Title

Niemann-Pick C1 protein facilitates the efflux of the anticancer drug daunorubicin from cells according to a novel vesicle-mediated pathway

Authors and Affiliations

Yuping Gong, Muralikrishna Duvvuri, Michael B. Duncan, Jian Liu and Jeffrey P. Krise

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence KS 66047 (YP, MD and JPK). The Division of Medicinal Chemistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 (MBD and JL).

Running title

Niemann-Pick C1 and drug efflux

Address correspondence to

Jeffrey P. Krise, Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS 66047. Tel. 785-864-2626; Fax 785-864-5736; E-Mail: krise@ku.edu

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Non-standard abbreviations

RND, resistance nodulation division; NPC1, Niemann-Pick C1; MDR, multidrug resistance

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Cellular and Molecular

Abstract

Niemann-Pick C1 (NPC1) is a late endosomal/lysosomal membrane protein originally reported on for its role in cholesterol trafficking in mammalian cells. NPC1 has been recently shown to share significant structural homology with a family of prokaryotic permeases and was proposed to play a role in intracellular drug transport; however, the mechanism for this has not been fully understood. We provide evidence here that is consistent with NPC1's involvement in a vesicle-mediated clearance of the anticancer agent daunorubicin from cells. In experiments with human fibroblasts, we demonstrate that lysosomal efflux of daunorubicin, as well as dextran molecules, are significantly reduced in cells with mutated and dysfunctional NPC1 compared to wild type fibroblasts. Furthermore, we show that NPC1 is implicated in a lysosomal drug sequestration phenotype exhibited by the multi-drug resistant (MDR) human leukemic HL-60 cancer cell line. Evaluations of cholesterol trafficking, NPC1 mRNA levels and protein expression are all consistent with a loss of NPC1 activity that is associated with the emergence of the MDR phenotype in this cell line. Collectively, this work proposes a novel role for NPC1 in a vesicle-mediated pathway responsible for the clearance of drugs from cells and provides an explanation for a drug sequestration phenotype exhibited by the MDR HL-60 cell line.

Introduction

NPC1 is a protein well known for its role in intracellular trafficking of LDL-derived free unesterified cholesterol from late endosomes-lysosomes to the plasma membrane and other organelles (Liscum, 2000; Garver and Heidenreich, 2002). Cells with defective NPC1 characteristically display hyper-accumulation of cholesterol in lysosomes. Interestingly, recent reports have suggested that NPC1 shares significant homology with the RND family of prokaryotic permeases and have postulated that this protein may possess drug transport activity (Davies et al., 2000; Tseng et al., 1999). Despite this report, little was known regarding the substrate specificity or the role this protein may play in intracellular drug transport.

Cancer cells exposed to chemotherapeutic agents frequently become resistant to the cytotoxic effects of drugs after prolonged exposure. This phenomenon has been termed MDR because cells often become resistant not only to the administered drug but also to structurally unrelated drugs that are subsequently administered. Overall, the MDR phenotype is complex and multifactorial (Gottesman and Pastan, 1993).

Our laboratory focuses on evaluating intracellular drug sequestration mechanisms that have been shown to contribute to MDR (Duvvuri et al., 2005). We have recently shown that the MDR HL-60 human leukemic cancer cell line can sequester different drugs into different organelles through independent mechanisms (Gong et al., 2003). In this work we demonstrated that the weakly basic anticancer drug daunorubicin specifically accumulates within lysosomes of the MDR cell line; a process that did not appear to occur in drug-sensitive cells. The lysosomal sequestration is thought to reduce the drug's effectiveness by limiting its availability to enter the nuclear compartment that houses the drug's target molecules, which include DNA and topoisomerase II (Binaschi et al., 2001).

We and others have shown that the sequestration of weakly basic drugs into lysosomes of MDR cancer cell lines is driven by a pH-partition-type mechanism (Gong et al., 2003; Simon et al., 1994). In the MDR HL-60 cell line, we have previously shown that the lysosome and cytosol pH values are 5.1 and 7.1, respectively (Gong et al., 2003). When weakly basic drugs, such as daunorubicin, with pKa values near neutrality partition across the plasma membrane and diffuse into the cell cytosol, they exist, to a significant degree, in an unionized, membrane-permeable state. Upon entering the luminal domain of

acidic organelles such as the lysosomes, these drugs experience an acute shift in their ionization and exist predominantly in an ionized, membrane-impermeable state. As a result, steep pH gradients across intracellular compartments can drive significant accumulations of weakly basic drugs. The reason daunorubicin fails to accumulate significantly within lysosomes of drug sensitive HL-60 cells is because of defective acidification capacity associated with the lysosomal compartment (Gong et al., 2003).

Interestingly, daunorubicin remains visibly sequestered in the lysosomes of MDR HL-60 cells long after the drug is removed from the culture media. This observation is in disagreement with the proposed function of NPC1 in drug efflux from this compartment (Davies et al., 2000). We reasoned that either daunorubicin was not a substrate for NPC1 mediated efflux from lysosomes or perhaps NPC1 was not functioning properly on lysosomes of MDR cancer cells.

We describe experiments here that are consistent with daunorubicin being a substrate for NPC1-mediated efflux from lysosomes of human fibroblasts. We also provide evidence that suggests NPC1 mediates the efflux according to a vesicle-mediated process rather than directly translocating drugs across lipid bilayers, which is the mechanism ascribed to traditional drug transporter proteins such as P-glycoprotein. Finally, we establish that the emergence of MDR in HL-60 cells is associated with malfunctions in NPC1 activity. The implications of these findings with regard to the intracellular trafficking of drugs in both normal and MDR cancer cells are discussed.

Materials and Methods

Cell Lines and Growth Conditions. The human acute promyeloid leukemia cell line HL-60 and its doxorubicin-selected drug resistant variant (MDR HL-60) were kindly provided by Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). These cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes, 1 mM sodium pyruvate and 1% penicillin-streptomycin (Invitrogen Corp., Carlsbad, CA). Human $NPC1^{+/+}$ (CRL-2076) and $NPC1^{-/-}$ (GM-03123A) fibroblasts were kindly provided by Dr. William Garver (Department of Pediatrics, University of Arizona). Human fibroblasts were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were maintained at 37°C in a humidified 5% carbon dioxide atmosphere and were maintained at 60–80% confluence prior to experimentation.

NPC1 Western Blot and Real-Time RT-PCR. For Western blot analysis, HL-60 cells were collected and homogenized in a buffer consisting of 100 mM Tris pH 8.0, 2 mM ethylenediaminetetraacetic acid, 0.3 M sodium chloride, and protease inhibitor cocktail consisting of 0.1 mM phenyl-methyl sulfonyl fluoride and 1 μg/mL each of aprotin, leupeptin and pepstatin (Sigma, St. Louis, MO). Cell lysates were resolved with 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Blots were incubated with a rabbit polyclonal antibody against human NPC1 (gift from Dr. William Garver, University of Arizona) and with a mouse monoclonal antibody against β-actin (Sigma). For RT-PCR, 10 μg of RNA from designated cells lines were extracted using RNeasy spin columns (Qiagen, Valenca, CA) and were treated with 10 units of DNase (Promega, Madison, WI) according to the manufacturer's protocol. RNA was then converted to cDNA, using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and 1 μL of the reaction mixture was used to detect NPC1 levels. Real-time RT-PCR was based on TaqMan® Probe-based fluorogenic 5' nuclease chemistry. The primer/probe set (Assay ID: Hs00264835_m1) used to measure NPC1 RNA levels covers the exon 9 and 10 junction of the gene (NM_000271) and was purchased from Applied Biosystems. NPC1 expression in test samples was normalized using rRNA as an endogenous control. A 1000-fold dilution of 1 μL of the cDNA was used for the rRNA control experiments. Real-time

PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The PCR conditions were as follows: Stage 1, 1 cycle at 50°C for 2 minutes; Stage 2, 1 cycle at 95°C for 10 minutes; Stage 3, 40 cycles at 95°C for 0.15 minutes followed by 60°C for 1 minute. NPC1 gene expression in MDR HL-60 cells relative to drug sensitive HL-60 cells was based on the normalized threshold cycle (C_T) value of each sample and was determined using 7500 Real-Time PCR System Sequence Detection Software version 1.2.1. Statistical significance of differences in mRNA levels was evaluated with a two-tailed student's t-test using SigmaPlot 2001 (SPSS Inc., Chicago, IL) at a significance level of 0.05.

Cell Imaging Studies. Cells were viewed with a fluorescence microscope (Leica Diaplan, Leitz Weltzar, Germany) with 100X (HL-60 cell lines) or 40X (NPC1 fibroblasts) objectives. Differential magnifications were used to correct for large differences in cell sizes. Images were captured using an Orca ER camera (Hamamatsu Corp., Bridgewater, NJ) controlled by SimplePCI imaging software (Compix Inc., Norcross, GA). For daunorubicin localization studies, all cells were cultured in medium supplemented with 2 μM daunorubicin (Sigma) for 1 hour and subsequently washed twice with ice-cold phosphate buffered saline pH 7.4 (PBS) before mounting on a coverslip and imaging. Designated fibroblasts were incubated in culture media supplemented with 50 μM nocodazole (Sigma) for 4 hours prior to the addition of 2 μM daunorubicin for 1 hour. For filipin staining, cells were collected and fixed in PBS containing 3% paraformaldehyde for 30 minutes and washed with PBS prior to incubation in 50 mM ammonium chloride (in PBS) for 15 minutes and washed in PBS again. Cells were then incubated with PBS containing 10% goat serum and 50 μg/mL filipin (Sigma) for 1 hour and washed thrice with PBS prior to imaging. Lysosomal pH determination in the fibroblasts was carried out using a ratiometric approach previously described by Diwu and coworkers using LysoSensor Yellow/Blue DND-160 (Molecular Probes, Eugene, OR) as a probe (Diwu et al., 1999).

Dextran Secretion Assay. Designated cell lines were incubated in culture medium supplemented with 50 μCi/mL [^{3}H]-dextran (70,000 MW, 182 μCi/mg, American Radiolabeled Chemicals Inc., St. Louis, MO) at 37°C for 1 hour and washed with PBS four times. A fraction of treated cells were homogenized in a solution containing 100 mM Tris pH 8.0, 2 mM ethylenediaminetetraacetic acid and 0.3

M sodium chloride and subjected to liquid scintillation counting to determine initial [^{3}H]-dextran loading. The remaining cells were returned to [^{3}H]-dextran-free warmed cell culture medium (37°C) and the amount of [^{3}H]-dextran secreted into the medium at indicated time points was recorded. To determine the amount of non-specifically associated [^{3}H]-dextran that was released into the medium at each time point, the experiment was repeated at 4°C following the initial incubation with [^{3}H]-dextran for 1 hour at 37°C. These values were subtracted from total amount of [^{3}H]-dextran released into the medium at 37°C for each time point. The cumulative amount secreted into the culture medium was expressed as a percent of the initial amount contained in the cells at the start of the experiment. Quantification of [^{3}H]-dextran in samples was performed using a liquid scintillation counter (Beckman, LS 6000IC) and Econoscint (LPS, Laboratory Products Inc.) liquid scintillation cocktail. Statistical significance of differences in amount of [^{3}H]-dextran secreted at 60 min was evaluated with a two-tailed student's t-test using SigmaPlot 2001 (SPSS Inc., Chicago, IL) at a significance level of 0.05.

Results

Assessment of NPC1 Functional Activity. To assess the functional activity of NPC1 in cholesterol trafficking, we examined cells labeled with the cholesterol-binding antibiotic filipin. As expected, normal fibroblasts ($NPC1^{+/+}$) showed a characteristic diffuse fluorescence pattern indicating normal clearance of cholesterol from late endosomes/lysosomes and thus normal function of the protein (Fig. 1A). Conversely, $NPC1^{-/-}$ fibroblasts revealed excessive buildup of cholesterol in intracellular compartments indicating defects in the protein's functional capacity. We next compared these results to those obtained from labeling experiments with drug sensitive and MDR HL-60 cells. In Fig. 1B the drug sensitive HL-60 cell line appears to have normal cholesterol distribution whereas the MDR HL-60 cell line shows enhanced accumulation of cholesterol in intracellular compartments. Further, treatment with progesterone, an agent that is known to cause a NPC1 phenotype (te Vruchte et al., 2004), resulted in excessive intracellular accumulation of cholesterol in both $NPC1^{+/+}$ and drug sensitive HL-60 cells (see Supplemental Fig.1). Together, these results suggest a correlation between the emergence of MDR in HL-60 cells and defects in NPC1 activity as revealed through cholesterol trafficking defects.

Previous studies have shown that when NPC1 loses functional capacity in cells it is typically overexpressed to compensate for the reduced activity (Watari et al., 2000; Patel et al., 1999). We therefore comparatively evaluated NPC1 RNA and protein levels in drug sensitive and MDR HL-60 cells. Western blot analysis of cell lysates revealed that NPC1 was significantly overexpressed in the MDR cell line relative to the drug sensitive cell line (Fig. 2A). Moreover, real-time RT PCR revealed that NPC1 mRNA levels are approximately three times greater in MDR cell line relative to drug-sensitive cell line (Fig. 2B). The RNA and protein expression results are consistent with the filipin staining experiments and suggest that NPC1 loses functional activity when the drug sensitive HL-60 cell line acquires the MDR phenotype.

Correlating NPC1 Functional Activity with Daunorubicin Clearance from Lysosomes. The previous studies support a correlation between loss of NPC1 activity and the emergence of MDR. To

evaluate whether daunorubicin is a substrate for NPC1 mediated efflux from lysosomes, the cellular distribution of daunorubicin in $NPC1^{+/+}$ and $NPC1^{-/-}$ fibroblasts were assessed using fluorescence microscopy. Fig. 3A shows that daunorubicin remains sequestered in intracellular compartments of $NPC1^{-/-}$ fibroblasts, while daunorubicin is efficiently cleared from intracellular compartments of $NPC1^{+/+}$ cells. Similarly, MDR HL-60 cells, which we have shown to have impaired NPC1 function, fail to clear daunorubicin from intracellular compartments (Fig. 3B). Moreover, treatment of $NPC1^{+/+}$ cells with progesterone resulted in lysosomal retention of accumulated daunorubicin (see Supplemental Fig.2). Alternatively, progesterone treatment did not have any effect on daunorubicin distribution in $NPC1^{-/-}$ cells, which do not have functional NPC1. Together, these results suggest that daunorubicin clearance from intracellular compartments requires the cell to possess functional NPC1.

Mechanism for NPC1-Mediated Clearance of Daunorubicin from Intracellular Compartments. There are two general mechanisms by which NPC1 could conceivably facilitate the clearance of daunorubicin from intracellular compartments such as lysosomes. The first possibility is that NPC1 could directly interact with a drug molecule and facilitate its transfer across the lysosomal lipid bilayer. A second mechanistic possibility is that NPC1 could be involved in the trafficking of an intracellular vesicle containing the drug to the plasma membrane for release. We reasoned that evaluation of daunorubicin sequestration in the presence of nocodazole, a disrupter of the cell's microtubule network, would help differentiate between the previously stated mechanistic possibilities. A direct comparison of daunorubicin cellular distribution with and without nocodazole pretreatment in $NPC1^{+/+}$ and $NPC1^{-/-}$ fibroblasts is shown in Fig. 4 and reveals that daunorubicin efflux is significantly reduced in the presence of the microtubule disruptor with $NPC1^{+/+}$ fibroblasts. Alternatively, nocodazole had no significant effect on $NPC1^{-/-}$ fibroblasts other than slightly altering organelle morphology. Collectively, these observations are consistent with NPC1 playing a role in clearing lysosomes of the drug by a vesicle-mediated transport pathway.

To further evaluate this mechanistic possibility, we examined whether efflux of [^{3}H]-dextran molecules from lysosomes could be correlated with the functional capacity of NPC1 in the fibroblasts and in HL-60 cells. To achieve this we specifically localized [^{3}H]-dextran to lysosomes using a pulse-chase technique and measured the release of dextran into the medium at indicated times (Fig. 5). We found that the dextran secretion rate was significantly decreased in both cell lines that have impaired NPC1 function, namely the NPC1$^{-/-}$ fibroblast and the MDR HL-60 cell line relative to cell lines with normal NPC1 function (Fig. 5). Together, these results are consistent with daunorubicin clearance from lysosomes through an NPC1-dependent vesicle-mediated secretory type mechanism.

Discussion

The results presented here provide evidence that NPC1 mediates the clearance of the anticancer agent daunorubicin from cells. Previously, proteins belonging to the ATPase binding cassette (ABC) superfamily of drug transporters were thought to be the principal class of proteins possessing drug transport activity. Such transporters (i.e., P-glycoprotein), are known to directly bind with drug molecules and facilitate their transfer across lipid bilayers of cells (Borges-Walmsley et al., 2003). Results presented here are consistent with NPC1 mediating drug efflux according to a different mechanism, which involves a vesicle-mediated transport pathway. Importantly, our results further establish that MDR cancer cells can acquire defects in NPC1 function, which provides a mechanistic basis for an intracellular drug sequestration phenotype exhibited by these cells.

The observation that the intracellular compartmentalization of daunorubicin differs with respect to functional activity of NPC1 in a pair of human fibroblasts supports a role for this protein in the intracellular trafficking of daunorubicin. Our results suggest that NPC1 participates in clearing intracellular compartments of drugs that have accumulated there by alternative mechanisms.

The accumulation of weakly basic drugs, such as daunorubicin, in acidic intracellular vesicles has been previously established to occur according to a pH partition-type mechanism (DeDuve et al., 1974). Therefore, in order for daunorubicin to be sequestered into lysosomes according to this mechanism the luminal pH of the organelle must be low. To confirm that both NPC1 fibroblasts had acidic lysosomes, we measured the pH values associated with them and found them to be 4.4 ± 0.1 and 4.6 ± 0.3 for $NPC1^{+/+}$ and $NPC1^{-/-}$ fibroblasts, respectively (results from ten experiments – see *Materials and Methods*). Therefore, theoretically, both of these cell lines should sequester daunorubicin in lysosomes to a similar degree according to a pH-partition-type mechanism. The fact that the $NPC1^{+/+}$ cell line did not visibly retain daunorubicin in the lysosomes suggested that NPC1 was able to efficiently clear the drug from this compartment. $NPC1^{-/-}$ fibroblasts, on the other hand, were unable to clear the compartmentalized drug. These results established a role for NPC1 in the clearance of drug from acidic vesicles such as lysosomes.

We have previously established that the accumulation of daunorubicin in the lysosomes of MDR HL-60 cells occurs through a pH partition-type mechanism and not through the action of ABC type drug transporters (Gong et al., 2003). This is important to note because we and others have shown that intracellular drug sequestration of some drugs can be facilitated by drug transporters such as P-glycoprotein and the multi-drug resistance associated protein MRP1 in some MDR cell lines (Gong et al., 2003; Shapiro et al., 1998). We have also previously shown that the reason that daunorubicin does not sequester in lysosomes of drug sensitive HL-60 cell line is because they have defective acidification of their lysosomes (Gong et al., 2003).

Having established a role for NPC1 in drug clearance from intracellular vesicles we next sought to elucidate the mechanism. Prior to completing this work, the mechanistic basis for NPC1's role in drug transport was poorly understood and controversial. We considered two general mechanisms by which NPC1 could mediate the clearance of daunorubicin from intracellular vesicles (see Fig. 6 for a diagram). First, NPC1 could be involved in a vesicle-mediated exocytosis of lysosomal contents, which would result in clearance of daunorubicin from the cells (see pathway 2, Fig. 6). This possibility is supported by the work of Nuefeld and coworkers who suggested that NPC1 can participate in non-discriminate transport of lysosomal cargo to the plasma membrane (Neufeld et al., 1999). Second, NPC1 could be involved in direct transport of daunorubicin from the lumen of the lysosomes to the cell cytosol. From the cytosol the drug could passively permeate out of the cell (see pathway 1, Fig. 6). This possibility would be predicted from the work of Davies and colleagues, who proposed that NPC1 participates in the transport of weakly basic compounds such as acriflavine across lipid bilayers of intracellular compartments (Davies et al., 2000).

We reasoned that evaluation of daunorubicin sequestration in the presence of nocodazole, a disrupter of the cell's microtubule network, would help differentiate between the previously stated mechanistic possibilities. The $NPC1^{+/+}$ fibroblast was chosen for this evaluation because 1) the lysosomes are acidic and thus daunorubicin should be accumulating within them according to a pH- partition-type mechanism and 2) this cell line also has functional NPC1 and is able to clear the lysosomes of sequestered drug. In

essence, this cell line is believed to have accumulation and clearance mechanisms operating simultaneously, the end result is that daunorubicin is not visibly sequestered into perinuclear vesicles (Fig. 4). Nocodazole treatment would be expected to result in enhanced retention of daunorubicin in lysosomes of $NPC1^{+/+}$ fibroblasts if a vesicle-mediated event was responsible for its clearance. Alternatively, nocodazole treatment would not be expected to influence lysosomal clearance if NPC1 was directly transporting daunorubicin across the lysosomal lipid bilayer into the cell cytosol. The fact that nocodozale treatment reduced the clearance of daunorubicin from intracellular compartments is consistent with NPC1 playing a role in a vesicle-mediated clearance of the drug (pathway 2, Fig. 6).

NPC1's role in a vesicle-mediated transport for daunorubicin clearance was further evaluated using a lysosomal dextran secretion assay (Fig. 5). Dextran molecules were specifically localized to lysosomes, the same compartment that sequesters daunorubicin. Unlike daunorubicin, dextran molecules are very large and hydrophilic and cannot cross lipid bilayers by passive diffusion. As a result, dextran molecules can only be released from cells through vesicle mediated type transport events (Straubinger et al., 1983). The release of dextran molecules into the media surrounding the cells was found to be significantly reduced in cells with dysfunctional NPC1. These results further support NPC1's role in mediating lysosomal clearance of drugs according to a vesicle mediated pathway.

The results from cholesterol trafficking studies with filipin and NPC1 RNA and protein expression suggested that NPC1 protein lost its functional activity when HL-60 cells developed a MDR phenotype. This finding helps rationalize why MDR HL-60 cells retain accumulated drug in the lysosomal compartment (Fig. 3B). As previously stated, daunorubicin accumulation in the MDR HL-60 cells occurs according to a pH partition-type mechanism. The finding that NPC1 is not functioning properly in this cell line explains why daunorubicin fails to be efficiently cleared from the lysosomes. A similar daunorubicin sequestration phenotype is observed with $NPC^{-/-}$ fibroblasts that also have low lysosomal pH and dysfunctional NPC1, like the MDR HL-60 cells.

In summary, this work establishes a role for NPC1 in a novel vesicle-mediated efflux pathway for the clearance of drugs from cells. This finding has broad implications and significantly improves our

understanding regarding drug efflux mechanism. It is reasonable to assume that all cells, under normal conditions, have low lysosomal pH and functional NPC1 to varying degrees. The combination of these two features can be expected to result in an efficient sequestration and secretion pathway responsible for enhancing the clearance of drugs from cells. Weakly basic drugs that are susceptible to ion-trapping in lysosomes would be expected to be substrates for this novel efflux pathway. Considering the fact that a large percentage of commercially available drugs are weak bases, the scope of potential substrates could be quite large. This work also established a correlation between the emergence of MDR in HL-60 cancer cells with defective NPC1 functional activity. This observation allowed us to arrive at a mechanistic explanation for a unique drug sequestration phenotype observed with these cells.

It is important to note that we believe it is unlikely that the loss NPC1 function in MDR cells would directly contribute to the resistance to daunorubicin. This is because drug sequestered in lysosomes would be considered to be ineffective regardless of whether or not it is secreted. Moreover, at the present time it is uncertain how prevalent this relationship between MDR and NPC1 loss of function is. From our unpublished findings it appears that not every MDR cell line has a loss of NPC1 activity. The relationship may be dependent on the MDR selection conditions among other factors and more work will be required to establish this.

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Footnotes

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Send reprint requests to:

Jeffrey P. Krise

Department of Pharmaceutical Chemistry

236B Simon Laboratories

2095 Constant Ave

The University of Kansas

Lawrence

KS 66047

Figure Legends

Fig. 1. The emergence of MDR is associated with intracellular cholesterol trafficking defects indicative of malfunctions in NPC1 activity. Cells were stained with filipin (50 μg/mL for 1 hour) to visualize the cellular distribution of unesterified cholesterol. A, as a control, filipin staining shows normal cholesterol distribution in $NPC1^{+/+}$ fibroblasts and a defective trafficking in $NPC^{-/-}$ fibroblasts revealed by hyper-accumulation of cholesterol in intracellular compartments. B, drug sensitive HL-60 cells appear to have normal cholesterol trafficking whereas MDR HL-60 cells have developed an apparent defect in NPC1 function resulting in enhanced accumulation of cholesterol in intracellular compartments. Each of the micrographs is representative of at least 10 images.

Fig. 2. Comparative evaluations of NPC1 protein and mRNA levels in drug sensitive and MDR HL-60 cell lines. A, Western blot analysis of whole cell lysates from drug sensitive HL-60 and MDR HL-60 cell lines reveals that NPC1 (top panels) is overexpressed in MDR HL-60 cells as compared to drug sensitive HL-60 cells. As a control for protein loading actin levels are shown in the bottom panels. B, real-time RT-PCR analysis reveals an approximately threefold increase in mRNA levels from MDR HL-60 cell lines compared to drug-sensitive HL-60 cells. mRNA levels are represented as mean ± SD of three values for each cell line (*$P<0.001$ by student's t-test).

Fig. 3. Daunorubicin clearance from intracellular compartments requires functional NPC1. Cells were incubated with 2 μM daunorubicin for 1 hour before viewing under the microscope. A, the fluorescent anticancer agent daunorubicin does not visibly accumulate in perinuclear compartments of $NPC1^{+/+}$ fibroblasts but does so in $NPC1^{-/-}$ fibroblasts. B, MDR HL-60 cells retain daunorubicin in intracellular

compartments whereas the drug sensitive HL-60 cells do not. Each of the micrographs is representative of at least 10 images.

Fig 4. Daunorubicin clearance from intracellular compartments requires intact microtubule network. Designated cells were treated with 50 μM nocodazole for 4 hours prior to incubation with 2 μM daunorubicin for 1 hour. With intact microtubules $NPC1^{+/+}$ fibroblasts are able to efficiently clear daunorubicin from intracellular compartments. When $NPC1^{+/+}$ fibroblasts are treated with the microtubule network disrupting agent nocodazole daunorubicin containing intracellular compartments become visible indicating that an intact microtubule network is necessary for efficient daunorubicin clearance. Alternatively, nocodazole pretreatment did not have any significant effect on daunorubicin sequestration in $NPC^{-/-}$ fibroblasts. Each of the micrographs is representative of at least 10 images.

Fig. 5. [^{3}H]-dextran secretion assay reveals that cells with defective NPC1 function (MDR HL-60 and $NPC1^{-/-}$) display a decreased rate of lysosomal secretion relative to cells with normal NPC1 activity (HL-60 and $NPC1^{+/+}$). A, comparison of lysosomal [^{3}H]-dextran secreted into the medium for HL-60 and MDR HL-60 cell lines as a function of time. B, comparison of lysosomal [^{3}H]-dextran secreted into the medium for $NPC1^{+/+}$ and $NPC1^{-/-}$ fibroblasts as a function of time. The total amounts of [^{3}H]-dextran in cells at the start of the experiments were 15192, 13694, 21188 and 23573 DPM for the $NPC1^{-/-}$, $NPC^{+/+}$, HL-60 and MDR HL-60 cells, respectively. Values represent mean ± SD (n=3) for each time point. Differences in amount of [^{3}H]-dextran secreted at 60 min by related cell lines were statistically significant by student's t-test (*$P<0.001$ for NPC cell lines and **$P<0.001$ for HL-60 cell lines).

Fig 6. Diagram illustrating daunorubicin accumulation in lysosomes and two possible pathways for NPC1-mediated clearance of the drug. Daunorubicin free base (D) partitions across organelle lipid bilayers into the lumen where it is predominantly ionized (DH^+) due to the low pH environment. There

are two general pathways for removal of DH^+ from the organelle and cell. In pathway 1, DH^+ directly binds to NPC1 which facilities its transfer across the lipid bilayers into the cell cytosol where it can diffuse out of the cell. In pathway 2, NPC1 mediates the trafficking of the drug-containing vesicle to the plasma membrane where drug is released into the extracellular space.

Figure 1

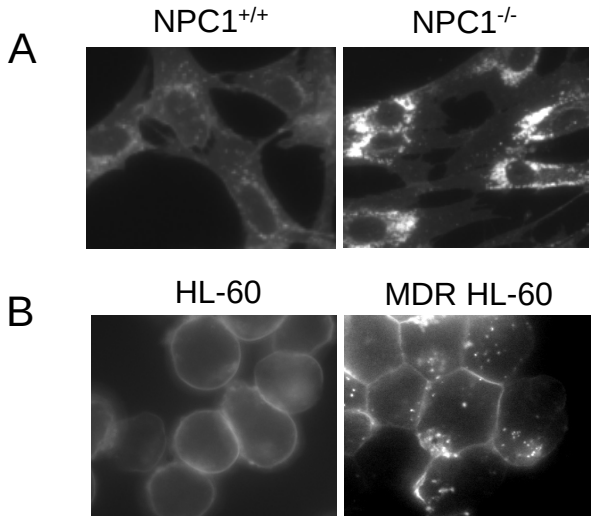

Figure 2

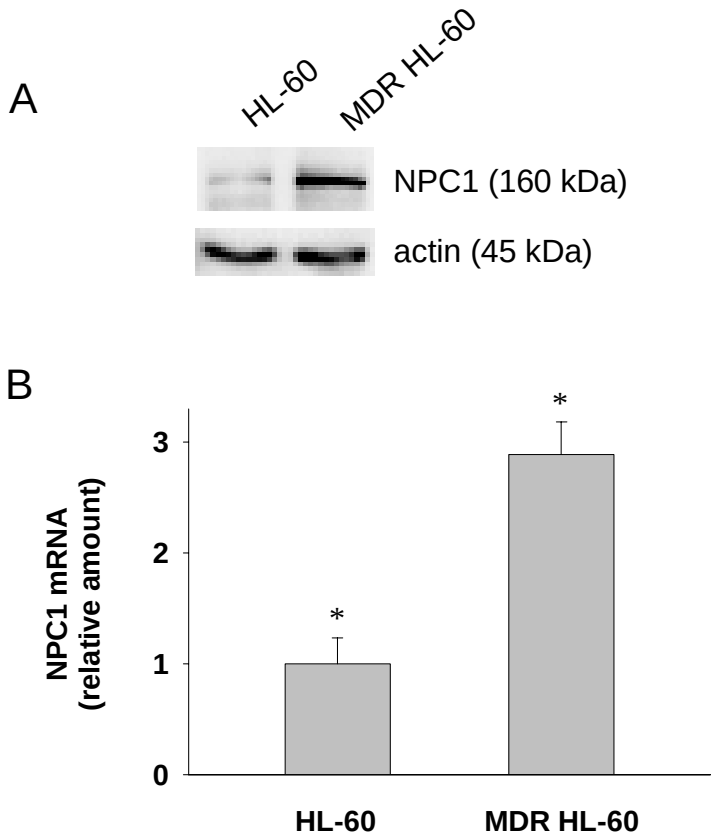

Figure 3

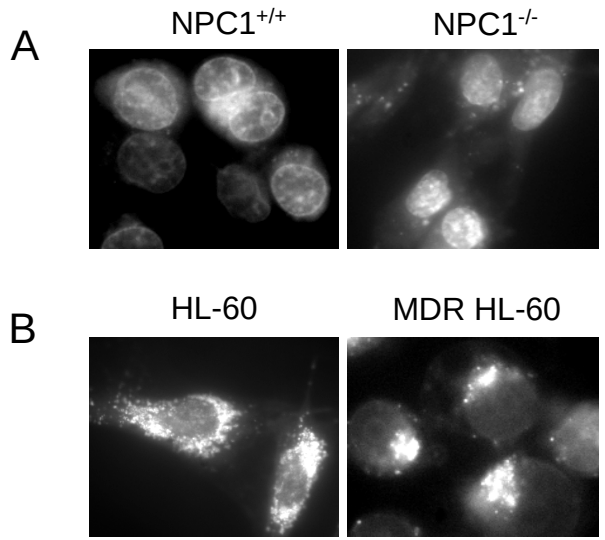

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Figure 4

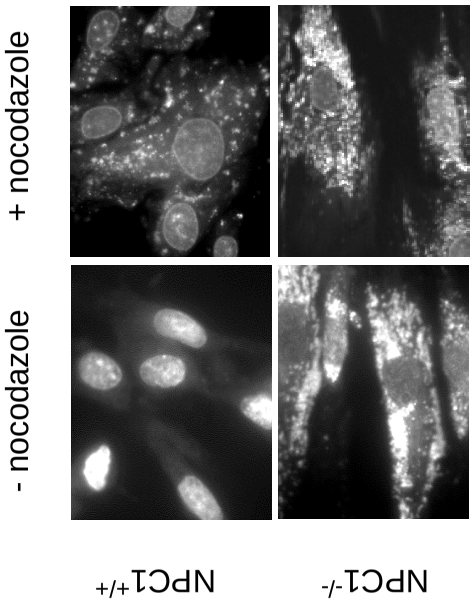

Figure 5

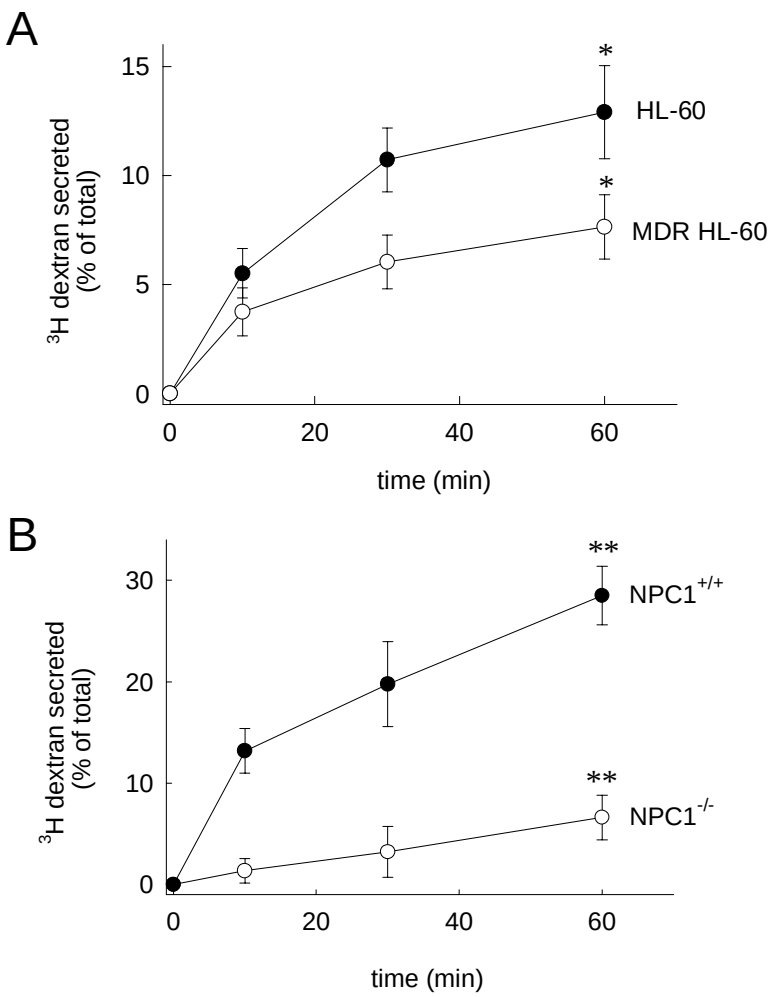

Figure 6