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CDK4/6-inhibiting drug substitutes for p21 and p16 in senescence

Duration of cell cycle arrest and MTOR activity determine geroconversion

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CDKN1A (p21) and CDKN2A (p16) inhibit CDK4/6, initiating senescence. According to our view on senescence, the role of p21 and p16 is to cause cell cycle arrest, whereas MTOR (mechanistic target of rapamycin) drives geroconversion to senescence. Recently we demonstrated that one of the markers of p21- and p16-initiated senescence is MEK-dependent hyper-elevation of cyclin D1. We noticed that a synthetic inhibitor of CDK 4/6 (PD0332991) also induced cyclin D1-positive senescence. We demonstrated that PD0332991 and p21 caused almost identical senescence phenotypes. p21, p16, and PD0332991 do not inhibit MTOR, and rapamycin decelerates geroconversion caused by all 3 molecules. Like p21, PD0332991 initiated senescence at any concentration that inhibited cell proliferation. This confirms the notion that a mere arrest in the presence of active MTOR may lead to senescence.

Introduction

In cell culture, cellular senescence is defined as an irreversible state. To reach this state, cells first need to be arrested. At first, this arrest is reversible.[1] However, over time, active MTOR and MEK/MAPK drive geroconversion (conversion to irreversible senescence), leading to a large morphology (hypertrophy), hyperfunctional and hyper-secretory phenotypes, hyper-elevated cyclin D1, and loss of replicative and regenerative potential (RP).[2-7] For example, induction of ectopic p21 and p16 in HT1080 cells (HT-p21 and HT-p16 cells) causes cell cycle arrest, which is reversible during 2 days.[8-10] If p21 and p16 switched off, then most cells can recover and resume proliferation.[8-10] It is most important that, during p21- and p16-induced arrest, MTOR and MEK are still active[3,7] and drive cellular growth in size (hypertrophy) and loss of regenerative/replicative potential (RP). In the presence of IPTG, which induces ectopic p21 and p16, cells can remain senescent for a seemingly unlimited period of time. When IPTG is removed, senescent cells either cannot resume proliferation or die. This system allows one to observe reversibility vs. irreversibility by switching on and off p21 by simple removal of IPTG. However, this model is restricted to one cell line. To study cellular aging in any cell line, including normal and primary cells, one needs a removable drug, which acts on non-transfected cells (note: HT-p21 and HT-p16 have IPTG- inducible exogenous gene). Majority of agents that induce arrest, such as doxorubicin, are not easily removable, and they are toxic or DNA damaging. Given that p21 and p16 inhibit CDK 4/6, we chose a small-molecule CDK 4/6 inhibitor, PD0332991[11,12] However, it is believed that p21 and p16 cause senescence not by simply inducing arrest, but by running a putative senescence program including interaction with cytoplasmic proteins and trans-regulation of numerous genes. In our view on senescence, the role of p21 and p16 is mere inhibition of CDK 4/6, while MTOR causes geroconversion to senescence.

Results

PD0332991 and p21 cause cyclin-D1-positive senescence

First we compared the effects of PD0332991 and IPTG-induced p21 in HT-p21 cells. Like IPTG, PD0332991 did not inhibit phosphorylation of the MTOR target p70 S6K (on either Thr389 or Thr421/Ser424 phosphorylation sites) and ERK1/2 (**Fig. 1A**). IPTG and PD0332991 equally hyper-induced cyclin D1 and E, as seen on days 1 and 3 (**Fig. 1A**). This confirmed that cyclin D1 hyper-induction is a common marker of geroconversion, regardless of which CDK inhibitor used (p21, p16 or the synthetic small molecule PD0332991). Thus, PD0332991 and IPTG caused identical effects, even though PD0332991 is a direct inhibitor of CDK4/6 and IPTG is acting via induction of ectopic p21 (**Fig. 1A**). Second, we compared the effects of rapamycin and U0126 on cyclin D1 induction. U0126 was more potent in inhibiting cyclin D1 in senescent cells. As expected,

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unlike rapamycin, U0126 inhibited phosphorylation of the mostly "MEK-dependent site" (Thr421/Ser424) on p70S6K and predominantly on day 1 (**Fig. 1A**), while rapamycin completely inhibited phosphorylation of "MTOR site" (Thr389) and partially "MEK site" (Thr421/Ser 424) (**Fig. 1A**). In contrast, only U0126 inhibited ERK phosphorylation, a target of MEK (**Fig. 1A**). We conclude that hyper-induction of cyclin D1 is mostly regulated by MEK, and just partially via MTOR. Like IPTG, PD0332991 caused senescent morphology in HT-p21 cells (**Fig. 1B**). This senescent morphology was partially suppressed by co-treatment with rapamycin (**Fig. 1B**). Next, we investigated replicative potential (RP) of PD0332991-arrested cells after the drug was washed out, allowing quiescent cells to proliferate and form colonies, whereas senescent cells could not resume proliferation[13] (**Fig. 1C and D**). Yet, PD0332991 (at non-toxic concentrations) did not arrest every single cell and therefore senescent/quiescent cells coexisted with proliferating cells, which rapidly overgrew in the culture (**Fig. 1D**, right panel, no Noco). To avoid overgrowth of non-arrested cells during treatment with PD0332991, we treated cells for the last 2 days with nocodazole to kill off proliferating cells, thus leaving intact only PD0332991-arrested cells (**Fig. 1C–D**), as described previously.[13] This method revealed that replicative potential of PD0332991-arrested cells was low and comparable with that of IPTG-treated cells (**Fig. 1D** left panel, + Noco). Co-treatment with rapamycin increased RP of both IPTG- and PD0332991-treated cells (**Fig. 1D**), confirming that PD0332991-induced senescence was MTOR-dependent. Furthermore, we investigated if the duration of treatment with PD0332991 affects the extent of geroconversion using a lower (0.5 μM) concentration of the drug. In agreement with data shown in **Figure 1D**, most cells were able to resume proliferation after 3.5 days of treatment with 0.5 μM PD0332991 (**Fig. S1A**). By day 7, however, most cells (~90%) acquired senescent morphology (**Fig. S1B**), and cells lost their RP (**Fig. S1A**).

Time- and MTOR-dependent senescence caused by PD0332991 in MEL 10 cells

We next investigated the effect of PD0332991 in MEL10 cells, which are prone to senescence. MEL10 cells are easily arrested by nutlin-3a, etoposide, and U0126. These cells are senescence-prone not only because they are easily arrested, but also because they possess a resilient MTOR pathway, and none of the drugs

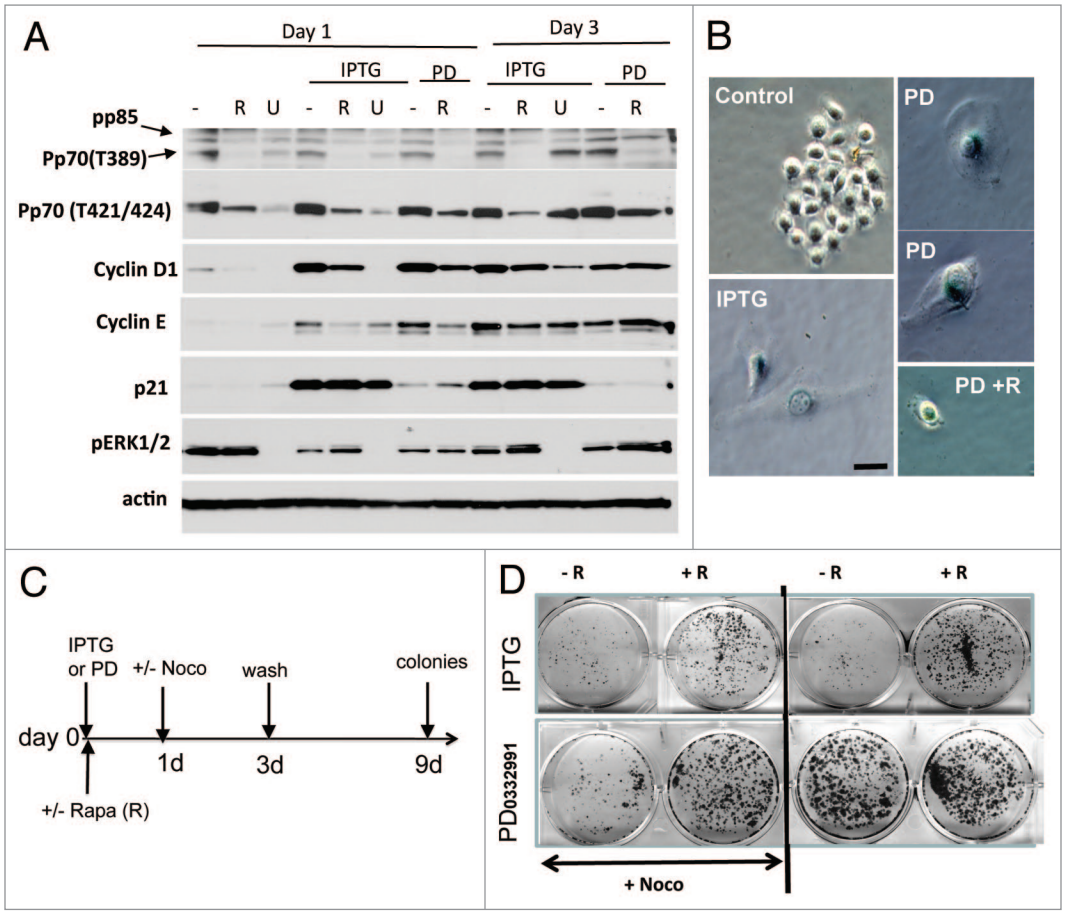

Figure 1. Comparison of p21- and PD0332991-induced senescence in HT-p21 cells. (**A**) Immunoblot analysis. Cells were treated with IPTG and 1 μM PD0332991 with or without 500 nM Rapamycin or 10 μM U0126. Cells were lysed on day 1 and 3 and immunoblotting was performed with the indicated antibodies.(**B**) Beta-Gal staining. Cells were treated with IPTG and 1 μM PD0332991 with or without 500 nM Rapamycin. (**C**) RP: Schema of experiment for measuring RP, presented in (**D**). (**D**) RP in IPTG- and PD0332991- treated HT-p21 cells. Cells were treated with IPTG or 1 μM PD0332991 with or without 500 nM rapamycin (R). After 1 day, 200 nM nocodazole was added in half of the wells (+Noco). After 3 days of drug treatment, drugs were washed out and cells were allowed to recover for 9 days and colonies were stained with crystal violet.

used (nutlin, etoposide, or U0126) inhibit it.[7,14] This creates a condition for geroconversion. At a wide range of concentrations, PD0332991 caused dephosphorylation of retinoblastoma protein (Rb) at Ser780, which is phosphorylated by CDK4/6, indicating that PD0332991 inhibited its target (**Fig. 2**). A longer exposure of the blot revealed residual phosphorylation of Rb at Ser 780 at concentrations of 0.125–0.5 μM, indicating that some cells were not arrested on day 1. Importantly, PD0332991 did not cause pS6 dephosphorylation at any concentration used, thus creating a situation for geroconversion to occur. In fact, at all concentrations, PD0332991 induced cyclin D1, an early marker of geroconversion.[7] This marker was decreased by co-treatment with rapamycin, which blocked S6 phosphorylation. Importantly rapamycin did not restore but even decreased Rb phosphorylation (see overexposed blot), consistent with continuous arrest of the cell cycle. In agreement, PD0332991 inhibited proliferation at the same concentrations, and rapamycin did not restore proliferation, but in turn, it was cytostatic by itself (**Fig. 3A and C**). We also determined RP of the arrested cells after 3.5 and 7 days of treatment with PD0332991 (**Fig. 3B and D**). Loss of RP was prominent at 1–2 μM PD0332991 after 3.5 days treatment (**Fig. 3B**), and it was prevented by co-treatment with rapamycin. By day 7, cells treated with 0.5 μM PD0332991 completely lost RP, which was prevented by rapamycin (**Fig. 3D**). Thus, geroconversion was dependent on the duration of treatment. After 3.5 days, cells started to acquire senescent morphology, which was not prominent, consistent with some retention of RP (**Fig. 4**). By day 7, all cells were senescent (**Fig. 4**), consistent with loss of RP (**Fig. 3D**). Rapamycin prevented both senescent morphology (**Fig. 4**) and loss of RP (**Fig. 3D**) in MEL10 cells treated with PD0332991.

Time- and MTOR- dependent senescence caused by PD0332991 in normal RPE cells

Next we extended our observations to normal RPE cells. At a wide range of concentrations PD0332991 inhibited Rb phosphorylation at CDK4/6 site Ser780 (**Fig. 5A**). In agreement, at the same concentrations, PD0332991 inhibited cell proliferation (**Fig. 5B**), but it did not inhibit the MTOR pathway (**Fig. 5A**). Cyclin D1 was induced as a marker of geroconversion. In agreement, RPE cells showed decreased replicative potential (RP, **Fig. 5C**). Rapamycin preserved RP in PD0332991-treated RPE cells (**Fig. 5D**). Finally, we confirmed that U0126 prevented cyclin D1 induction during geroconversion, but it did not inhibit proliferation and Rb phosphorylation (**Fig. 5A**).

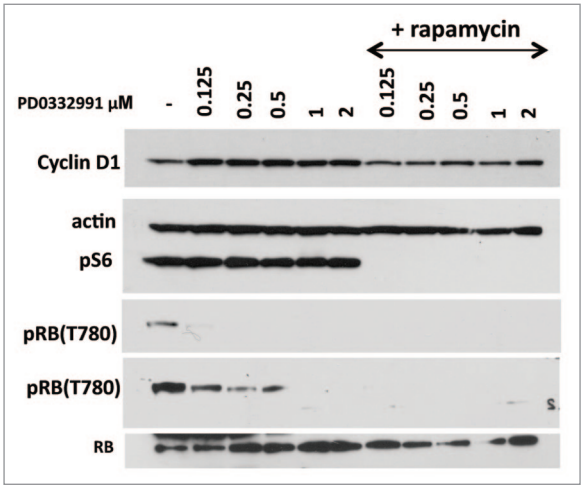

Figure 2. Immunoblot analysis of PD0332991-induced senescence in MEL10 cells. Cells were treated with indicated concentrations of PD0332991 with or without 10 nM rapamycin. After 1 day, cells were lysed and immunoblotting was performed with the indicated antibodies.

Discussion

Here we showed that the synthetic inhibitor of CDK4/6, p21, and p16 caused almost identical senescence phenotypes in HT-p21 cells. While inhibitors of CDKs cause cell cycle arrest, MTOR and MEK determine geroconversion and are responsible for the acquisition of hallmarks of senescence. p21 and p16 affect some targets besides CDKs, which were considered to be related to the senescence program. Although PD0332991 may have other off-target effects, only CDKs are the common targets for p21, p16, and PD0332991. p21, p16, and PD0332991 do not inhibit MTOR, which, in turn, drives senescent phenotype, and rapamycin decelerates geroconversion caused by all 3 molecules.

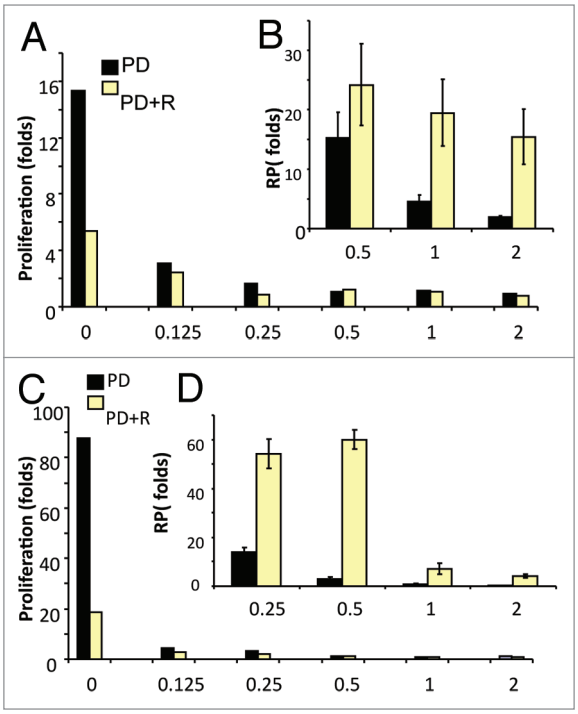

Figure 3. The effect of PD0332991 on proliferation and replicative potential (RP) in MEL10 cells. Cells were treated with the indicated concentrations of PD0332991 with or without 10 nM rapamycin. (**A and B**) After 3.5 days, proliferation (**A**) was measured by cell counting, as fold increase compared with initial (seeding) cell numbers. In parallel sets (**B**), drugs were washed out, and cells were allowed to proliferate, and RP was measured after 7 days of culture, as described in "Materials and Methods". (**C and D**) After 7 days treatment, proliferation (**A**) was measured by cell counting, as fold increase compared with initial (seeding) cell numbers. In parallel sets (**B**), drugs were washed out, and cells were allowed to proliferate, and RP was measured after 7 days of culture, as described in "Materials and Methods".

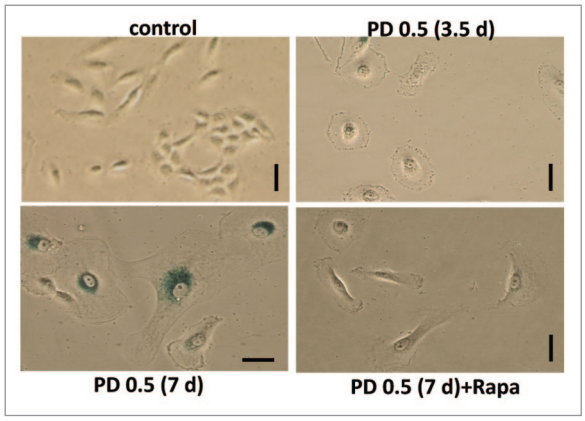

Figure 4. Senescent morphology of PD0332991-treated MEL10 cells. MEL10 cells were treated with 0.5 μM PD0332991 with or without 10 nM rapamycin. After 3.5 and 7 days, cells were stained for beta-Gal. Bar: 100 μm.

This confirms the notion that a mere arrest in the presence of active MTOR may lead to geroconversion to senescence. Gerogenic conversion (geroconversion) without cell cycle arrest is associated with cancer[15,16] and not coincidentally PI3K/MTOR and MEK/MAPK pathways are almost always activated in cancer.[17-25] p16 is considered a marker of senescence,[26-29] although it is rather a marker of cell cycle arrest, which may (or may not) be associated with senescence. A combination of cell cycle arrest (p16 or p21) with elevated cyclin D1 and active MTOR (phospho S6-positivity) may be a precise marker of gerogenic conversion (**Fig. 6**). Such gerogenic cells drive organismal aging,[30] and rapamycin, which suppresses geroconversion,[2-7,31-42] also delays age-related diseases and extends lifespan in mice.[43-63] Whether inhibitors acting downstream of MTOR could suppress geroconversion is under initial investigation.[64]

Material and Methods

Cell lines and reagents

HT-p21 cells, derived from HT1080 human fibrosarcoma cells (ATCC) were previously described.[8-10] In HT-p21 cells, p21 expression can be turned on or off using isopropyl-thio-galactosidase (IPTG).[8,9] HT-p21 cells were cultured as described previously.[5,6] Melanoma MEL10 (formally, SK-MEL-147) were described previously.[7,14] Normal retinal pigment epithelial RPE cell lines were obtained from (ATCC) were maintained in MEM plus 10% FBS. PD0332991 was purchased from Selleckchem. Rapamycin was obtained from LC Laboratories. IPTG (Invitrogen) was used in cell culture at final concentration 50 μg/ml. U0126 was purchased from Sigma-Aldrich.

Immunoblot analysis

Immunoblot analysis was performed as described previously.[5] The following primary antibodies were used: mouse anti-phospho p70S6K (Thr389), rabbit anti-phospho Thr389 p70S6K and anti-phospho Thr421/Ser424 p70S6K, rabbit anti-phospho ERK ½ (Thr202/Tyr204), and anti-phospho Ser780 RB were purchased from Cell Signaling Biotechnology; mouse

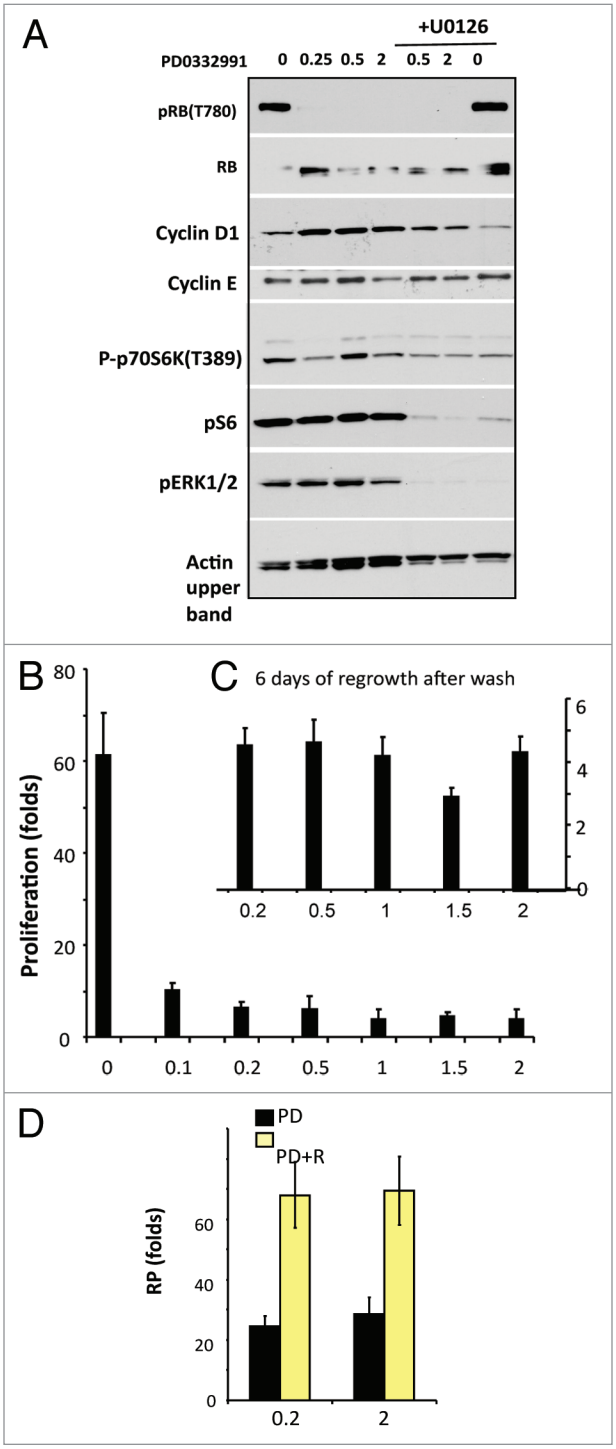

Figure 5. The effect of PD0332991 on RPE cells. **(A).** Immunoblot analysis: Cells were treated with the indicated concentrations of PD0332991 with or without 10 μM U0126. Cells were lysed after 24 h. (**B and C)** Proliferation (**B**) and RP: replicative potential (**C**). After 7 days treatment proliferation **(B)** was measured by cell counting. In parallel set (**C**), drugs were washed out and cells were allowed to proliferate. RP was measured by counting after 6 days of culture. (**D**) The effect of rapamycin on RP of PD0332991-treated RPE cells. Cells were treated with 0.2 or 2 μM PD0332991 with or without 5 nM rapamycin (R). After 5 days treatment drugs were washed out and RP was determined.

anti-cyclins D1 and E and anti-RB were obtained from Santa Cruz Biotechnology; mouse anti-p21 and rabbit anti-actin were purchased from BD Biosciences and Sigma-Aldrich, respectively. Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies were purchased from Cell Signaling Biotechnology.

SA-β-Gal staining

Beta-Gal staining was performed using Senescence-galactosidase staining kit (Cell Signaling Technology) according to manufacturer's protocol.

RP (regenerative/replicative potential) assay

Cells were plated at low density, treated with senescence-inducing agents (IPTG or PD0332991) as indicated in figure legends. Cell numbers were determined at the end of treatment (initial cell numbers) and drugs were removed by washing. Cells were incubated in fresh drug-free medium for several days, as indicated in figure legends, and then final cell numbers were determined. RP was calculated as a ratio between final and initial cell numbers: a fold-increase in cell numbers after the drugs were washed out.

Colony formation assay

HT-p21 cells were plated at low density, treated with IPTG or PD0332991 with or without rapamycin. In some experiments, after 1 day of treatment nocodazole was added as indicated in schema in **Figure 1C**. Then, drugs were washed out, and cells were incubated in fresh drug-free medium for 6–7 days. Plates were fixed and stained with 1.0% crystal violet (Sigma-Aldrich).

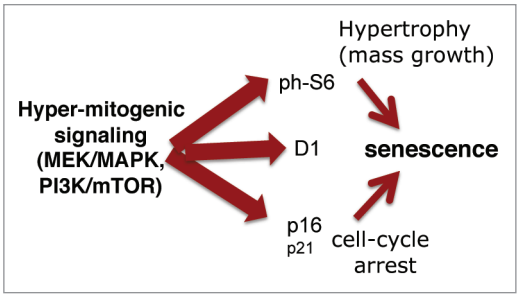

Figure 6. Hyper-mitogenic signaling and markers of senescence. When the cell cycle is arrested, active MAPK and MTOR pathways drive geroconversion from arrest to senescence. Although p16 expression is commonly considered as a marker of senescence,[26-29,65] p16 merely causes cell cycle arrest. It is hyper-mitogenic signaling via MAPK and MTOR that over-stimulates arrested cells, causing senescence. (Note: p16 can be also associated with aggressive cancer[66] and may have anti-aging activities[67]). However, p16 is induced by hyper-mitogenic signaling (Ras, Raf, MAPK, and PI-3K/mTOR),[68-76] rendering p16 a proxy marker of hyper-mitogenic signaling and senescence (senescence = hyper-mitogenic signaling plus cell cycle arrest). The most reliable marker of senescence could be a combination of p16, cyclin D1 (Mek-dependent), and phospho-S6 (MTOR-dependent).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26130

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