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CDK9 Inhibition Induces a Metabolic Switch that Renders Prostate Cancer Cells Dependent on Fatty Acid Oxidation

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Abstract
Cyclin-dependent kinase 9 (CDK9), a key regulator of RNA-polymerase II, is a candidate drug target for cancers driven by transcriptional deregulation. Here we report a multi-omics-profiling of prostate cancer cell responses to CDK9 inhibition to identify synthetic lethal interactions. These interactions were validated using live-cell imaging, mitochondrial flux-, viability- and cell death activation assays. We show that CDK9 inhibition induces acute metabolic stress in prostate cancer cells. This is manifested by a drastic down-regulation of mitochondrial oxidative phosphorylation, ATP depletion and induction of a rapid and sustained phosphorylation of AMP-activated protein kinase (AMPK), the key sensor of cellular energy homeostasis. We used metabolomics to demonstrate that inhibition of CDK9 leads to accumulation of acyl-carnitines, metabolic intermediates in fatty acid oxidation (FAO). Acyl-carnitines are produced by carnitine palmitoyltransferase enzymes 1 and 2 (CPT), and we used both genetic and pharmacological tools to show that inhibition of CPT-activity is synthetically lethal with CDK9 inhibition. To our knowledge this is the first report to show that CDK9 inhibition dramatically alters cancer cell metabolism.

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Introduction
Uncontrolled growth of cancer cells requires significant metabolic reprogramming to satisfy the requirements of rapidly dividing cells. Most cancers have increased appetite for glucose, which allows evaluation of the tumor-burden using glucose-positron emission tomography (PET) [1]. Prostate cancer, the most common cancer in males in the USA [2], is not always readily detectable using PET-imaging, but can instead be visualized using 11C-acetate due to higher dependency of prostate cancer cells on lipids for energy production [3,4].

Normal prostate tissue and prostate cancer have specific metabolic features that are distinct from the rest of the body. In the untransformed state, cells of the prostate gland accumulate high levels of citrate due to prostate-specific accumulation of zinc [5,6]. High zinc concentration inhibits m-aconitase activity and citrate oxidation, truncating the TCA cycle and reducing ATP production [7]. Some of the prostate-specific metabolic features may be utilized to sensitize prostate cancer cells to other treatments.

Cell division requires a substantial amount of ATP and continuous synthesis of macromolecules, especially nucleic acids and lipids. Direct crosstalk between metabolism and the cell-cycle regulators, cyclin-dependent kinases (CDKs), could integrate nutrient

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availability with cell proliferation. There is clear evidence of such crosstalk in the literature. For example, increased glucose uptake can activate oncogenic signaling in nonmalignant cells [8]. Conversely, CDK1, the major regulator of the G2/M cell cycle transition is also targeted to mitochondria, where it phosphorylates proteins of the complex I electron-transport machinery to promote ATP production [9]. In addition, CDK4 and CDK6, regulators of the G1-S transition, can also directly regulate glucose metabolism [10,11]. Increased activity of CDKs is observed in most cancers, which has fostered development of CDK inhibitors for cancer therapy.

Regulation of cell cycle initiation and progression is ultimately dictated through transcriptional regulation. A specific class of CDKs phosphorylate the RNA-polymerase II (RNA-Pol II) carboxy-terminal domain (CTD) to regulate its activity [12,13]. CDK7 and CDK9 are the major RNA-Pol II CDKs. CDK7 promotes transcription initiation, while the switch to productive elongation is dependent on CDK9. CDK7 additionally functions as a CDK-activating kinase and inhibition of its activity will directly affect both cell cycle CDKs and RNA-Pol II. On the other hand, CDK9 has a well-defined primary function, and several reports suggest that this enzyme could be an attractive target for prostate cancer therapy. First, CDK9-mediated phosphorylation regulates the activity of the androgen receptor, the major drug-target in prostate cancer [15]. Second, CDK9 is required for RNA-Pol II pause-release, a process that is frequently deregulated in cancer cells [16,17]. Third, compounds targeting CDK9 induce apoptosis in cancer cells, in part through decreased expression of the anti-apoptotic protein MCL1 [9]. In addition, CDK4 and CDK6, regulators of the G1-S transition, can also directly regulate glucose metabolism [10,11]. Increased activity of CDKs is observed in most cancers, which has fostered development of CDK inhibitors for cancer therapy.

In this study, we show that the pan-CDK inhibitor AT7519 induces acute metabolic stress in prostate cancer cells. Metabolite profiling showed that AT7519 treatment led to accumulation of acyl-carnitines. We identify CDK9 as the key mediator of the observed metabolic effects in prostate cancer cells and show that simultaneous inhibition of CDK9 and enzymes required for acyl-carnitine production (CPT1 and CPT2) is lethal to prostate cancer cells. In brief, our study reports a CDK9 inhibition-induced adaptive metabolic response in prostate cancer cells, and thereby identifies a candidate compound combination for prostate cancer therapy.

Materials and Methods

Cell Culture and Manipulations

LNCaP and PC3 cells were obtained from ATCC and maintained as recommended by the provider. AT7519 (used in a dose of 0.5 μM unless otherwise indicated) and Etomoxir were purchased from Selleckchem. Perhexiline (used in a dose of 10 μM unless otherwise indicated) was purchased from Sigma. NVP2 was from MedChem Express. Viability assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Growth rate and cell death activation were evaluated using the Incucyte instrument according to manufacturer’s instructions. For detection of cell death activation, we used IncuCyte Caspase-3/7 Green Reagent for Apoptosis (Essen Biosciences). Cell cycle analysis was performed using the Propidium Iodide Flow Cytometry Kit (Abcam, ab139418) and the BD FACSCanto instrument (BD Biosciences). Knockdown experiments were performed using RNAiMax reagent (Sigma). CPT1 targeting siRNAs were from ThermoFisher Scientific (siCPT1a s3467 and siCPT2 s3468).

Seahorse Metabolic Flux Analysis

Metabolic flux analysis was performed using a Seahorse XFe 96 instrument. An equal number of LNCaP cells were plated and allowed to attach for 1 day. Cells were treated with inhibitors for 24 hours and subjected to flux analysis. In brief, cells were changed to base media containing 1 mM pyruvate, 2 mM glutamine and 10 mM glucose 45 minutes before the assay. Cartridge was equilibrated overnight and loaded with Oligomycin 2 μM, FCCP 1 μM and rotenone/antimycin A 0.5 μM prior to the assay. Results were analyzed using Seahorse Wave software.

Protein Profiling

Samples for western blotting were prepared as previously described [19]. Samples for the reverse-phase protein array profiling were prepared using the same protocol. Antibodies used are as follows: from Cell Signaling Technology, CI-PARP (9541), p-H2AX (9718), p-h3ser10 (9701), CPT1A (12252), h3 (9715), HES1 (11988), CDK1 (9116), p-RNA Pol II-Ser 2 (13499), MCL1 (5453 T) and MYC (5605S). Actin (ab69490), CPT2 (ab181114) and GAPDH (9485) antibodies were from Abcam.

mRNA Profiling

RNA isolation was performed using the illustraMiniSpin-kit (GE Healthcare) according to manufacturer’s instructions, except luciferase RNA (Promega, cat # L4561) was added into cell lysis buffer. cDNA was synthesized using the qScript cDNA Synthesis Kit (Quintabio). Primers used are as follows: MYC F- TACCC TCTCAACGCAGCACG, R- TCTTGACATTCTCCTCGGTG; MCL1 F- TGCTTCCGAAAATCGACATCA, R- TAGGCCACA AAGGCACCAAAAG and Luciferase F-TACAACCCCGACATCTTCGA, R- GGAAGTTCACCGGCGTCAT.

Metabolomic Profiling

LNCaP cells were allowed to attach for 1 day and treated for 1 day. For harvesting, cells were washed with PBS, trypsinized, counted and centrifuged 4000 rpm for 5 minutes at 4 °C, washed with PBS, centrifuged again, pellet was washed with water (cells not solubilized), cells were frozen using liquid nitrogen and stored at −80 °C until analysis. Targeted metabolomics was purchased as a service from FIMM Metabolomics/Lipidomics/Fluxomics Unit (Helsinki Finland).

Results

CDK Inhibitor AT7519 Induces Acute Metabolic Stress in Prostate Cancer Cells

Cyclin-dependent kinase (CDK) inhibitors suppress cell cycle progression, but there has been no study on how the prostate cancer cell proteome is altered in response to CDK inhibition. In order to characterize these changes, we used AT7519, a pan-CDK-inhibitor that most potently targets CDK9 [20].

We wanted to identify both immediate and sustained changes in the proteome using reverse-phase protein arrays (RPPA), and therefore analyzed two time-points, 4 and 24 hours after treatment with AT7519. In order to identify adaptive changes that enable cell survival, we selected a dose (500 nM) that decreased the proliferation rate of cells but did not induce substantial cell death as measured by
live-cell imaging and PARP cleavage, respectively (Supplementary Figure 1, A and B). We hypothesized that proteins whose expression changes rapidly and in a sustained manner report on the most essential adaptations for cancer cell survival. We identified two targets whose levels were significantly increased at both time points, the phosphorylated form of AMP-activated protein kinase (AMPK) and the serine/threonine-protein kinase A-Raf (Figure 1 and Suppl. Table 1). Lopez-Mejia et al. (2017) have reported that CDK4-mediated phosphorylation of AMPK increases glycolysis and decreases fatty acid oxidation in mouse embryonic fibroblasts [21]. However, the RPPA-data showed that inhibition of CDK-activity using AT7519 induced phosphorylation of AMPK in prostate cancer cells. AMPK is one of the key regulators of cellular energy homeostasis and is canonically activated in response to an increased AMP/ATP ratio [22]. As detailed in the introduction, prostate cancer cells have unique metabolic features, and we speculated that phosphorylation of AMPK in response to AT7519 might represent a prostate cancer-specific metabolic response. We therefore focused on AMPK, and first confirmed by western blotting that AMPK phosphorylation is indeed induced in a dose-dependent manner in response to AT7519 treatment (Figure 1B). Activation of AMPK in response to AT7519 suggested that the treatment might decrease cells’ ability to produce ATP.

We directly measured the cellular oxygen-consumption rate (OCR) to assess if AT7519 affects the rate of ATP production. AT7519 dose-dependently inhibited mitochondrial oxygen consumption and ATP production (Figure 1, C and D). Basal respiration-, maximal respiration- and proton leak-dependent OCR were also decreased, but coupling efficiency was maintained. These data indicate that the activity of the mitochondria decreases in response to AT7519 treatment, but the mitochondria remain functional.

We have so far shown that a low dose of AT7519 induces acute metabolic stress in prostate cancer cells. The dose selected led to a strong decrease in cells’ ability to produce ATP but did not induce apparent cell death. We therefore hypothesized that cells activate alternative metabolic strategies to enable survival in the presence of AT7519.

**AT7519 Induces Accumulation of Acyl-Carnitines**

We performed metabolite profiling to probe the possibility that AT7519 affects metabolites involved in ATP generation. Targeted mass spectrometry was used to quantify ~100 different metabolites, including amino acids, nucleotides, neurotransmitter intermediates, choline metabolites and enzyme cofactors (Figure 2A). We noted a prominent decrease in nucleotide metabolites, including uracil (−51%), hypoxanthine (−40%), inosine (−29%) and guanosine (−20%). In addition, AT7519 induced a striking increase in the abundance of the nine different acyl-carnitines measured (Figure 2, A and B). Carnitine serves as an acyl-group carrier across the mitochondrial membrane in a process that delivers lipids to mitochondria for fatty acid oxidation [23]. The metabolite profiling

![Figure 1.](image-url)
data suggest that AT7519 reprograms cells to rely more on fatty acid oxidation.

We tested if the accumulation of acyl-carnitines in response to AT7519-treatment is important for cancer cell survival. Addition and removal of the lipid chain to and from carnitine is catalyzed by carnitine palmitoyltransferases (CPT) 1 and 2, which are localized in the outer and inner mitochondrial membranes, respectively [23] (Figure 2C). These enzymes are required for acyl-carnitine production and fatty acid oxidation, and we tested if they are part of the adaptive response to AT7519. Knockdown of either CPT1 or CPT2 sensitizes cells to AT7519 treatment. KD was performed for 4 days as indicated, after which cells were treated with AT7519 for 3 days and viability was assessed using the CellTiter-Glo assay. Data shown is an average of at least 3 biological replicates with SEM and t-test was used to evaluate the statistical significance (*<.05, **<.01).

In summary, AT7519-treatment leads to depletion of nucleotide intermediates and accumulation of acyl-carnitines, and knockdown of the CPT enzymes sensitizes cells to AT7519 (Figure 2, D and E).

In order to probe if AT7519 renders prostate cancer cells dependent on lipids for ATP synthesis, we pre-treated cells with AT7519 and measured mitochondrial oxygen consumption in response to acute treatment with Perhexiline, a clinically approved CPT inhibitor [24]. Indeed, acute treatment with Perhexiline decreased the mitochondrial oxygen consumption of cells pretreated with AT7519 (Figure 3A). This result is consistent with our model that AT7519 renders prostate cancer cells more dependent on lipids for ATP synthesis and this dependency can be targeted using Perhexiline.

We next assessed the effects of combination treatment with AT7519 and CPT inhibitors on viability of prostate cancer cells. Perhexilene and Etomoxir, another FAO-inhibitor [25], dose-dependently decreased prostate cancer cell viability as assessed by ATP generation, and co-treatment with AT7519 additively enhanced their effects (Supplementary Figure 2, A and B). Etomoxir is an irreversible inhibitor of CPT1 and the compound has been used to inhibit fatty acid oxidation in both normal and cancer cells, however,

**Figure 2. AT7519 induced accumulation of acyl-carnitines promotes prostate cancer cell survival.**

A) Targeted metabolite profiling of LNCaP cells treated as indicated for 24 hours. Data shown is an average of four biological replicates with SEM. Only metabolites whose abundance changed at least 20% in comparison to DMSO are shown. Metabolite levels were quantitatively measured using mass spectrometry and normalized to cell count. Control sample was set to 100% and treatments were also normalized to this. Student’s t-test was used to assess the statistical significance (*<.05, **<.01).

B) Measurement of the absolute amount of carnitines. Data shown is an average of 4 biological replicates with SEM and t-test was used to evaluate the statistical significance (*<.05).

C) Schematic presentation of acyl-carnitine transport across the mitochondrial membrane.

D) Knockdown (KD) of CPT1 and CPT2 sensitizes cells to AT7519 treatment. KD was performed for 4 days as indicated, after which cells were treated with AT7519 for 3 days and viability was assessed using the CellTiter-Glo assay. Data shown is an average of at least 3 biological replicates with SEM and t-test was used to evaluate the statistical significance (*<.05, **<.01).

E) The efficacy of CPT1 and CPT2 KD was evaluated using western blotting.

**AT7519 and Inhibitors of Fatty Acid Oxidation are Synthetically Lethal to Prostate Cancer Cells**

In order to probe if AT7519 renders prostate cancer cells dependent on lipids for ATP synthesis, we pre-treated cells with AT7519 and measured mitochondrial oxygen consumption in response to acute treatment with Perhexiline, a clinically approved CPT inhibitor [24]. Indeed, acute treatment with Perhexiline decreased the mitochondrial oxygen consumption of cells pretreated with AT7519 (Figure 3A). This result is consistent with our model that AT7519 renders prostate cancer cells more dependent on lipids for ATP synthesis and this dependency can be targeted using Perhexiline.
CDK9 inhibition induces a metabolic switch

Simultaneous Targeting of CDK9 and Fatty Acid Oxidation is Synthetically Lethal to Prostate Cancer Cells

As with any studies involving chemical probes, it is important to test additional compounds that inhibit the same proposed targets, if possible. We found that Perhexiline and Etomoxir, as well as CPT knockdown, had similar effects on cells when combined with AT7519 (Figure 2D and Supplementary Figure 2, A and B). AT7519 is a pan-CDK inhibitor that most potently targets CDK9 [20], and we therefore hypothesized that CDK9 is the primary target for the combinatorial lethality between AT7519 and Perhexiline. In 2018, a novel CDK9 inhibitor, NVP2, was identified and reported to have 700-fold selectivity over other kinases [33]. We found that treatment with NVP-2 led to a rapid (24 hours treatment), dose-dependent depletion of the cellular ATP-levels at low nanomolar concentrations (Figure 3F). In addition, and similar to AT7519, NVP2-treatment caused activation of AMPK and resulted in down-regulation of CDK1 and up-regulation of HES1 (Figure 3G). We noted that 10 nM NVP2 had only a modest effect on the phosphorylation of serine-2 of RNA Pol II CTD with clear effect on the ATP levels (Figure 3, F and G). It is possible that the ATP generation is very sensitive to CDK9 inhibition and in order to further probe if NVP2 decreases transcription also in low doses, we treated cells with NVP2 for 4 hours, isolated total RNA using cell lysis buffer that contains spike-in luciferase RNA for normalization and evaluated the transcript levels of MYC and MCL1, both of which have been reported to be affected by CDK9 inhibition [33,34]. Importantly, NVP2 decreased MYC and MCL1 mRNA levels by over 50% (Figure 3H). Based on these data, inhibition of CDK9 activity leads to a rapid decrease in transcription, which decreases mRNAs that have short half-lives and are associated with fast proliferation of cancer cells. In a longer time-frame, CDK9 inhibition leads to a decrease in the metabolic activity of cells, manifested by decreased OCR and ATP generation (Figure 1D). These data indicate that the highly specific CDK9 inhibitor NVP2 causes similar effects as the pan-CDK inhibitor AT7519, confirming CDK9 as a major target for AT7519 in prostate cancer cells.

Next, we assessed if inhibition of CDK9 is sufficient to induce synthetic lethality in combination with Perhexiline. Perhexiline additively enhanced the effect of NVP2 on LNCaP cell viability (Supplementary Figure 4A) and combination of 5 nM NVP2 with Perhexiline led to a complete growth arrest (Figure 3A). A low dose of NVP2 did not induce cell death on its own, but potentiated the effect of Perhexiline by over 60-fold, as measured using caspase-activation, which indicates that certain anti-proliferative effects of CDK9 inhibition are greatly enhanced by Perhexiline (Figure 3J). CDK9 inhibition has been reported to sensitize cancer cells to apoptosis through decreased expression of MCL1 and MYC [33,35,36]. Combining NVP2 with Perhexiline led to over 50% decrease in MYC levels but MCL1 was only modestly affected (Supplementary Figure 4B). In addition, and similar to the Perhexiline+AT7519 combination, we observed that Perhexiline enhances NVP2-induced DNA damage (Figure 3B and Supplementary Figure 4B). These data imply that the combination of CDK9 and FAO inhibitors does not induce apoptosis through down-regulation of anti-apoptotic proteins but rather through induction of excessive DNA damage; however, to fully define the signaling pathways involved will require a comprehensive future study.

Next, we moved on to evaluate the effect of combining CDK9 and FAO inhibitors in PC3 cells. The combination of NVP2 with Perhexiline decreased cell viability, induced cell death and caused growth arrest also in PC3 cells (Supplementary Figure 5). We wanted to further assess the ability of Perhexiline to sensitize prostate cancer cells to a clinically relevant CDK9 inhibitor. For these experiments, we selected TG02/SB1317, a compound that is currently in clinical trials [34,37]. First, we identified a dose for TG02 that had only a modest effect on cell viability as a single agent (Supplementary Figure 6A). Combining this low dose of TG02 with Perhexiline led to a complete loss of proliferation of both LNCaP and PC3 cells (Supplementary Figure 6B). To conclude, Perhexiline can enhance the effects of CDK9 inhibition on prostate cancer cell proliferation. These data confirm the model that CDK9 inhibition causes...
metabolic stress to prostate cancer cells, rendering them dependent on CPT activity, which can be targeted to achieve synthetic lethality.

**Discussion**

CDKs are the key regulators of cell cycle and RNA-Pol II activity, and in this study we discovered that inhibition of CDK9 induces acute metabolic stress in prostate cancer cells. This is, to our knowledge, the first study to report that RNA-Pol II CTD kinase inhibition affects metabolism. Our work highlights the complex interplay between metabolism, transcription and the proteome, and shows the value of multiplatform profiling of cancer cell response to cytostatic compounds. Overall, we found that CDK9 inhibition triggers a metabolic switch in prostate cancer cells so they rely more on CPT enzymes for growth.

Development of normal prostate tissue requires significant remodeling of mitochondrial metabolism. As a result, cells of the prostate gland do not complete the TCA cycle and secrete high levels of citrate\(^5,6\). This prostate-specific metabolic feature is important to promote sperm survival. However, the lack of selection pressure to maintain functionally proficient mitochondria may lead to accumulation of mitochondrial mutations and defective mitochondria. Indeed, mitochondrial genomes of normal prostate cells contain numerous mutations that are not found in other tissues of the same person\(^38\). Certain cancer-associated mitochondrial mutations are
associated with aggressive disease [39], and specific mitochondrial mutations have also been reported to confer a growth advantage to otherwise isogenic prostate cancer cells [40]. Some mitochondrial mutations found in prostate cancer may also render cells more sensitive to inhibitors that affect mitochondrial function.

In this study we unexpectedly found that CDK9 activity plays a crucial role in mitochondrial function in prostate cancer cells. One report has previously linked CDK9 activity to mitochondrial dysfunction. In that case, chronic activation of CDK9 due to over-expression of the cyclin-T1 partner was shown to result in mitochondrial dysfunction in cardiac tissue [41]. Here we have found that inhibiting CDK9 activity decreased oxidative phosphorylation, rendering cells more dependent on lipids for ATP production (Figs. 1D and 3A). Therefore, inhibiting CPT activity in combination with CDK9 inhibition results in lethality in cell culture (Figure 3, D and J). Notably, the observed increase in acyl-carnitines in response to AT7519 may reflect an increased dependence on FAO or decreased ability to deacetyl (Figure 2B). The latter seems more likely as we also observed a concomitant decrease in OCR (Figure 1D). Nevertheless, it may be possible to combine CDK9 and CPT inhibitors for therapeutic purposes but testing this will require challenging animal studies.

The basal transcription machinery is operational in every cell, however, inhibition of the kinases that phosphorylate RNA Pol II carboxy-terminal domain (CTD) is more toxic to cancer cells than normal cells and compounds targeting the CTD kinases are in clinical trials [34,36,42,43]. The activity of RNA Pol II is regulated at multiple levels by CDK7, CDK9 and CDK12, and transcription of different genes appears to be more reliant in the activity of only some of these kinases [13]. For example, inhibition of CDK7 and CDK9 down-regulates the expression of mRNAs with short half-life, such as the anti-apoptotic mitochondrial gene MCL1 and the oncogenic transcription factor MYC [35], as observed also in our current study (Supplementary Figure 4B). On the other hand, CDK12 appears to be particularly important for the expression of long genes, including many genes involved in the DNA damage response [44]. We observed here that the pan-CDK inhibitor AT7519 causes suppression in mitochondrial OCR and sensitizes cells to inhibition of FAO (Figs. 1D, 3, D and E). A more thorough characterization of cancer cells’ response to inhibition of specific CDKs is likely to reveal additional synthetic lethal interactions.

In conclusion, here we show that inhibition of CDK9 activity leads to suppression of mitochondrial activity and ATP production in prostate cancer cells. In the future, it will be important to identify the key mediators of this response. In more general terms, CDK9 inhibitors could be combined with other inhibitors of the key energy-sensor pathways to induce apoptosis in prostate cancer cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.05.001.

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Declarations of Interest
None.

References

Figure 3. CDK9 inhibitors are synthetically lethal with inhibitors of fatty acid oxidation. A) AT7519 renders prostate cancer cells sensitive to Perhexiline as measured using oxygen-consumption rate (OCR). A Seahorse Xfe 96 analyzer was used to measure OCR of cells upon acute injection of perhexiline (indicated with an arrow). LNCaP cells were pre-treated with AT7519 for 24 hours as indicated. Data shown is an average of 3 biological replicates with SEM; t-test was used to evaluate statistical significance (*<.05, **<.01). B) Combination of 0.5 μM AT7519 with 10 μM Perhexiline induces DNA damage and cell death in LNCaP cells. Cells were treated as indicated for 24 hours and western blotting was used to detect the proteins of interest. Densitometry was used to evaluate the abundance of each protein. C) Combination of AT7519 with Perhexiline increases the sub-G1 population of cells. Cell cycle distribution was assessed using propidium iodide staining and flow cytometry. Data shown is an average of two biological replicates with SEM. D) Activation of cell death in response to AT7519 and Perhexiline treatments. The cumulative activation of Caspases 3 and 7 was recorded using live-cell imaging until 36 hours and normalized to cell confluency. Data shown is an average of four biological replicates with SEM and t-test was used to assess the statistical significance (**<.01).


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