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The histone deacetylase inhibitor cambinol prevents acidic pH_e-induced anterograde lysosome trafficking independently of sirtuin activity

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ABSTRACT

Common features of the solid tumor microenvironment, such as acidic extracellular pH and growth factors, are known to induce the redistribution of lysosomes from a perinuclear region to a position near the plasma membrane. Lysosome/plasma membrane juxtaposition facilitates invasion by allowing for the release of lysosomal proteases, including cathepsin B, which contribute to matrix degradation. In this study we identified the sirtuin 1/sirtuin 2 (SIRT1/2) inhibitor cambinol acts as a drug that inhibits lysosome redistribution and tumor invasion. Treatment of cells with cambinol resulted in a juxtanuclear lysosome aggregation (JLA) similar to that seen upon treatment with the PPARγ agonist, troglitazone (Tro). Like Tro, cambinol required the activity of ERK1/2 in order to induce this lysosome clustering phenotype. However, cambinol did not require the activity of Rab7, suggesting that this drug causes JLA by a mechanism different from what is known for Tro. Additionally, cambinol-induced JLA was not a result of autophagy induction. Further investigation revealed that cambinol triggered JLA independently of its activity as a SIRT1/2 inhibitor, suggesting that this drug could have effects in addition to SIRT1/2 inhibition that could be developed into a novel anti-cancer therapy.

1. Introduction

Metastatic disease accounts for ~90% of cancer-associated deaths. Invasion through the basement membrane allows tumor cells to access sites beyond the tissue of origin which is a precondition for metastasis. Although there are protease-independent mechanisms of invasion, it is widely accepted that protease activity greatly contributes to the invasive potential of tumor cells [1,2]. Specifically, the release of lysosomal cathepsins and matrix metalloproteinases are thought to contribute to tumor invasion by promoting degradation of the extracellular matrix. Lysosomes are acidic vesicles that normally function as degradative organelles at the endpoint of the endosomal pathway and are rich in hydrolases and proteases, including cathepsins B, D, and L. Several studies have shown that cathepsin expression is enhanced in invasive cancer cells and secreted cathepsin B can be found at sites of invadopodia formation, supporting the notion that release of lysosome proteases promotes an invasive phenotype [3–7].

Lysosomes traffic along microtubules and actin filaments via the activity of ATP-dependent kinesin and dynein motor complexes [8]. The position of lysosomes within cells can be influenced by extracellular stimuli, resulting in anterograde (plus end) or retrograde (minus end) trafficking. Tumor cells with lysosomes positioned closer to the plasma membrane secrete more proteases and are more invasive when compared to cells with lysosomes clustered near the center of the cell over the microtubule organizing center (MTOC) [9–13]. Several stimuli that are commonly found within the extracellular tumor microenvironment, such as growth factors and acidic pH, drive anterograde lysosome

Abbreviations: SIRT, sirtuin; NHE, sodium proton exchanger; Tro, troglitazone; JLA, juxtanuclear lysosome aggregation; EIPA, ethyl-isopropyl amiloride; LAMP-1, lysosome associated membrane protein 1; HDAC, histone deacetylase; pH_e, extracellular pH; MTOC, microtubule organizing center

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movement, which is critical for invasion [12–14]. In solid tumors, the extracellular pH (pH_e) can reach as low as 6.0 as a result of reduced blood flow, enhanced sodium hydrogen exchanger (NHE) activity, and anaerobic glycolysis (Warburg Effect) [15]. The acidic pH_e has been shown to enhance tumor invasiveness both *in vitro* and *in vivo* [16,17]. Acidic pH_e also stimulates anterograde lysosome trafficking and previous studies have found that this lysosome redistribution is necessary for cathepsin B release and tumor cell invasion in response to acidic pH_e, which is mediated by NHE activity [12]. We previously identified troglitazone (Tro), an antidiabetic PPARγ agonist, and ethyl-isopropyl amiloride (EIPA), an NHE antagonist, as novel inhibitors of anterograde lysosome trafficking. Tro treatment induces a juxtanuclear lysosome aggregation (JLA) in an ERK/Rab7/RILP-dependent manner and inhibits acidic pH_e and growth factor-mediated tumor invasion [10].

Previous studies strongly suggest that inhibition of anterograde lysosome trafficking and protease release could be therapeutically beneficial to slow tumor invasion [10–13]. Due to clinical toxicity associated with Tro treatment, we have begun to use a high-content imaging approach to screen for compounds that also prevent anterograde lysosome trafficking (manuscript in preparation). Our collaborators previously characterized Sirtuin 1 (SIRT1), one of seven class III histone deacetylase (HDAC), as a major contributor to cancer cell migration and invasion [18,19]. Sirtuins are NAD+ dependent deacetylases, are the mammalian homologs of yeast SIR2 (silent information regulator 2), and have many histone and non-histone targets [20]. As sirtuins are often upregulated in cancer, a number of inhibitors have been generated to facilitate the study of the role of individual sirtuins in disease. Cambinol is a β-naphthol compound that is thought to specifically inhibit the deacetylase activity of SIRT1/2. It is well tolerated in mice and has potent antitumor activity *in vivo* [21]. To assess the role of SIRT1/2 activity in cancer cell lines, our collaborators performed a microarray analysis to characterize the gene expression changes between control-treated and cambinol-treated cells (unpublished data) and found that Rab7 mRNA was overexpressed in cambinol treated cells. Rab7 is a small GTPase that specifically localizes to lysosomes, recruits dynein motors to lysosomes *via* its effector RILP, and drives JLA, resulting in decreased tumor invasion [11,22].

The upregulation of Rab7 in response to cambinol treatment suggests that sirtuins may play a role in lysosome trafficking. The goal of this study was to determine the effects of cambinol treatment on lysosome positioning. We found that cambinol was a potent inhibitor of acidic pH_e-mediated anterograde lysosome trafficking. In addition, we showed that the JLA phenotype occurs independently of Rab7 or changes in gene expression, highlighting the distinct differences in mechanism of action compared to Tro. Additionally, we found that cambinol induces JLA independently of its activity as a SIRT1/2 inhibitor, suggesting that cambinol may have effects on other cellular targets. The ability to alter lysosmal trafficking makes cambinol a viable therapeutic agent for slowing tumor progression.

2. Materials and methods

2.1. Cell culture

The human prostate cancer cell lines DU145 ATCC (Manassas, Va) and PPC1 (a generous gift from Dr. Brianna Williams, LSU-Health Shreveport, originally obtained from Brothman [23]) were maintained in RPMI 1604 with 10% FBS and 1% Penicillin–Streptomycin. The human glioma cell line A127 (ATCC) was maintained in DMEM with 10% FBS and 1% Penicillin–Streptomycin. All cells were cultured at 37 °C and 5% CO_2 and passaged upon reaching 80% confluence. For experiments involving treatment with acidic media, cells were treated with RPMI 1604 containing 130 mM NaCl and 10 mM $NaHCO_3$+2% FBS. The pH was adjusted to 6.4 prior to the start of the experiment. SIRT1 and NonTarget shRNA expressing cells were maintained under 3.6 μg/mL Puromycin (Fisher, Waltham, MA).

2.2. Reagents and antibodies

Cambinol, Actinomycin D, TSA, Rapamycin, and Nicotinamide were purchased from Sigma (St. Louis, MO). AK1 and AGK2 were purchased from ChemBridge (San Diego, Ca). EX527 was supplied by Selleck Chemicals (Houston, TX). UO126 was purchased from Calbiochem (Billerica, MA) and used at 10 μM. LAMP-1 antibody (h4A3) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and used at a 1:200 dilution. GM130 and EEA1 antibodies (BD Biosciences, San Jose, CA) were used at 1:50. Mitotracker Red (Molecular Probes, Carlsbad, CA) was used according to the manufacturer's protocol. The SIRT1 (B-10) and Ac-tubulin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX) and was used at 1:1000. pMEK1/2 S271/221, p70 S6 Kinase Thr389, pErk1/2 T202/Y204, Ac-p53 Lys382, LC3I/II, and total Erk1/2 antibodies (Cell Signaling Technology, Beverly, MA) were used at 1:1000 for western blot. Anti-tubulin antibody (NeoMarkers, Fremont, CA) was used at 1:100 for immunofluorescence (IF) and 1:20,000 for western blot analysis. Secondary antibodies include Dylight 594 donkey anti-mouse 1:100 (Jackson IR, West Grove, PA) for immunoflorescence, and HRP-conjugated anti-mouse and anti-rabbit for western blot (GE Healthcare, Pittsburgh, PA) used at 1:5000. Phalloidin 488 (Invitrogen, Carlsbad, CA) was used at 1:200.

2.3. Immunofluorescence

Cells were seeded at 50% confluence on 22 mm coverslips in 6-well dishes. Following experimental treatment, cells were fixed in 4% PFA (pH 7.2) for 20 min at room temperature. Cells were then washed once with Phosphate Buffered Saline (PBS) (Corning, Manassas, VA) and then incubated with primary antibody for one hour at room temperature in BSP (0.25% bovine serum albumin and 0.1% Sapponin in PBS). Cells were washed twice with PBS then incubated for 1 h with secondary antibody in BSP for one hour at room temperature, protected from light. Cells were washed once with PBS, and then incubated with phalloidin in BSP for 20 min at room temperature protected from light. Cells were washed three times with PBS and coverslips were mounted using DAPI with SlowFade gold anti-fade reagent (Invitrogen). For LC3-GFP-mCherry IF, cells were fixed for 7 min in ice-cold methanol, washed with PBS, and coverslips were mounted using DAPI with slow fade gold reagent. Images were taken on a 40× (Olympus UPlanFl 40×/0.75) or 60× (UPlan Apo 60×/0.90) objective using an Olympus BX50 microscope, Roper Scientific Sensys Camera, and MetaView software. Images were pseudocolored and merged using ImageJ.

2.4. Western blot analysis

Cell lysates were harvested in boiling Laemmli buffer containing 2% β-mercaptoethanol and then boiled for five minutes. SDS-PAGE was performed loading equal volume of each sample. Protein was transferred to PVDF membrane, blocked in 5% milk in TBST and incubated with the indicated antibodies overnight at 4 °C. Anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare) were diluted at 1:5000 in 5% milk TBST. Membrane was developed using Pierce ECL 2 Western Blotting Substrate (Thermo) and Premium Blue X-Ray Film (Phenix). Representative blots are shown.