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Research Article

Apoptosis Cellular Models in Cancer Therapeutics

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ABSTRACT

Apoptosis, one of the major regulated cell death pathways, is a highly regulated suicide mechanism used for the elimination of damaged and unwanted cells in multicellular organisms. Derailed apoptosis has been observed in two extremes of the disease spectrum, for example, cancer (too little apoptosis) and acute myocardial infarction (AMI; too much apoptosis). Using human cellular models and patient samples, we have previously shown that human apolipoprotein L6 (ApoL6), when overexpressed intracellularly, induces mitochondria- and reactive oxygen species (ROS)-mediated apoptosis. ApoL6 also blocks Beclin 1-initiated autophagy in both human colorectal cancer cells (DLD-1) and atherosclerotic lesion-derived cells. We speculated that small compounds enhancing ApoL6-induced apoptosis are candidate drugs to treat cancer. In the present study, we use our established human cellular models, high throughput and targeted screening strategies, and well-defined assays to identify nifedipine, L-proline, L-tryptophan, and picolinic acid as anti-apoptotic agents, which would be candidate drugs for treating diseases such as AMI. We also identified fulvestrant and L-lysine, two compounds that can further enhance ApoL6-induced apoptosis in cancer cells.

Introduction

Apoptosis, one of the major programmed and regulated cell death pathways, is a decidedly regulated and persistent suicide mechanism used for the elimination of damaged and unwanted cells in multicellular organisms. In general, apoptosis can be initiated extrinsically (through death receptors, e.g., Fas) or intrinsically through mitochondria or endoplasmic reticulum. Execution of apoptosis is dependent on caspases, a group of cysteine-activated aspartate-specific proteases. In general, activation of initiator caspases, such as caspase 9, is associated with mitochondria- and reactive oxygen species (ROS)-mediated apoptosis whereas activation of caspase 8 frequently mediates death receptor-mediated apoptosis. Derailed apoptosis has been observed in two ends of the disease spectrum, for example, cancer (too little apoptosis) and atherosclerosis (too much apoptosis). Cancer cells frequently possess defects in the genetic and biochemical pathways of apoptosis [1, 2].

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Colorectal cancer (CRC) is one of the most common cancer types, with an estimated 147,950 new cases and over 53,200 deaths during 2020 in the USA (http://www.cancer.org), and over 1.2 million new cases and 600,000 deaths worldwide (http://www.who.int). Anticancer treatment of CRC usually leads to cell death through apoptosis. A major cause of chemotherapy failure is the evolution of resistance to the apoptotic effects of anticancer drugs. Most patients with disseminated CRC die after their metastases become resistant to the available chemotherapeutic agents. Defective apoptosis and altered metabolism (in part through derailed/unhealthy autophagy) are two hallmarks of tumorigenesis and major components in cancer therapeutic resistance [3]. It is paramount, therefore, to use the genetically and biochemically well-established mechanisms involved in the restoration of apoptosis and the inactivation of autophagic survival in cancer cells to circumvent drug resistance. Conversely, atherosclerosis (ATH) is a multifactorial, chronic inflammatory disease and the principal cause of acute myocardial infarction (AMI) and stroke [4]. The progression of atherosclerosis results from a dynamic interplay involving inflammation, apoptosis, autophagy, and necroptosis in cardiovascular (CV) cells.

Mounting evidence suggests that chronic inflammation contributes to atherogenesis and the inflammatory infiltrate in human ATH lesions promotes atherogenesis by releasing proinflammatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF) and interleukin-1 (IL-1) that regulate apoptosis and autophagy of CV cells. Apoptosis initiated by inflammatory cytokines plays a pivotal role in the plaque rupture and thrombosis of atherosclerotic lesions, which can lead to AMI. Increasing evidence suggests that apoptosis plays a pivotal role in AMI because ischemia, the major cause of heart cell death, induces ROS generation and causes apoptosis [5-7]. It has been shown that up to 6 hours following the initial ischemia, most cell death occurs via apoptosis. After that, necrosis predominates, which is an irreversible process. Thus, it is believed that apoptosis happens prior to necrosis during most of the cell and tissue death, including AMI. In addition, it is known that when apoptosis occurs in smooth muscle cells (SMCs) in the thin fibrous cap of advanced lesions, the plaque is prone to rupture, thereby triggering thrombosis and myocardial infarction. Therefore, apoptosis is a major contributing factor to plaque instability and AMI, and the target of early AMI treatment. Blocking apoptosis in SMCs could prevent the devastating consequences of AMI. In fact, all cell types present in the heart can undergo apoptosis [4, 8, 9].

We recently demonstrated that apolipoprotein L6 (ApoL6) regulates apoptosis in IFNγ-initiated, Fas-mediated apoptosis in SMCs, causing plaque instability, and is a potential therapeutic target for treating atherosclerosis and therefore AMI. We showed that ApoL6 is highly expressed and partially co-localized with activated caspase 3 in activated SMCs in atherosclerotic lesions. In addition, overexpression of ApoL6 promotes ROS generation, caspase activation, and subsequent apoptosis, which can be blocked by pan caspase inhibitor and ROS scavenger DPI (Diphenylene iodonium). Knockdown of ApoL6 expression by siApoL6 suppresses INFγ- and Fas-mediated apoptosis. Furthermore, ApoL6 binds Bcl-XL, one of the most abundant anti-death proteins in LDCs (human atherosclerotic lesion derived cells)**,** initiating apoptosis [5, 10, 11]. To the best of our knowledge, ApoL6 is the first BH3-only protein that, when expression is upregulated, simultaneously promotes apoptosis and blocks autophagy, and therefore is an attractive target for treating atherosclerosis/AMI and cancer. It is becoming evident that healthy and regulated autophagy plays an important role in counteracting apoptosis [1, 2, 12].

In this study, we aimed to identify and characterize FDA-approved repurposing drugs, and amino acids and their metabolites that can block or enhance ApoL6-induced apoptosis in model cells. We used Tet-off, ApoL6-inducible (DLD-1.ApoL6) cells as the cell-based system of apoptosis in our high-throughput and targeted drug screening. The candidate drugs were further characterized in the blocking of ROS generation, caspase activation, and in the regulation of autophagic flux.

Materials and Methods

I Cell Lines and Reagents

DLD-1.ApoL6 cells, a previously established, Tet-off inducible cell line, were cultured in D.20 medium, comprised of McCoy's 5A supplemented with 10% FBS, 1X antibacterial antimycotic solution, 400 μg/ml G418, 250 μg/ml hygromycin B and 20 ng/ml Doxycycline (Dox; D.20). Cells were switched to induction medium, D.1 (same medium as D.20 but with 1 ng/ml Dox) when needed. All medium components were purchased from Gibco (Thermo Scientific) [10, 11]. A library of L-amino acids, their derivatives and their immediate metabolites was assembled using individual components purchased from Sigma-Aldridge.

II Prestwick Repurposing Drug Library

The Prestwick Chemical Library contains 1,200 small molecules with a high degree of drug-likeness, of which ~700 are previously FDA-approved drugs. The active compounds were selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans. The Prestwick Chemical Library was designed to reduce the risk of "low quality" hits, reduce the cost of the initial screening, and accelerate lead discovery. This library has been successfully used in screening antagonists of anti-apoptotic Bcl-2 family members at Center for Molecular Discovery at UNM [13, 14]. The compounds were initially dissolved in DMSO at concentration of 1 mM.

III A High-Throughput DLD-1.ApoL6 Cell-Based Platform

A 96-well high-throughput platform was developed according to our published protocol [10, 11]. In brief, 1×10^5 DLD-1.ApoL6 cells/well were seeded in 96-well plates in D.20 medium. The compounds were supplied as 1 mM DMSO stock solutions in 96-well plates and were added to the assay plates using a BioMek FXP robot (Beckman Coulter) equipped with a 96 channel pipetting head to transfer 2 μl of the sample to make the final concentrations of: first screening 10 μM; secondary screening 1 μM. After pretreating cells with compounds for 3 hrs, one group of plates with D.20 medium containing compounds was replaced with D.1 medium containing compounds and incubated for 8 hrs at 37 °C, 5% CO2, 95% humidity. Controls were treated with 0.1% DMSO, which was used as the solvent for the tested compounds. Compounds were graded by the degree to which they block the death of DLD-1.ApoL6 in D.1 medium, that is, by their ability to block ApoL6-induced apoptosis to 50% (EC50) compared to controls. All experiments were performed in triplicate.

IV Cytotoxicity Assays

Cell viability was determined using crystal violet staining and tetrazolium dye colorimetric test (MTT) assays. In brief, cells were seeded into 96-well plates and treated with drug in different concentrations. After 48-hr incubation, 20 µl MTT (5 mg/ml) was added into each well for 4-hr incubation and then 150 µl DMSO was added into each well in order to solubilize the blue-purple crystals of formazan afterwards. The absorbance was then measured using a BioTek Synergy H4 plate reader at 570 nm previously described [14]. EC50 was plotted and shown by a dotted line in order to divide these compounds into ±50% viability post-treatment. In addition, apoptosis was quantified morphologically by nuclear condensation and fragmentation using Hoechst-33342 staining as previously described [10]. Cell survival assays were also performed using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, G7570) according to the manufacturer's instructions.

V Assay of Caspase 9 Activation, p62, and LC3 by Immunoblotting

To prepare total cellular extracts, cells were rinsed twice with PBS and gently scraped off from the culture dish in PBS with a rubber policeman. Total cellular extracts were obtained by dissolving and sonicating cells in RIPA solution (1% sodium deoxycholate, 0.1% SDS, 1% triton X-100, 0.01 M Tris-Cl, pH 8.0, 0.14 M NaCl). To prepare cytosolic fractionation, cells were gently scraped off from the culture dish, centrifuged down at 200 g for 5 min, washed twice with 1.0 ml of cold PBS and resuspended in 200-300 µl of buffer A (250 mM sucrose, 20 mM HEPES-KOH (pH 7.4), 10 mM KCl, 1.5 mM Na-EGTA, 1.5 mM Na-EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and protease inhibitors. Cells were incubated on ice for 30 min and then disrupted by Dounce homogenizer. Homogenates were centrifuged at 800g for 10 min. Supernatants were further centrifuged at 22,000g for 15 min. The resulting supernatants were used for immunoblotting analysis. Protein concentrations were measured using the BCA protein assay reagent Kit (Pierce). Proteins were separated using 10 % SDS-PAGE (20 µg protein/lane) and transferred to PVDF membranes (Millipore) which were then incubated with polyclonal anti-ApoL6, anti-activated caspase-9 antibody (1:1,000) (Cat. # 9501S; Cell Signaling technology), anti-p62 (610832; BD Transduction Laboratory) anti-LC3 (MBL), and anti-β-actin (Oncogene Research Products). Subsequently, blots were incubated with goat anti-rabbit or anti-mouse HP-labeled secondary antibodies (1:5,000 for both) (Bio-Rad) [11, 15].

VI Assay of ROS Generation

A live cell, high-throughput, microplate-based ROS assay that utilized the cell permeable substrate, 2',7'-dichlorofluorescin diacetate, (DCF-DA) a reliable fluorogenic marker for ROS detection was used as previously described [10]. Upon ROS generation, the highly fluorescent dye, 2',7'-dichlorofluorescein is produced, with EX (excitation): 495 nm and EM (emission): 530 nm. In brief, DLD-1.ApoL6 cells were plated in 96-well tissue culture plates at a density of 100,000 cells/well and then loaded with DCF-DA at 20 µM final concentration for 30 min before treating with the indicated compounds at 2, 10 and 50 µM in D.0 for 8 hrs. Both end point and area-scan readings were taken using the Synergy H4 Plate Reader at 485 nm with emission at 528 nm using a 20 nm bandwidth, according to the manufacturer's instruction.

VII Statistical Analysis

Each test plate contains positive control wells (containing 1 µM DPI) and negative control wells (containing D.20 or D.1 medium only) was used to calculate a Z' score for the plates on the basis of assay signal, cell viability, in those wells. The Z' score is a measurement of scoring assay quality that reflects both assay signal dynamic range and data variation associated with the signal measurements. Data is presented as means ± standard deviation (SD) of n experiments/ samples. Z' scores less than 0.05 are considered significant.

Results

Using our well characterized cellular model, DLD-1.ApoL6, and a well-defined drug screening and assay workflow, we identified the drugs from the Prestwick and amino acid libraries that influenced ApoL6-induced apoptosis in DLD-1.ApoL6 cells (Figure 1) [10, 11]. First, we developed a cost-effective quantitative assay to monitor cell death in the 96 well-based system and utilized HTS drug screening platform to analyze the Prestwick library for blocking and/or enhancing apoptosis.

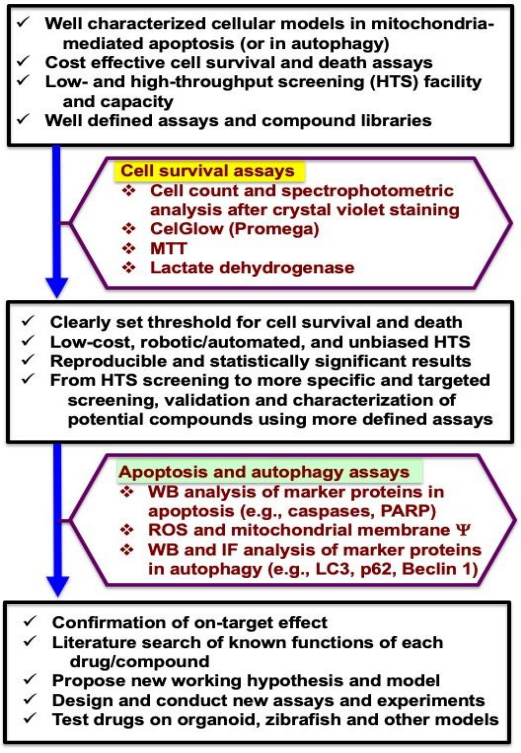

Figure 1: Schematic representation of the logics and models in screening, identification, and characterization of compounds that alter apoptosis.

After 3 rounds of HTS with a fixed drug concentration of 10 µM, we were able to identify doxycycline (Dox) and its analogue, tetracycline, from the library as inhibitors of ApoL6-induced apoptosis. Importantly, this result validated our screening system, because by design, in the presence of Dox the gene expression of ApoL6 is turned off so there is

no induction of cell death. It also demonstrated that apoptosis was indeed mediated through ApoL6 overexpression (Figure 2A). For a positive control, we have previously shown that ApoL6 overexpression promotes ROS generation and caspase 9 activation, subsequently causing apoptosis, which can be blocked by a ROS reducer, Diphenylene iodonium (DPI) [11]. Thus, we first confirmed that DPI protected DLD-1 cells from apoptosis induced by ApoL6 (well D11 in Figure 2B; DPI, Figure 2C).

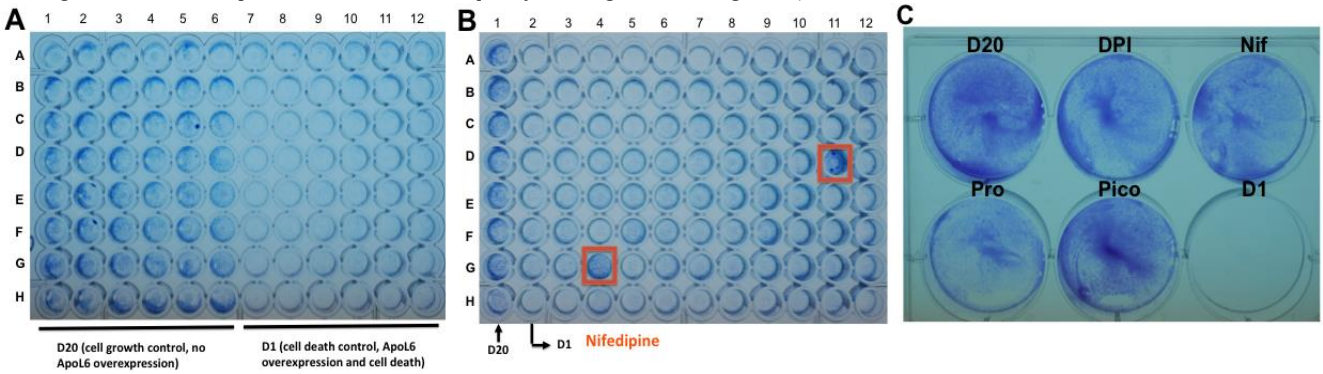

Figure 2: Establishment of drug screening platform and identification of small molecules that block ApoL6-induced apoptosis. **A)** Defined cell growth and death of DLD-1.ApoL6 cells in 96-well plates. Cells grown overnight were treated with D.20 or D.1 for 8 hrs and then stained with crystal violet solution (0.5%). D.20 was designated as the normal/control medium (positive control in term of cell growth), whereas D.1 was the induction medium, in which ApoL6 expression and cell death was induced. Columns 1 to 6 showed cell growth covering an average of 80% of the surface of each well. Columns 7 to 12 showed less than 10% of cells still covering/attaching to the surface of each well. **B)** Indicated drugs (10 μM each in initial screens) were robotically added to D.1 in columns 2 to 12. Cells were cultured for an additional 8 hrs and the attached/alive cells were stained, counted and assayed. **C)** DLD-1.ApoL6 cells were grown in a 6-well plate with the indicated treatments. DPI, Nif, Pro, and Pico were added in D.1 for 8 hrs.

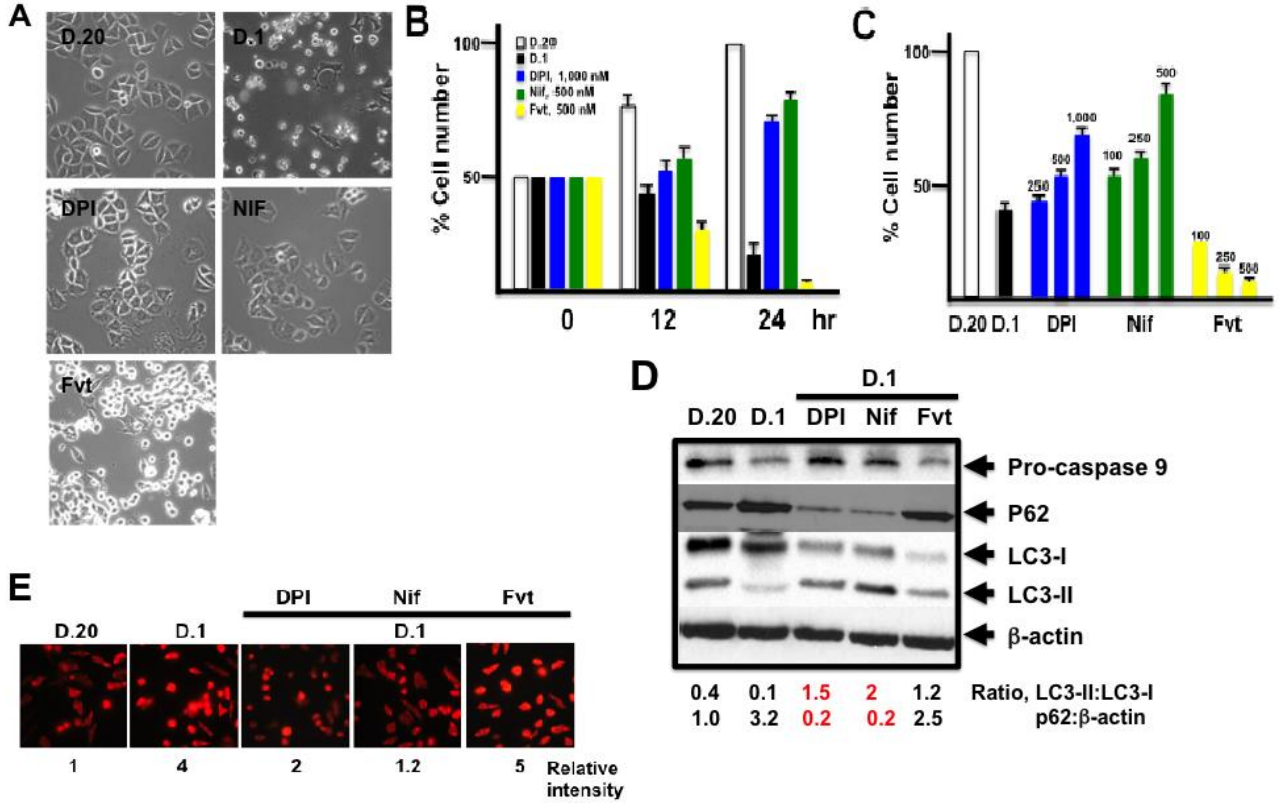

Figure 3: Effects of two approved drugs, Nif and fulvestrant (Fvt), on cell growth and autophagic flux in ApoL6-induced apoptosis in DLD-1 cells. **A)** Light microscopy analysis of DLD-1.ApoL6 cells grown in D.20, D.1, D.1 + 1μM Diphenylene iodonium (DPI), D.1 + .5 μM Nif (Nif), and D.1 + .5 μM Fvt (Fvt) for 8 hrs; **B)** Time-dependent treatment of DLD-1.ApoL6 cells in the indicated medium for 0, 12 or 24 hrs; **C)** Drug concentration-dependent treatment of DLD-1.ApoL6 cells in the indicated medium and concentrations; **D)** Western blot analysis of protein samples isolated from the cells of panel A. The same membranes were probed with the indicated primary antibodies raised against pro-caspase 9, p62, LC3, and β-actin. Ratios of LC3-II/LC3-I and p62/β-actin were also indicated. As shown, DPI and Nif induced autophagic flux; and **E)** Measurement of ROS generation of the cells indicated in panel A. DPI and Nif reduced, whereas Fvt increased, ROS generation in cells grown in D.1.

We then identified nifedipine (Nif) (Figure 2B & 2C), L-proline (Pro), L-tryptophan (Trp), and picolinic acid (Pico) (Figure 2C, Figures 3 & 4) as anti-apoptotic agents. In addition, we identified fulvestrant (Fvt), an anti-cancer drug, and L-lysine (Lys) can further enhance ApoL6-induced apoptosis in cancer cellsin D.20 using cell imaging and cell count assays (Figures 3 & 4), as indicated by > 64% of the growth of cells in D.20. We then conducted imaging and cell count assay demonstrating that DPI, NIF (Figure 3), Pro, Trp and Pico (Figure 4) protected DLD-1.ApoL6 cells from apoptosis in a concentration- and time-dependent manner (Figures 3 & 4), whereas Fvt and Lys caused more severe cell death in DLD-1.ApoL6 cells cultured in D.1 (Figures 3 & 4). We then evaluated the compounds according to their effects on caspase-9 activation (for apoptosis), p62 and LC3-II/LC3-I (for autophagic flux), and ROS generation (for apoptosis) in DLD-1.ApoL6 cells.

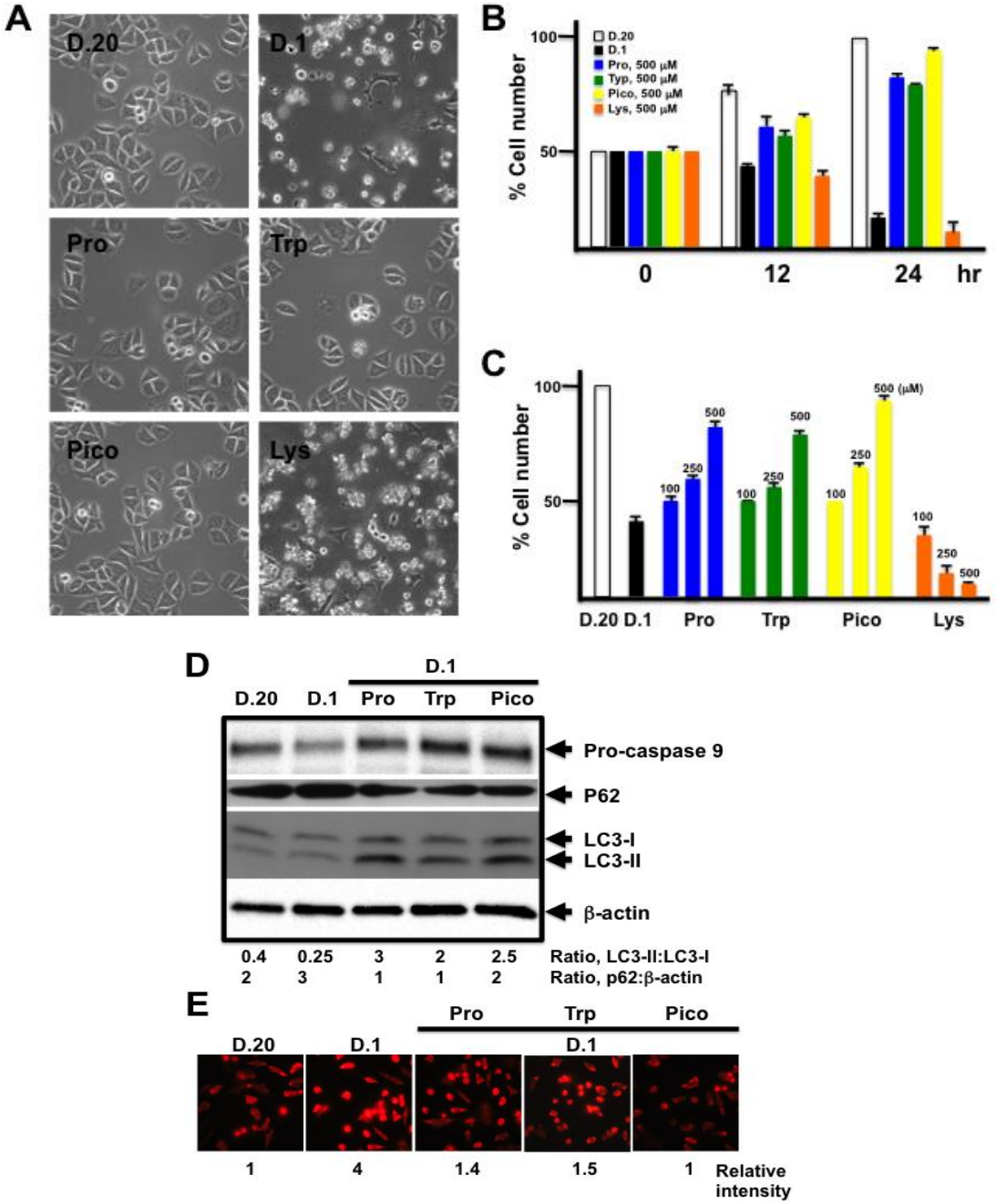

Figure 4: Effects of Pro, tryptophan (Trp), Pico, and lysine (Lys) on cell growth and autophagic flux in ApoL6-induced apoptosis in DLD-1 cells. **A)** Light microscopy analysis of DLD-1.ApoL6 cells grown in D.20, D.1, D.1 + .5μM Pro, D.1 + .5 μM Trp, and D.1 + .5 μM Lys for 8 hrs; **B)** Time-dependent treatment of DLD-1.ApoL6 cells in the indicated medium for 0, 12 or 24 hrs.; **C)** Drug concentration-dependent treatment of DLD-1.ApoL6 cells in the indicated medium and concentrations; **D)** Western blot analysis of protein samples isolated from the cells of panel A. The same membranes were probed with the indicated primary antibodies raised against pro-caspase 9, p62, LC3, and β-actin. Ratios of LC3-II/LC3-I and p62/β-actin were also indicated. Taken together, this set of results indicated that Pro, Trp, and Pico induced autophagic flux; and **E)** Assay of ROS generation in the cells indicated in panel A. Pro, Trp and Pico reduced ROS generation in cells grown in D.1.

Immunoblot analysis showed that D.1 induced caspase 9 activation (the level or pro-caspase 9 is lower than that of D.20), p62 upregulation and LC3-II/LC3-I ratio reduction, demonstrating cellular apoptosis through the blocking of autophagic flux (Figures 3D & 4D). In contrast, DPI, Nif, Pro, Trp and Pico showed reduced caspase 9 activation (the level or pro-caspase 9 is higher than that of D.20 and D.1), p62 downregulation and LC3-II/LC3-I ratio increasing, demonstrating blocking of apoptosis through the induction of autophagic flux (Figures 3D & 4D). Furthermore, Fvt and Lys showed even greater caspase 9 activation, p62 upregulation and LC3-II/LC3-I ratio reduction, indicating an additive effect on ApoL6-induced cell death in DLD-1. Measurement of ROS generation of the cells indicated that DPI, Nif, Pro, Trp, and Pico reduced RSO intensity, whereas Fvt and Lys increased ROS generation in cells grown in D.1 (Figures 3E & 4E).

Discussion

In the present study we identified and characterized small compounds that modulate both apoptosis and autophagy in our 2-D cellular model. The goal was to use these molecules to treat diseases with dysregulated programmed cell death. Previously, the lack of functionally-defined, apoptosis-Inducing cancer cell models for high throughput screening (HTS) is a bottleneck for drug identification and validation [16]. Now, we have developed a fine-tuned cell-based system to assay ROS and caspase-dependent apoptosis using DLD-1.ApoL6, a stably transfected, APOL6 "Tet-Off" inducible colorectal cancer DLD-1 cell line harboring full-length cDNA of wild-type APOL6. We demonstrated that induction of ApoL6 in DLD-1.ApoL6 cells had a marked effect on cell death by profound reduction of cell number after 8 hrs culture in D.1 induction medium. In contrast, DLD-1.ApoL6 cells grown in normal D.20 exhibited normal growth. ApoL6-initiated cell death is mediated in part through ROS generation [10]. In a low-throughput experiment using a 6-well plate, cell death was first assayed by two methods: cell count and MTT assay. As shown in (Figure 1), there was ~60% cell death of cells grown in D.1 (Figure 2A). DPI (diphenylene iodonium, 1 μM), an antioxidant and NAD(P)H oxidase inhibitor, as a control, blocked ApoL6-induced apoptosis (Figure 2C).

After all the controls have established, we showed that Nif, a repurposed drug, and Pro, Trp, and Pico are inhibitors of ApoL6-induced, mitochondria- and ROS-mediated cell death and are autophagy inducers in this study. We and others have previously shown that canonical/healthy autophagy mainly functions as a survival mechanism in various cell types, including those originating from human atherosclerotic lesions and tumors. In fact, we observed that ApoL6 overexpression not only induced apoptosis but also inhibited Beclin-1 dependent autophagy [11]. Thus, it is logical to speculate that increasing autophagy counteracts mitochondria-mediated apoptotic cell death and can be considered for use in treating AMI, a major consequence of endothelial and SMC apoptosis. Interestingly, Nif, a L-type calcium channel blocker and a drug which has been used to treat hypertension and has been shown to improve outcomes in patients with cardiovascular disease, exhibits *in vitro* efficacy in blocking apoptosis in our apoptosis model cell lines; our results take into consideration Nif as a potential repurposed drug to treat ATH in heart disease patients [16-18]. Consistently, induction of autophagy by Nif has been shown by other groups previously [19, 20].

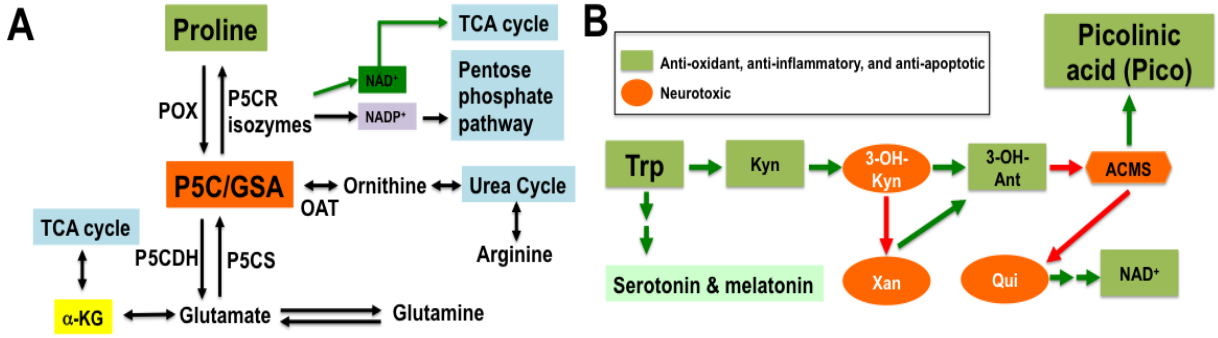

Figure 5: Pro, Trp, and their metabolites involve in energy metabolism and anti-oxidation. **A)** Proline metabolism in mammals involves four other amino acids, glutamate, ornithine, arginine, and glutamine, and 5 enzymatic activities, Δ1-pyrroline-5-carboxylate (P5C) reductase (P5CR), proline dehydrogenase/proline oxidase (PRODH/POX), P5C dehydrogenase (P5CDH), P5C synthase (P5CS), ornithine-δ-aminotransferase (OAT). With the exception of OAT, which catalyzes a reversible reaction, the other 4 enzymes are unidirectional, suggesting that proline-related metabolism is purpose-driven, tightly regulated, and compartmentalized. This five-amino-acid system also links with three other essential metabolic systems, namely the TCA cycle (through α-ketoglutarate (α-KG)), the urea cycle, and the pentose phosphate pathway (through NADP+). **B)** The Kynurenine pathway of tryptophan (Trp) catabolism plays an essential role in the generation of functional compounds that modulate a variety of biological functions. For example, Trp, kynurenine (Kyn), 3-hydroxy anthranilic acid (3-OH-Ant), and Pico are potent anti-oxidants, whereas 3-hydroxykunurenine (3-OH-Kyn), xanthurenic acid (Xan), A-amino-β-carboxymuconate-ε-semialdehyde (ACMS), and quinolinic acid (Qui) are neurotoxic.

Furthermore, our results are consistent with those reported for the biological functions of apoptosis and autophagy in cells and further emphasize the importance of research into the role of autophagy in the therapeutics of atherosclerosis [1, 2]. Pro metabolism in humans involves four other amino acids, glutamate, ornithine, arginine, and glutamine (Figure 5A). This five-amino-acid system also links with three other essential metabolic systems, namely the TCA cycle (through α-ketoglutarate (α-KG)), the urea cycle (through ornithine), and the pentose phosphate pathway (through NADP+), essential for energy production, detoxification and nucleic acid biosynthesis [21, 22]. Trp catabolism through the kynurenine (Kyn) pathway plays an essential role in the generation of functional compounds that modulate a variety of biological functions (Figure 5B). For example, Trp, Kyn, 3-hydroxy anthranilic acid (3-OH-Ant), and Pico are potent anti-oxidants [23, 24].

To further investigate the effect of these 4 small molecule compounds with respect to mechanism and efficacy means, we are in the process of developing 2-D, and 3-D spheroid models of LDC-ApoL6 cells and animal models such as zebrafish and the pig [25, 26]. We also plan to characterize and validate the combinational treatment of Nif, Pro and Pico individually and in combination of their activity and specificity in blocking apoptosis, promoting autophagic survival and mitigating inflammatory response using our DLD-1.ApoL6 and LDC-ApoL6 cell models.

Our screening also has identified compounds, such as fulvestrant (Fvt) and L-lysine (Lys), which enhance apoptosis. Fvt, also known as Faslodex, is a selective estrogen receptor downregulator/degrader (SERD) that binds, blocks and degrades the estrogen receptor (ER), leading to complete inhibition of estrogen signaling through the ER [25]. Fvt was introduced to clinical practice in 2002, initially with the indication to treat postmenopausal women with hormone-receptor-positive advanced breast cancer. Currently, the standard dose is 500 mg, which is administered with a loading dose. Fvt received a new FDA indication in December 2016, in combination with palbociclib, both in pre/peri/postmenopausal women with breast cancer progressing after endocrine therapy [27, 28]. We showed that Fvt individually enhanced ApoL6-induced apoptosis in DLD-1, a p53-null colorectal cancer cell line. This observation of a re-sensitization of cancer cells to chemotherapy-induced apoptosis supports the need for a detailed study of the mechanisms by which Fvt boosts mitochondria-mediated apoptosis, which in this study is initiated by ApoL6. Such knowledge is critical to formulating a multi-targeted chemotherapeutic treatment approach to re-sensitize and synergistically kill cancer cells.

Cancer is a heterogeneous, highly adaptive, and constantly evolving disease, driven in large part by the targeted therapeutic agents designed specifically to suppress the disease. The challenges of therapeutic intervention at any one of these stages is likely to necessitate a different strategy which may not only address the existing target(s) but those that emerge following mutations or the development of resistance. As DLD-1.ApoL6 cells are biochemically defined, p53-null, tumorigenic cell lines undergo mitochondria-mediated apoptosis when single-gene for ApoL6 is induced by the Tet-off process. We are in the process of using these cells to generate *in vitro* 3-D organoids and tumor xenografts in athymic mice to model drug resistance and treatment *in vivo* [25]. Interestingly, we have shown previously that ectopic expression of ApoL6 induces apoptosis, in part, via the release of cytochrome c along with Second Mitochondria-Derived Activator of Caspase (SMAC) from the mitochondria and the activation of caspases 8 and 9 [10]. SMAC is an antagonist of Inhibitors of Apoptosis (IAPs), which are crucial regulators of apoptosis as well as inflammatory-related necroptosis [29, 30]. Thus, SMAC and its mimetics may induce both apoptosis and necroptosis when caspases are inactivated in certain cellular context. Therefore, it is logical to speculate that ApoL6 in certain cell types under inflammatory stress might also regulate necroptosis. The further investigation is warranted [29, 30]. In fact, Murphy *et al.* have recently shown that ApoL6 can induce dichotomous cell death involving both apoptosis and necroptosis in various cell types under various physiological conditions [31].

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Conflicts of Interest

None.

Compliance with Ethical Standards

This article does not involve any human participants and animal work.

Abbreviations

ApoL6: Apolipoprotein L6
DPI: Diphenylene Iodonium
Fvt: Fulvestrant
LDCs: Atherosclerotic Lesion Derived Cells
Lys: L-Lysine
Nif: Nifedipine
Pro: L-Proline
Pico: Picolinic Acid
Trp: L-Tryptophan

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