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Identification of endoplasmic reticulum stress-inducing agents by antagonizing autophagy: a new potential strategy for identification of anti-cancer therapeutics in B-cell malignancies

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Abstract

The endoplasmic reticulum (ER) plays a vital function in multiple cellular processes. There is a growing interest in developing therapeutic agents that can target the ER in cancer cells, inducing a stress response that leads to cell death. However, ER stress-inducing agents can also induce autophagy, a survival strategy of cancer cells. Therefore, by inhibiting autophagy we can increase the efficacy of the ER stress-inducing agents. Nelfinavir, a human immunodeficiency virus (HIV) protease inhibitor with anti-cancer properties, can induce ER stress. Nelfinavir's effects on chronic lymphocytic leukemia (CLL) are yet to be elucidated. Herein we demonstrate that nelfinavir induces ER morphological changes and stress response, along with an autophagic protective strategy. Our data reveal that chloroquine, an autophagy inhibitor, significantly increases nelfinavir cytotoxicity. These results identify a novel strategy potentially effective in CLL treatment, by repositioning two well-known drugs as a combinatorial therapy with anti-cancer properties.

Keywords

Nelfinavir; autophagy; ER stress; drug screening; chronic lymphocytic leukemia

Introduction

Cancer remains a major cause of death in the United States, and therefore represents a major area of need for new therapeutics. A recent cancer therapeutic target that has generated

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Supplementary material available online
Supplementary Figure and movies showing additional results

interest has been the unfolded protein response (UPR), which is activated under conditions of endoplasmic reticulum (ER) stress [1,2]. One downstream consequence of the activation of ER stress is the induction of autophagy [3]. Utilizing primary tumor cells from patients with chronic lymphocytic leukemia (CLL), we recently demonstrated that ER stress induced by either thapsigargin or flavopiridol promoted ER stress with modest cytotoxicity but also autophagy [4]. The antagonism of autophagy in this setting using chloroquine or siRNA directed at ATG5/ATG7 greatly enhanced tumor cell killing. These findings support autophagy as a primary mechanism of resistance to ER stress-inducing agents in CLL. It is noteworthy that other kinase inhibitors used in the clinic for hematologic malignancies also promote ER stress, and their efficacy is enhanced by agents that antagonize autophagy [5]. Based on this, we hypothesized that therapeutics that may produce ER stress would be better identified when co-administered with an inhibitor of autophagy. To test this hypothesis prior to embarking on a large screening effort, we pursued this strategy with a known therapeutic already in the clinic for a non-cancer indication. This strategy is termed repositioning, and offers the potential to rapidly transition therapeutics to clinical practice for a new indication. Nelfinavir (NFV) is a common human immunodeficiency virus (HIV) protease inhibitor approved by the US Food and Drug Administration (FDA) for therapeutic use in patients with HIV in 1997, with very good tolerability. Recently, NFV has been shown to have potential activity as an anti-cancer agent in several solid tumors, in part through mediation of ER stress [6 – 8]. Herein, we utilized NFV to determine whether the addition of chloroquine, an agent that prevents lysosomal degradation of the autophagosomes, results in enhanced killing [9,10]. Our data demonstrate that treatment with NFV alone does not result in significant viability loss, whereas co-treatment with chloroquine induces significant cytotoxicity. Collectively these results suggest that novel screening of both approved and unapproved therapeutics suspected of mediating ER stress in combination with chloroquine may enhance the identification of promising therapeutic agents.

Materials and methods

Patients, cell separation, culture conditions and reagents

Cells from patients with previously diagnosed CLL as defined by the modified National Cancer Institute (NCI) criteria [11] were obtained with written, informed consent. CD19-positive cells from patients with CLL and normal volunteers were selected and maintained in culture as previously described by our group [12]. Rapamycin was purchased from EMD-Calbiochem. NFV, chloroquine, thapsigargin, tunicamycin, flavopiridol and F-ara-A (9- β -D-arabinofuranosyl-2-fluoroadenine 5'-phosphate) were obtained from Sigma-Aldrich.

Confocal immunofluorescence microscopy of fixed cells

Cells were incubated 6h with the reagents and next washed in phosphate-buffered saline (PBS) and adhered to a microscope slide by centrifugation in a Cytospin3 (Shandon) centrifuge. Immediately after cytopsin, cells were fixed in cold acetone. Next, the cells were incubated in blocking solution (2% bovine serum albumin in PBS) and stained for LC3, calnexin (Cell Signaling) and Lamp2 (Santa Cruz Biotechnology) by incubating separately with the respective primary antibodies overnight, at 4°C, followed by incubation with fluorescent secondary antibodies (Invitrogen): AlexaFluor 594 (red) anti-rabbit for LC3 and calnexin and AlexaFluor 488 (green) anti-mouse for Lamp2. Nuclei were stained blue with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories). Images were collected with a $\times 60$ objective and $\times 4$ digital zoom using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope at the Ohio State University Campus Microscopy and Imaging Facility.

Quantification of immunofluorescence data

Integrated intensity was measured using Metamorph v.7.0. To calculate the number of “dots” and to quantify colocalization, we used a method previously described by our group [4].

Live cell imaging and quantification

CLL cells were seeded in compartmentalized, CELLview glass bottom, 35 mm dishes (Greiner Bio-One GmbH) that had previously been treated with concanavalin A (Sigma-Aldrich). Approximately 3h prior to live cell imaging, cells were seeded at a density of 3×10^5 cells/well, in low serum OptiMEM medium (Invitrogen) containing 1 μ M ER-Tracker Red (Invitrogen) and dimethylsulfoxide (DMSO) only (vehicle control), NFV (5.2 μ M), thapsigargin (1 μ M) or F-ara-A (5 μ M). An image series through the cells (z-stack) was acquired sequentially using an excitation wavelength of 561 nm every 30 min for a period of 12h for each treatment group. Images were acquired using a Visitech Infinity3 multibeam, confocal scanner equipped with a Hamamatsu C9100 EM-13 camera and an Olympus IX81 microscope using a $\times 100$, 1.40 NA UPlanApo objective lens. Cells were maintained at 37°C in a 5% CO₂, highly humidified atmosphere during imaging. All images were captured using identical camera settings. Movies were produced from each z-stack image series using MetaMorph software (Ver. 7.7.6.0; Molecular Devices). Image intensity analysis of image stacks from each treatment group was performed using MetaMorph software. Integrated fluorescence intensity values were adjusted to compensate for the variability between treatment groups (i.e. cell number per well, differences in amount of dye added per well). Standardization of the data was accomplished by dividing the fluorescence intensity values at each time point by the value at time 0.

Immunoblot analysis

Proteins were extracted from whole cell lysates (50 μ g/lane), separated on polyacrylamide gels and transferred onto a nitrocellulose membrane. Antibodies used for immunoblots included LC3, p62/SQSTM1, Grp78 (Cell Signaling) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Santa Cruz Biotechnology). Species-appropriate secondary antibodies were obtained from BioRad Laboratories. Following antibody incubations, proteins were detected using a chemiluminescent substrate (BioRad Laboratories). Quantification was done using FluorChem Q (Alpha Innotech).

Cell viability

After exposure to agents, cells were washed with PBS and stained with annexin V – fluorescein isothiocyanate and propidium iodide (PI) diluted in 1 $\times$ binding buffer (BD Biosciences). Cell viability was assessed by flow cytometry using a Beckman-Coulter Cytomics FC500 cytometer. Data were analyzed with the CXP Cytometer software package (Beckman-Coulter). A total of 10 000 cells were counted for each sample. For all conditions, cells were incubated with the respective drugs for 24h or 48h continuously.

Real-time quantitative polymerase chain reaction

RNA was extracted using TRIzol reagent (Invitrogen) and purified using Qiagen RNeasy columns (Qiagen). cDNA was prepared with a SuperScript First-Strand Synthesis System (Invitrogen). Real-time quantitative polymerase chain reaction (RT-PCR) was performed using primers purchased from Applied Biosystems (IRE1 Hs00176385_m1, CHOP Hs00358796_g1, XBP1 Hs02856596_m1, GRP78 Hs99999174_m1). Detection was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). Average relative expression (treatment compared with vehicle) was normalized to the internal control gene TBP (Applied Biosystems; 4326322E-1010010).

Statistical analyses

For assessments of the interaction between NFV and chloroquine (CQ) on autophagy related protein intensity, outcomes were natural log-transformed to stabilize variability among conditions and mixed effects models were then applied to the data. Linear mixed effect models were also used to test for significant interactions between NFV and CQ on CLL cell viability and to test for NFV effects on ER stress-related gene expression after the data were normalized to internal controls. Main effects and differences were estimated from this model. Holm's procedure was used to control for multiple comparisons. *p*-Values of less than 0.05 were considered significant for single comparisons or after adjustment for multiple comparisons. All analyses were performed using SAS/STAT software, Version 9.2 (SAS Institute, Inc).

Results

Nelfinavir induces increase of ER stress markers in CLL primary cells

Previous work by our group has shown that ER stressinducing agents such as thapsigargin and flavopiridol promote autophagy in CLL cells that is protective against cell death. In this setting, antagonizing autophagy was shown to enhance cytotoxicity of both thapsigargin and flavopiridol. Given that the majority of new drug screens are performed with a viability assessment, we hypothesized that co-culture with an autophagy-inhibiting agent might enhance the identification of novel modest ER stressinducing agents. To test this hypothesis, we initially used the HIV protease inhibitor NFV, which has been shown to induce ER stress in other systems. We incubated CLL primary cells from 14 patients with NFV (at concentrations equivalent to plasma levels attained in patients with HIV) for 6h and examined levels of gene expression for four ER stress markers: *XBPI*, *IRE1*, *GRP78* and *CHOP* using RT-PCR analysis [Figures 1(A) – 1(D)]. We observed statistically significant ($p = 0.0001 - 0.0005$) elevated levels of *XBPI*, *IRE1*, *GRP78* and *CHOP* in CLL cells treated with NFV 5 μ M and 10 μ M; NFV 1 μ M did not induce a significant increase in these ER stress markers ($p = 0.1652$ for *XBPI*, $p = 0.7226$ for *IRE1*, $p = 0.1104$ for *GRP78* and $p = 0.6991$ for *CHOP*). All samples showed a much more dramatic increase in the presence of thapsigargin, which can induce cell death through activation of caspases downstream of *CHOP* [13,14]. Thapsigargin was used as control for ER stress induction; it inhibits the SERCA (sarcoplasmic/endoplasmic reticulum calcium – ATPase) pump, which causes calcium release from ER into the cytoplasm, thus perturbing calcium homeostasis. Cells from patients with CLL were used also to perform immunoblots for Grp78 (immunoblot shown is representative of three experiments); we observed an increase in Grp78 protein levels with NFV [Figure 1(E)], consistent with results from RT-PCR analysis [Figure 1(C)]. CLL cells were treated for 4, 8 and 12h with vehicle (DMSO), F-ara-A (5 μ M), flavopiridol (2 μ M), NFV (1, 5 or 10 μ M), thapsigargin (1 μ M) or tunicamycin (2 μ g/ μ L). Tunicamycin was used as an additional positive control; it induces ER stress by inhibiting N-linked glycosylation of lipids and tyrosine incorporation, a different mechanism from thapsigargin and flavopiridol, which act through disturbance of calcium homeostasis. Collectively, these data demonstrate that NFV does mediate ER stress in CLL cells, however at a modest level when compared to the thapsigargin control.

ER stress induced by nelfinavir associates with morphological changes in CLL cells

Time lapse live cell imaging of primary CLL cells revealed an increase in the fluorescence intensity of ER Tracker Red (Invitrogen) as well as formation of ER Tracker Red positive vesicular structures in endoplasmic reticulum after 4h of NFV treatment. The vesicles were visible in cells treated with thapsigargin, a classic ER stress inducer, used here as a positive control (see movies A: vehicle, B: NFV, C: thapsigargin, D: F-ara-A and quantification in Supplementary Data, Supplementary Figure 1 to be found online at <http://>

informahealthcare.com/1al/doi/10.3109/10428194.2013.781168). The quantification of live cell microscopy data revealed a higher accumulation of the ER Tracker Red positive structures in the endoplasmic reticulum in the presence of NFV as compared to thapsigargin, suggesting that the morphological changes induced by NFV might not be strictly related to the level of ER stress induced in the CLL cells. Additional CLL samples would be needed to further confirm and understand the nature of the changes observed in the ER structure. The morphological changes observed by live cell microscopy are consistent with previous studies which have shown that ER stress induces swelling of the endoplasmic reticulum as well as dysfunction of ER adenosine triphosphate (ATP)-sensitive potassium channels [6,15,16]. Next we performed confocal immunofluorescence microscopy for fixed CLL cells to determine whether treatment of cells with NFV results in an accumulation of calnexin protein, which would indicate an increase in the level of misfolded proteins within the ER lumen. Cells were also probed for Lamp2, a marker for lysosomes. As with thapsigargin treated CLL cells, we observed an accumulation of calnexin in a time dependent manner in cells treated with NFV, suggesting an accumulation of misfolded proteins inside the lumen of endoplasmic reticulum caused by ER stress (Figure 2). We co-stained cells for Lamp2 together with calnexin to test whether exposure to NFV might induce degradation of endoplasmic reticulum through the autophagy pathway, previously described as endoplasmic reticulumphagy or reticulophagy [17,18]. We observed relatively small areas of co-localization between calnexin and Lamp2 that could suggest endoplasmic reticulum fragments were engulfed into autophagosomes and consequently into lysosomes.

Nelfinavir induces autophagy in CLL primary cells

Given the finding of ER stress in CLL cells following NFV treatment, we hypothesized that autophagy may also be induced with this treatment. Indeed, previous studies have shown that NFV treatment results in accumulation of autophagosomes in other cancer cell lines [19]. To determine whether autophagy is induced following NFV treatment in CLL cells, confocal microscopy was used to detect an increase in LC3, a common marker for autophagosomes. We observed a significant accumulation of autophagosomes after 4h of incubation [as shown in Figure 3(A) and quantified in Figures 3(B) and 3(C)]. Autophagosome accumulation was further increased by treating cells with a combination of NFV and chloroquine. Lamp2 was examined as a marker of lysosomes. During regular autophagy flux, autophagosomes and lysosomes fuse together, allowing the autophagosome cargo to be digested by the lysosomal enzymes [20]. The co-localization of LC3 and Lamp2 in the presence of NFV indicates that this agent does not inhibit autophagosome – lysosome fusion and degradation, as seen with chloroquine, an inhibitor of late stages of the autophagy process. We observed an inhibition of LC3 – Lamp2 co-localization in the presence of chloroquine, confirming the efficacy of the chloroquine concentration used, equivalent to the plasma concentration attained in patients with malaria. LC3 is an intracytoplasmic protein required for the formation of autophagosomal membranes. LC3 is cleaved by Atg4 protease, resulting in LC3I (cytosolic), with a glycine residue at the C-terminus. The C-terminus is next conjugated with phosphatidylethanolamine, yielding LC3II, which localizes on both sides of the autophagosome membrane [21]. The LC3 antibody used for immunofluorescence detects both LC3I and LC3II; however, LC3II is the form visible by fluorescence confocal microscopy and appears as punctae distributed throughout the cytoplasm. To confirm that the autophagy flux is not hindered by NFV, we performed immunoblot analysis using CLL cells that were exposed to the drugs for 24h [Figure 3(D)]; at this late time point, the autophagosomes formed in the presence of NFV and rapamycin should be degraded by fusion with the lysosomes. However, there should be an accumulation of autophagosomes in the presence of chloroquine due to the inhibition of autophagosome degradation. Analysis of LC3 by immunoblot revealed two bands: LC3I (18 kDa) and LC3II (16 kDa); the amount of LC3II correlated with the number of

autophagosomes. We observed a thick LC3II band in the presence of NFV + CQ and rapamycin + CQ (rapamycin is a common autophagy inducer, used here as a positive control) due to chloroquine, which inhibits autophagosome degradation through the lysosomal pathway [9]. These data confirm that NFV does not inhibit autophagy, and therefore the NFV-induced accumulation of autophagosomes observed by immunofluorescence at this early time point (4 h) is due to autophagy stimulation and not due to inhibition of autophagosome degradation. To further test whether the autophagosome accumulation observed in the presence of NFV is due to induction of autophagy and not due to inhibition of autophagosome degradation, we performed immunoblot analysis for p62/SQSTM1 protein, a protein degraded by the autophagy pathway and a useful tool in examining autophagy flux [22]. As shown in Figure 3(E), p62/SQSTM1 does not accumulate with NFV treatment alone and accumulates only when chloroquine is added. These data suggest that the autophagosome accumulation observed with NFV is due to an increase of autophagosome formation and not due to inhibition of their degradation.

Nelfinavir does not mediate direct cytotoxicity in CLL primary cells unless autophagy flux is inhibited by chloroquine

Given that NFV mediates a modest ER stress response and also autophagy in CLL cells, we hypothesized that direct cytotoxicity with this agent alone would be modest. To examine this, we assessed CLL cell viability following exposure to NFV. The reported concentration range of NFV in HIV-positive patients receiving this for treatment is 5.2 – 10 μ M [7]. CLL cells were therefore treated *in vitro* with NFV at concentrations of 1, 5.2 and 10 μ M for 24 and 48 h. As shown in Figure 4, we observed no significant decrease in cell viability in the presence of NFV alone. Given the protective role of autophagy in ER stress-mediated killing in CLL cells, we hypothesized that co-treatment of CLL cells with an inhibitor of autophagy flux would enhance the cytotoxicity observed with NFV. As shown in Figure 4, while NFV and chloroquine alone do not promote cytotoxicity, we observed a significant decrease in cell viability in the presence of the NFV – chloroquine combination.

Collectively, these data provide support for broadening screening of prospective ER stress-inducing agents with inhibitors of autophagy as a strategy to enhance the identification of structural leads that can be modified to enhance potential cytotoxicity against cancer cells.

Discussion

Herein, we have demonstrated a potential method to enhance the identification of therapeutic agents mediating ER stress in primary CLL cells. Previous work by our group using two ER stress-inducing agents, flavopiridol and thapsigargin, demonstrated prompt induction of autophagy, which is protective against CLL cell death. As most cancer screening assays use death or diminished proliferation as a successful read-out, we hypothesized that agents inducing modest ER stress with compensatory autophagy may not be identified as promising therapeutics using this approach. As such, screening of ER stress-inducing agents for cytotoxicity is done both with new targeted molecules and also for screening re-purposing compounds; the relevance of this could extend to different types of therapeutics. We utilized the anti-HIV drug NFV administered at physiologic concentrations to test this hypothesis, as it has been shown to mediate ER stress and also autophagy in other cancer systems. We confirmed that NFV also mediates modest ER stress and autophagy in CLL cells. Notably, NFV did not mediate direct cytotoxicity against CLL cells as a single agent. However, co-treatment with NFV and chloroquine dramatically induced direct killing of CLL cells *in vitro*.

Collectively, these data provide preliminary support for inclusion of a secondary screen using an autophagy inhibitor when studying small molecules that mediate ER stress as a

potential mechanism of cytotoxicity. For more limited libraries of compound screening such as has been applied with re-purposing compounds that are already FDA approved, inclusion of an autophagy-inhibiting molecule also appears to be supported.

Repositioning of old drugs to create new strategies for incurable malignancies is both time-saving and cost-reducing. This is particularly true when both therapeutics are off-patent and can be administered for low cost as an oral therapy. While many new therapeutics are coming forward for the treatment of CLL, their cost in general is prohibitive for many patients around the world outside of developed countries. The preliminary assessment of a known ER stress-inducing agent NFV using a secondary screen with chloroquine, where enhanced cytotoxicity is noted, provides justification for broadening this to a full examination of all compounds currently approved for clinical use. In addition, our data demonstrating that the combination of NFV and chloroquine has synergy against CLL cells provides rationale for moving this combination forward as a potential treatment of this disease. Both NFV and chloroquine are already approved for use in non-cancer indications and in general are well tolerated. In addition, these two agents have been administered together in populations diagnosed with both HIV and malaria with documented safety (patent 7553844 approved by US Patent and Trademark Office [23]). Based upon these data, we have begun to move forward with a combination trial of these two agents in CLL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Nelfinavir induces increase in ER stress markers. (A – D) Quantitative RT-PCR for ER stress marker genes (A) *XBPI* ($p = 0.0005$), (B) *IRE1* ($p = 0.0001$), (C) *GRP78* ($p = 0.0001$), (D) *CHOP* ($p = 0.0001$) in CLL cells ($n = 14$). Each symbol represents a sample from a patient with CLL; horizontal lines represent the median value. Cells were treated *in vitro* for 6h with vehicle (DMSO), NFV 1, 5, 10 μ M, thapsigargin 1 μ M and F-ara-A 5 μ M. (E) Immunoblot for Grp78 (78 kDa); primary CLL cells were treated *in vitro* for 4, 8 or 12h with vehicle (DMSO), F-ara-A 5 μ M, flavopiridol 2 μ M, NFV 1, 5, 10 μ M, thapsigargin 1 μ M, tunicamycin 2 μ g/ μ L. The immunoblot shown is representative of three experiments performed on cells from three patients with CLL. GAPDH was used as loading control.

Figure 2.

Nelfinavir induces endoplasmic reticulum morphological changes (A – E). Confocal fluorescence microscopy for calnexin (red: AlexaFluor 594) and Lamp2 (green: AlexaFluor 488) in primary CLL cells; CLL cells were incubated with vehicle, nelfinavir 5 μ M, thapsigargin 1 μ M and F-ara-A 5 μ M for 2, 4, 6, 8, 24 h. DAPI (blue) was used to stain nuclei. Images were collected with a $\times 60$ objective and a $\times 4$ optical zoom. Shown is one result representative of five experiments (five patients with CLL).

Figure 3.

Nelfinavir induces autophagy in CLL cells. (A) Confocal fluorescence microscopy in fixed CLL cells for LC3 (red: AlexaFluor 594) and Lamp2 (green: AlexaFluor 488). DAPI (blue) was used to stain the nuclei. Cells were incubated 6h with vehicle (DMSO), NFV 5 μ M, NFV 5 μ M + chloroquine 1 μ M, rapamycin 5 μ M, rapamycin 1 μ M + chloroquine 1 μ M. (B) Quantification of LC3 fluorescence intensity, $n = 5$. $p < 0.0001$. Average integrated intensity relative to the number of cells per field was measured in five microscopic fields per slide, in cells from five patients with CLL. (C) Quantification of LC3 – Lamp2 co-localization, $n = 5$. $p < 0.0001$. (D) Immunoblot for LC3I and LC3II in CLL cells. GAPDH was used as loading control. Shown is one immunoblot representative of three experiments. (E) Immunoblot for p62/SQSTM1 in CLL cells. GAPDH was used as loading control. Shown is one immunoblot representative of three experiments.

Figure 4.

Nelfinavir cytotoxicity is increased by inhibiting autophagy. Viability in CLL cells, $n = 10$, was measured by flow cytometry in cells stained with annexin V and PI. Values were normalized to vehicle. Cytotoxicity for NFV and CQ alone is not statistically significant relative to vehicle ($p > 0.3$); cytotoxicity for all three NFV – CQ combinations is significant ($p < 0.001$).