-2'-deoxyguanosine

AMPK	AMP-activated protein kinase
AR	aldose reductase
COX-IV	cytochrome c oxidase or complex IV
CRC	colorectal cancer
EGF	epidermal growth factor
HO-1	heme oxygenase-1
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1 alpha
mtDNA	mitochondrial DNA
NQO1	NAD(P)H Quinone Dehydrogenase1
Nrf1	nuclear respiratory factor 1
Nrf2	nuclear factor (erythroid-derived 2)-like 2
ROS	reactive oxygen species
SOD	superoxide dismutase
TFAM	Transcription Factor A mitochondrial

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Highlights

- Aldose reductase inhibitor increases the activation of Nrf2 and HO-1 in colon cancer cells.
- Fidarestat augments EGF-induced increase in the expression of Nrf2 and HO-1.
- Fidarestat increases EGF-induced increase in anti-oxidative proteins
- Aldose reductase inhibition enhances mitochondrial biogenesis.
- Aldose reductase inhibition prevents mitochondrial DNA Damage.

Fig. 1. Effect of AR inhibitor fidarestat on Nrf2 activation in CRC cells

(A) SW480, HT29, and HCT 116 cells were treated with fidarestat (10 μ M) for indicated times or pretreated with fidarestat for 24 h followed by incubation with EGF (10ng/mL) for an additional 30, 60, 120 and 240 mins. Equal amounts of nuclear proteins were subjected to Western blot analysis. (B) Nuclear localization of Nrf2 was determined by immunofluorescence of SW480 cells. The cells were immunostained with antibodies against Nrf2 followed by the addition of FITC (green)-labelled anti-rabbit antisera. DAPI (blue) staining was also performed to visualize the nuclei. The images shown are DAPI+FITC-labeled cells. (C) Nrf2-DNA binding activity was determined by ELISA in the nuclear extracts isolated from SW480 cells using a kit according to the manufacturer's instructions (Cayman Chemical). Data presented as mean $\pm$ SD (n=5). *p<0.05, **p<0.001 when compared with control and #p<0.001 when compared with the fidarestat-treated group. (D) Equal amounts of cytosolic proteins were subjected to Western blot analysis using antibodies against KEAP1 in SW480 cells. Blots represent one of three independent analysis and antibodies against β -actin or GAPDH were used as loading control.

Fig. 2. Fidarestat induces Nrf2 downstream proteins such as HO-1, NQO1, and SOD in CRC cells

The SW480 cells pre-treated with fidarestat (10 μ M) for 24 h followed by incubation with EGF (10 ng/ml) for an additional 24 h. **(A)** Equal amounts of cell extracts were subjected to Western blot analysis using specific antibodies against HO-1 and NQO1. Blots represent one of three independent analysis and antibodies against GAPDH were used as loading control. **(B)** The levels of HO-1 in the cell lysates were determined by ELISA kit as per manufacturer's instructions (Abcam). **(C)** The expression of HO-1 at the mRNA levels was determined by qPCR as described in the Methods section. **(D)** SOD activity was determined using ELISA kit according to the manufacturer's instructions (Cayman Chemical). **(E)** Catalase activity was determined by spectrophotometric rate reduction of H₂O₂ (units/ml enzyme) (Sigma -Aldrich). Data presented as mean $\pm$ SD (n=5). *p<0.05, **p<0.001 when compared with control and #p<0.001 when compared with the fidarestat-treated group.

Fig. 3. Effect of Fidarestat on Nrf2 and HO-1 expression in HT29 xenografts

HT29 cells were injected subcutaneously into nude mouse and when tumors reached a cross-sectional area of about 30mm², the mice were treated with AR inhibitor, fidarestat (50 mg/kg) in drinking water. At the end of the experiment, tumors were excised, and sections were cut as described by us previously [16]. The xenograft tumor sections were immunostained with antibodies against Nrf2 and HO-1. The immunoreactivity of the antibody was assessed by visualizing the dark brown stain in the tumor cells, whereas the non-reactive areas displayed only the background color. Photomicrographs of the stained sections were acquired using EPI-800 microscope (bright-field) connected to a Nikon camera (×400 magnification). Images represent one of three independent analysis.

Fig. 4. Fidarestat-regulated cell viability of cancer cells is mediated by Nrf2 activation

The untransfected, control and Nrf2-siRNA- transfected SW480 cells were treated with fidarestat $\pm$ EGF for 24h and cell viability was determined by MTT assay. Data represent mean $\pm$ SD (n = 6). *p<0.01 vs control; # p<0.01 vs EGF; NS=non-significant vs EGF-treated cells. The inset shows Western blots against Nrf2 and HO-1 in control and siRNA transfected cells. GAPDH= loading control.

Fig. 5. Effect of AR inhibition on EGF-induced mitochondrial biogenesis

(A) SW480 cells were pretreated with fidarestat for 24 h followed by stimulation with EGF for 3h. Total RNA was isolated and equal amounts of RNA were subjected to Q-PCR amplification using specific primers for Nrf1, TFAM, and PGC-1 α as described in the methods section. RT-PCR assays were performed with GAPDH gene as a normalizer. Data presented as mean $\pm$ SD (n=5). *p<0.05 when compared with control and #p<0.01 when compared with the EGF-treated group. (B) SW480 cells were pretreated with fidarestat for 24 h followed by stimulation with EGF for different time intervals (3, 6, 12, 24 h). Equal amounts of mitochondrial protein were subjected to Western blot analysis using antibodies against PGC-1 α , TFAM and COX-IV and antibodies against GAPDH served as a loading control. Blots represent one of three independent analysis. (C). Mitochondrial DNA damage was determined by measuring the levels of 8-Hydroxy-2'-deoxyguanosine (8-OHdG) in the mitochondrial DNA isolated from SW480 cells using ELISA kit according to the manufacturer's instructions (Cayman Chemical). **D and E** SW480 cells pretreated with fidarestat for 24 h followed by stimulation with EGF for indicated time periods. Equal amounts of protein in the cell lysates were subjected to Western blot analysis using specific antibodies against phospho- and total-AMPK α 1 and p53 (D); and phospho- and total-mTOR (E). Antibodies against GAPDH served as a loading control, and blots represent one of three independent analysis. Data presented as Mean $\pm$ SD (n=5). *p<0.05 when compared with control and #p<0.01 when compared with the EGF-treated group.