Pharmacologic activation of Δ133p53α reduces cellular senescence in progeria patients-derived cells

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Running title: Senescence by Δ133p53α-activating drugs in progeria cells

Abstract

Background: Patients with Hutchinson-Gilford progeria syndrome (HGPS) show accelerated aging phenotypes and have shortened lifespan, with implications in physiological aging processes as well. While therapeutic approaches targeting the disease-causing abnormal protein, progerin, have been developed, further efforts to explore mechanistically distinct and complementary strategies are still critical to better treatment regimens. We previously showed that lentiviral vector-driven expression of Δ133p53α, a natural inhibitory isoform of p53, rescued HGPS patients-derived fibroblasts from early entry into cellular senescence, which is a downstream event of progerin-induced DNA damage. We also performed a quantitative high-throughput screen (qHTS) of approved drug and investigational agent libraries, leading to the identification of celastrol and AZD1981 as compounds that upregulate Δ133p53α protein levels.

Methods: To investigate whether celastrol and ADZ1981 upregulate endogenous Δ133p53α in HGPS-derived fibroblasts and reduce their senescence-associated phenotypes, we performed western blot assays (Δ133p53α, progerin, and $p21^{WAF1}$, which mediates p53-induced senescence and is inhibited by Δ133p53α), senescence-associated β-galactosidase (SA-β-gal) staining, enzyme-linked immunosorbent assay (IL-6, which is a proinflammatory cytokine secreted from senescent cells), and qRT-PCR assays ($p21^{WAF1}$ and IL-6).

Results: Treatment with celastrol (0.1 μM for 24 h) or AZD1981 (10 μM for 24 h) reproducibly increased Δ133p53α expression and decreased $p21^{WAF1}$ expression in two strains of fibroblasts derived from HGPS patients. These compounds reduced the percentage of SA-β-gal-positive senescent cells and the secretion of IL-6 into culture medium in both of these fibroblast strains, irrespective of their different basal levels of senescence and IL-6 secretion. These compounds had no effect on the level of progerin.

Conclusion: Celastrol and ADZ1981 upregulate endogenous Δ133p53α and, reproducing the effects of its vector-driven expression, inhibit cellular senescence and IL-6 secretion in HGPS-derived fibroblasts. Their progerin-independent action suggests that they may synergize with currently available progerin-targeting therapies. This study also warrants further investigation of these compounds for potential applications in other diseases and conditions in which Δ133p53α-regulated senescence plays a role.

Keywords: p53 isoform, cellular senescence, IL-6, fibroblasts, aging, Hutchinson-Gilford progeria syndrome, progerin, pharmacologic activation

INTRODUCTION

The human *TP53* gene encodes not only the full-length protein consisting of 393 amino acids (simply referred to as "p53" in general and in this manuscript as well) but also at least a dozen of naturally occurring isoforms due to alternative RNA splicing and/or alternative usage of transcription and translation initiation sites [1]. Among these *TP53*-encoded isoforms, Δ133p53α is an N-terminally truncated isoform that is generated via an alternative transcription initiation from intron 4 and an alternative translation from the methionine codon 133 [1, 2]. In various types of normal human cells, the expression level of Δ133p53α is primarily regulated via protein turnover involving chaperone-assisted selective autophagy [3-5], which leads to diminished expression of endogenous Δ133p53α upon cellular senescence [2, 3, 5-9]. The retroviral or lentiviral vector-driven expression of Δ133p53α prevents or delays normal human cells from entering cellular senescence [2, 3, 5, 6, 8, 9], supporting the direct role of Δ133p53α in regulating human cell senescence. Of particular importance, Δ133p53α-expressing cells, not entering senescence, exhibit their inherent normal functions, such as neuroprotective activity of Δ133p53α-expressing astrocytes [5, 8-10] and anti-tumor activity of Δ133p53α-expressing T lymphocytes [11]. Although inhibition of p53 activities could lead to genome instability and increased oncogenesis [12, 13], Δ133p53α preferentially inhibits p53-mediated senescence in a dominant-negative manner, while maintaining or even enhancing p53-mediated DNA repair [7-9, 11, 14, 15]. This selective inhibitory nature of Δ133p53α suggests a potential therapeutic value of Δ133p53α in senescence-associated diseases and conditions with minimum risk of genome instability or oncogenesis. In an attempt to pharmacologically activate Δ133p53α for future therapeutic applications, we performed a cell-based quantitative high-throughput screen (qHTS) on approved drugs, investigational agents and chemical probe libraries using fluorescently

labeled Δ133p53α protein, which identified two compounds (celastrol and AZD1981) that upregulate Δ133p53α at the protein level in normal cells [16]. Celastrol, a pentacyclic quinone methide, is a natural product used in traditional Chinese medicine. The molecule has multiple molecular mechanisms, likely due to its thiol reactivity [17-19]. These activities include acting as an Hsp70 inducer and proteasome inhibitor [20, 21]. AZD1981 is a prostaglandin DP2 receptor antagonist developed for asthma [22].

Hutchinson-Gilford progeria syndrome (HGPS) is a genetic disease caused by a mutation in the *LMNA* gene, which produces an abnormal nuclear envelope protein called progerin [23]. Patients with HGPS exhibit premature aging phenotypes in childhood and have a short lifespan, with an average life expectancy of 13-15 years [24]. Although the occurrence of HGPS is very rare, this disease also has significant implications for natural aging processes at both the organismal and cellular levels, as well as for interventions against aging in healthy individuals [25, 26]. Similar to the premature aging phenotypes observed in patients with HGPS, their derived cells in culture (often fibroblasts) display a premature onset of cellular senescence accompanied by proinflammatory cytokine production, which is attributed to progerin-induced accumulation of DNA damage and hyperactivation of p53 [27-29]. Our previous study showed that lentiviral expression of Δ133p53α delayed the onset of cellular senescence and reduced the production of the proinflammatory cytokine IL-6 in fibroblasts derived from HGPS patients, concurrent with mitigated DNA damage and repressed expression of $p21^{WAF1}$, a p53-inducible gene that mediates cellular senescence [7]. In this study, we investigate whether treatment with celastrol or AZD1981 can reproduce the effects of lentiviral expression of Δ133p53α in HGPS-derived fibroblasts, exploring the potential therapeutic value of these compounds in HGPS.

METHODS

Cells and cell culture

A fibroblast strain AG11513, from an 8-year-old female HGPS patient, was obtained from Coriell Institute for Medical Research (https://catalog.coriell.org/). Another fibroblast strain HGADFN271, from a 1-year-3-month-old male patient, was obtained from Progeria Research Foundation (https://www.progeriaresearch.org/). These cells were grown in DMEM medium (Corning #15-013-CV) supplemented with 10% fetal bovine serum (Sigma-Aldrich #F0926), 1% L-glutamine (Thermo Fisher #25030081) and 1% penicillin/streptomycin (Thermo Fisher #10378016) at 37 °C under a humidified atmosphere of 5% CO_2. Cells were passaged at a split ratio of 1:3 (AG11513) or 1:4 (HGADFN271).

Compounds and treatment

Celastrol was purchased from Cayman Chemical (#70950) and dissolved in DMSO as 100 µM stock solution. AZD1981 was also purchased from Cayman Chemical (#20763) and dissolved in DMSO as 10 mM stock solution. Cells were treated with celastrol at a final concentration of 0.1 µM, AZD1981 at a final concentration of 10 µM, or DMSO vehicle control for 24 h in Figures 2, 3 and 4, as previously performed [16]. Following 4 days of cell culture, cells were harvested for assays mentioned below. In Figure 1, celastrol (0.1 µM), AZD1981 (10 µM) or DMSO was supplemented freshly every 3 or 4 days over 72 days, and the cumulative population doubling levels (PDLs) were calculated as previously described [7].

Western blot analysis

Preparation of protein lysates, gel electrophoresis, transfer to PVDF membranes, antibody incubation, and signal detection were performed as previously described [16]. Primary antibodies used were: rabbit polyclonal anti-Δ133p53 antibody MAP4 [2, 30], sheep polyclonal anti-p53

antibody SAPU [1, 30], anti-progerin antibody (Abcam ab153757), anti-p21^{WAF1} antibody (Cell Signaling #2947), and anti-GAPDH antibody (EMD Millipore, MAB374). The quantitative image analysis was performed using the ChemiDoc Imaging System (Bio-Rad) and the Image Lab software (Bio-Rad, ver. 6.1).

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal activity was stained using SA-β-Galactosidase Staining Kit (Cell Signaling #9860). Data of SA-β-gal-positive cells (%) were presented as mean ± S.D. from 3 biological replicates in 3 independent experiments. At least 100 cells were observed in each replicate.

Enzyme-linked immunosorbent assay (ELISA)

Quantification of IL-6 in culture media was carried out using Human IL-6 ELISA Kit (Sigma-Aldrich, RAB0306). Standard curves were drawn from recombinant IL-6 provided in the kit. Data were normalized to cell numbers and presented as mean ± S.D. from biological triplicates.

RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA samples were prepared using RNeasy Plus Micro Kit (QIAGEN #74034). Reverse transcription was carried out using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher #4368814). qRT-PCR assays were performed using Taqman Gene Expression Master Mix (Thermo Fisher #4369016). The primer/probe sets used were (Thermo Fisher): p21^{WAF1} (Hs00355782_m1), IL-6 (Hs00174131_m1), and GAPDH (Hs02758991_g1) as an internal control. All qRT-PCR data were mean ± S.D. from technical triplicate (n=3).

RESULTS

Cell proliferation of two fibroblast strains derived from HGPS patients and the effects of celastrol and AZD1981

In general, primary cells derived from HGPS patients are less proliferative and have a shorter replicative lifespan than those derived from normal donors. However, various factors such as age of patients, methods of strain establishment, and conditions of subsequent cell culture could significantly affect their proliferative potential and replicative lifespan. We therefore monitored two HGPS-derived fibroblast strains, AG11513 and HGADFN271, for long-term proliferation in cell culture (Figure 1A and Figure 1B, DMSO alone). These two strains in our laboratory, at the time of use in this study, were different in proliferative capacity and remaining replicative lifespan: AG11513 fibroblasts (derived from an 8-year-old patient) proliferated slowly and only had ~5 population doubling levels (PDLs) before proliferation arrest (Figure 1A, DMSO alone), while HGADFN271 fibroblasts (derived from a 1-year-3-month-old patient) still had ~11 PDLs remaining (Figure 1B, DMSO alone).

Treatment of these fibroblasts with celastrol or AZD1981, added fresh every 3 or 4 days, resulted in continuous proliferation beyond the point at which the control cells became proliferation-arrested (Figure 1A and Figure 1B, after 42 days). This extension of replicative lifespan appeared more pronounced in AG11513 fibroblasts, which had fewer PDLs remaining.

Celastrol and AZD1981 upregulate endogenous Δ133p53α protein in HGPS-derived fibroblasts

AG11513 and HGADFN271 fibroblasts were treated with celastrol (0.1 μM) or AZD1981 (10 μM) for 24 h, followed by 4 days of cell culture in the absence of compound and then harvested for western blot analysis. Both celastrol and AZD1981 were found to upregulate Δ133p53α protein levels by approximately 5- to 6-fold in these cells (Figure 2 and Supplementary Figure 1),

concurrent with decreased expression of $p21^{WAF1}$ (Figure 2), which is known to be repressed by Δ133p53α [2, 7, 15]. Neither compound affected progerin levels (Figure 2).

Celastrol and AZD1981 reduces cellular senescence in HGPS-derived fibroblasts

Consistent with the proliferative characteristics shown in Figure 1, SA-β-gal-positive senescent cells were more abundant in untreated AG11513 fibroblasts (~50%, Figure 3A) than in untreated HGADFN271 fibroblasts (~10%, Figure 3B). Despite this difference in basal senescence levels, treatment with celastrol or AZD1981 significantly reduced the percentage of SA-β-gal-positive senescent cells in both fibroblast strains. In AG11513, celastrol reduced it to ~20% and AZD1981 to ~30% (Figure 3A). In HGADFN271, both compounds resulted in only ~3% of SA-β-gal-positivity (Figure 3B). The mRNA expression levels of $p21^{WAF1}$, a mediator of cellular senescence, were also reduced in these treated fibroblasts (Figure 3C), confirming the protein levels shown in Figure 2. The inhibition of cellular senescence by celastrol and AZD1981 parallels the extension of replicative lifespan by these compounds (Figure 1).

Celastrol and AZD1981 reduces IL-6 secretion in HGPS-derived fibroblasts

In line with the difference in basal senescence levels (Figure 3A and 3B), the senescence-associated, proinflammatory cytokine IL-6 [31] was secreted into the culture medium at higher levels by untreated AG11513 fibroblasts (Figure 4A) than by untreated HGADFN271 fibroblasts (Figure 4B). Again, despite this difference in basal IL-6 secretion, celastrol and AZD1981 resulted in ~30% reduction in secreted IL-6 levels in both AG11513 and HGADFN271 fibroblasts (Figure 4A and 4B). The mRNA expression levels of IL-6 were also reduced in these fibroblasts treated with celastrol or AZD1981 (Figure 4C).

DISCUSSION

This is the first report of testing Δ133p53α-activating compounds in primary human cells from disease patients. Celastrol and AZD1981, when used to treat fibroblasts derived from HGPS patients, were confirmed to upregulate endogenous Δ133p53α levels. These compounds reduced cellular senescence and IL-6 secretion, which are major phenotypes prematurely occurring in these diseased cells, and extended their replicative lifespan. Celastrol and AZD1981 exhibited these effects regardless of the baseline levels of cell proliferation and senescence in these fibroblasts, suggesting that these compounds may act on both actively dividing and proliferation-arrested cells. Consistent with our previous findings with the lentiviral expression of Δ133p53α [2, 7, 15], compound-induced upregulation of Δ133p53α led to repressed expression of $p21^{WAF1}$, in turn leading to inhibition of cellular senescence. It should be noted that celastrol and AZD1981 did not affect the expression level of progerin. Considering that currently available therapies primarily target the synthesis, modification and protein turnover of progerin [32, 33], celastrol and AZD1981, acting independently of progerin regulation, may synergize with these therapies.

Further mechanistic studies are needed for both compounds. While AZD1981 has not been linked to cellular senescence or aging, celastrol has been reported to exert therapeutic effects in mouse and rat models of senescence- and aging-associated diseases and functional decline, such as neurodegenerative diseases [34], osteoarthritis [35], and skeletal muscle atrophy [36]. Since Δ133p53α is present only in humans and primates, but not in mice or rats [5, 37], celastrol could exhibit additional Δ133p53α-dependent therapeutic activities in human diseases beyond those

observed in rodent models. A potential synergy between Δ133p53α-activating compounds and senolytic or senomorphic drugs also merits exploration [10].

CONCLUSION

Celastrol and AZD1981, identified in our previous drug and investigational agent library qHTS, inhibit cellular senescence and IL-6 secretion in fibroblasts derived from HGPS patients, recapitulating the beneficial effects exerted by lentiviral expression of Δ133p53α. These compounds warrant further investigation for potential clinical translation.

DECLARATIONS

Authors' contributions

Performed data acquisition and analyses: Joruiz SM, Lissa D, von Muhlinen N; Made substantial contributions to conception and design of the study: Dranchak PK, Inglese J, Horikawa I, Harris CC; Wrote the manuscript: Joruiz SM, Lissa D, Horikawa I.

Availability of data and materials

All materials in this study and raw data related to this study are available upon request.

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authors and do not necessarily reflect the views of the NIH or the U.S. Department of Health and Human Services.

Conflicts of interest

Lissa D is a current employee at AstraZeneca but had no association with the company at the time of this project, and no participation from AstraZeneca was received in any form. All authors declared that there are no conflicts of interest.

Ethical approval and informed consent

Not applicable.

Figure legends

Figure 1. Cell proliferation in HGPS patients-derived fibroblasts AG11513 (A) and HGADFN271 (B) untreated and treated with celastrol or AZD1981. Both fibroblast strains were treated with 0.1 μM celastrol, 10 μM AZD1981, or DMSO alone (untreated control), which was supplemented freshly every 3 or 4 days. Cell proliferation was monitored by periodical cell counting for 72 days and the cumulative population doubling levels (PDLs) were calculated. Note the differences in cumulative PDLs between the two fibroblast strains.

Figure 2. Western blot analysis of HGPS patients-derived fibroblasts AG11513 and HGADFN271 treated with celastrol or AZD1981. The cells were treated with 0.1 μM celastrol, 10 μM AZD1981, or DMSO alone for 24 h, maintained in cell culture for 4 days in the absence of compound, and then harvested. In this figure, Δ133p53α was detected using a rabbit polyclonal antibody MAP4 [2, 30]. The original image obtained with MAP4 before cropping the bands, along with an image obtained using a sheep polyclonal anti-p53 antibody SAPU, which also detects Δ133p53α [1, 30], are shown in Supplementary Figure 1. The quantitative expression levels of Δ133p53α, $p21^{WAF1}$ and progerin, normalized to GAPDH and calculated relative to DMSO alone, are shown below the images.

Figure 3. Detection and quantification of senescent cells in HGPS patients-derived fibroblasts treated with celastrol or AZD1981. (**A** and **B**) AG11513 (A) and HGADFN271 (B) fibroblasts were treated as in Figure 2, followed by 4-day cell culture before staining for SA-β-gal activity. The quantitative data for the percentages of SA-β-gal-positive cells are presented as mean ± S.D. obtained from three independent experiments, each with biological triplicates. Note

the difference in vertical-axis values between (A) and (B). Representative images are presented in Supplementary Figure 2. (**C**) qRT-PCR assay of $p21^{WAF1}$ mRNA expression. Relative expression levels to DMSO alone are presented as mean ± S.D. from triplicated assays. Unpaired 2-tailed Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 4. Quantification of IL-6 secretion (A and B) and IL-6 mRNA expression (C) in HGPS patients-derived fibroblasts AG11513 and HGADFN271 treated with celastrol or AZD1981. The cells were treated as in Figure 2. (**A** and **B**) Following 4-day cell culture, culture media were collected for IL-6 ELISA and cell numbers were counted for normalization. The concentrations of secreted IL-6 (pg/ml) were normalized to cell numbers (per 1×10^4) and are presented as mean ± S.D. from biological triplicates. Note the difference in vertical-axis values between AG11513 (A) and HGADFN271 (B). (**C**) qRT-PCR assay of IL-6 mRNA expression. Relative expression levels to DMSO alone are presented as mean ± S.D. from triplicated assays. Unpaired 2-tailed Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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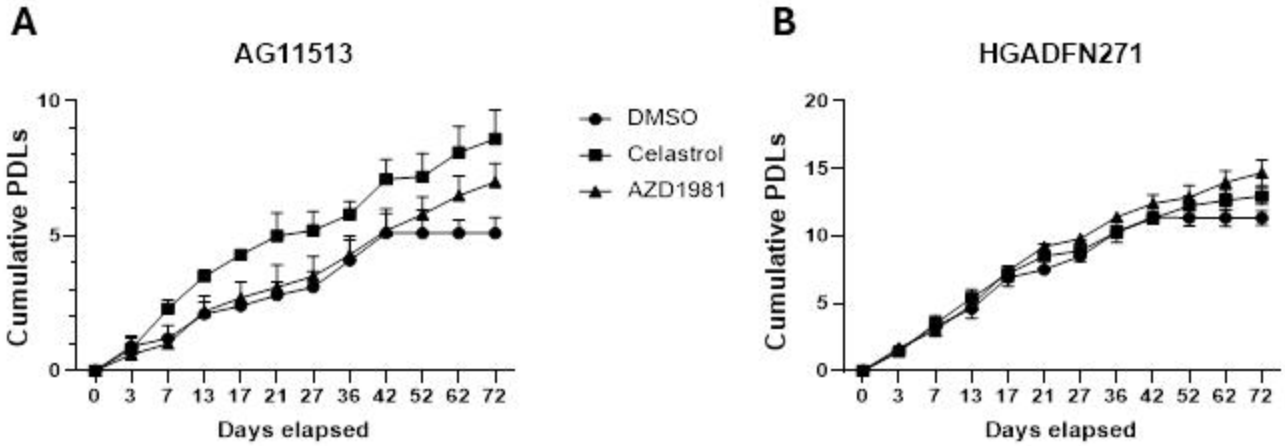
Figure 1


A
AG11513
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Days elapsed
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0 3 7 13 17 21 27 36 42 52 62 72

Figure 2

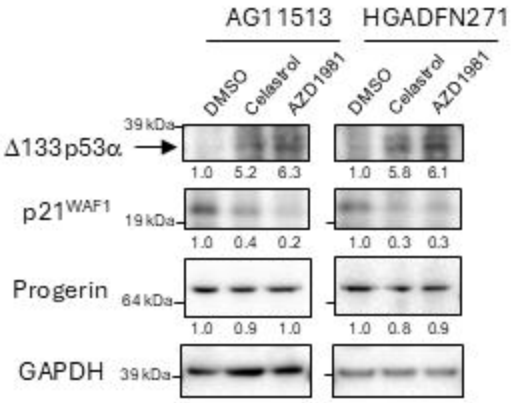

AG11513
HGADFN271
DMSO
Celastrol
AZD1981
DMSO
Celastrol
AZD1981
39 kDa
Δ133p53α
1.0 5.2 6.3
1.0 5.8 6.1
p21WAF1
19 kDa
1.0 0.4 0.2
1.0 0.3 0.3
Progerin
64 kDa
1.0 0.9 1.0
1.0 0.8 0.9
GAPDH
39 kDa

Figure 3

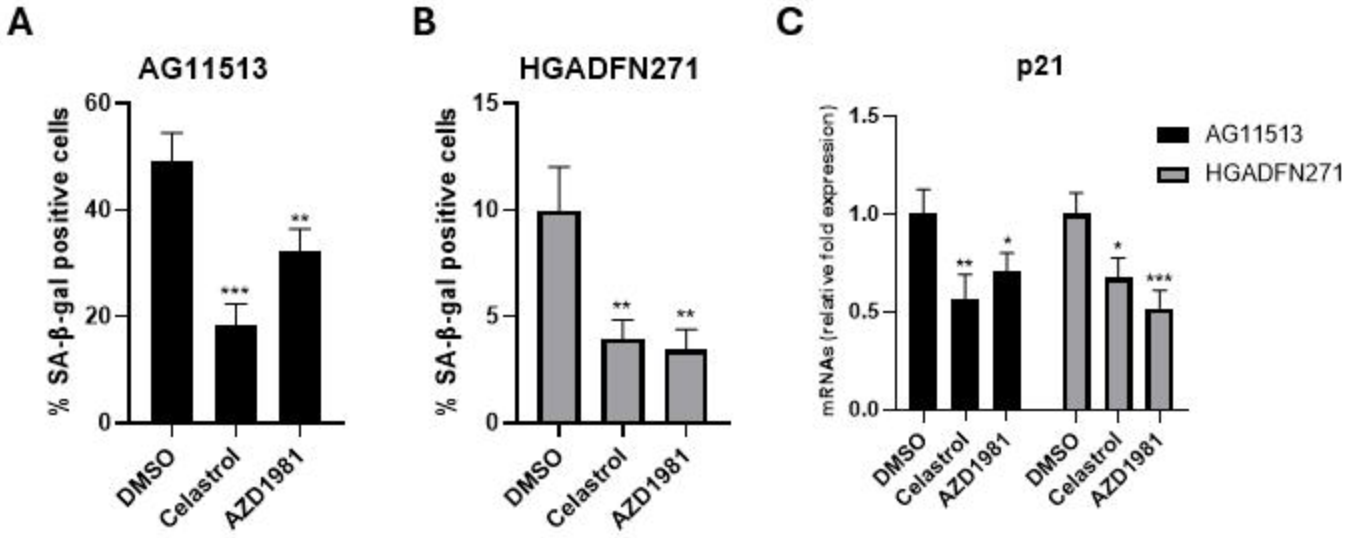
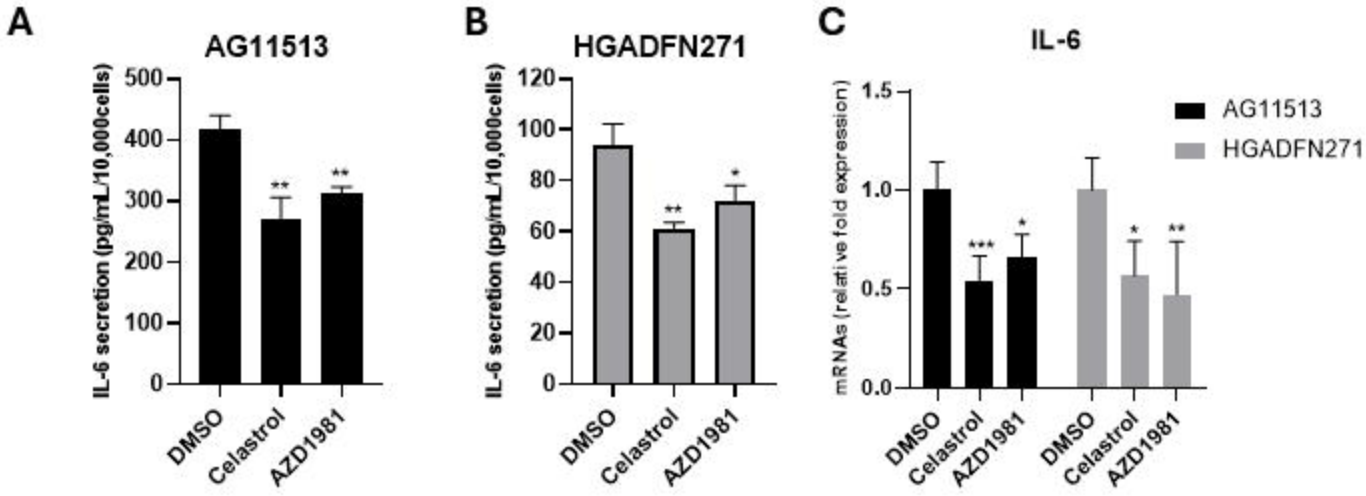

A
AG11513
% SA-β-gal positive cells
60
40
20
0
***
**
DMSO
Celastrol
AZD1981
B
HGADFN271
% SA-β-gal positive cells
15
10
5
0
**
**
DMSO
Celastrol
AZD1981
C
p21
mRNAs (relative fold expression)
1.5
1.0
0.5
0.0
**
*
*
***
AG11513
HGADFN271
DMSO
Celastrol
AZD1981
DMSO
Celastrol
AZD1981

Figure 4


A
AG11513
IL-6 secretion (pg/mL/10,000cells)
500
400
300
200
100
0
**
**
DMSO
Celastrol
AZD1981
B
HGADFN271
IL-6 secretion (pg/mL/10,000cells)
120
100
80
60
40
20
0
**
*
DMSO
Celastrol
AZD1981
C
IL-6
mRNAs (relative fold expression)
1.5
1.0
0.5
0.0
***
*
*
**
AG11513
HGADFN271
DMSO
Celastrol
AZD1981
DMSO
Celastrol
AZD1981