The Novel Anti-cancer Agent JNJ-26854165 Induces Cell Death Through Inhibition of Cholesterol Transport and Degradation of ABCA1.


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Abbreviations: 165, JNJ-26854165 (serdemetan). 165R, cells resistant to JNJ-26854165. ABCA1, ATP-binding cassette sub-family A member-1. ABCG1, ATP-binding cassette sub-family G member-1. CNT, centriole. DEL, deleted. DOXY, doxycycline. ERK, extracellular signal-regulated kinase. GFP, green fluorescent protein. HDL, high density lipoprotein. HDL-c, high density lipoprotein derived-cholesterol. HDM-2, human double minute 2. IC₅₀, median inhibitory concentration. LDL-c, low density lipoprotein derived-cholesterol. LDL-R, low density lipoprotein receptor. LW, Lipid Whorl. MCL, mantle cell lymphoma. MEFs, mouse embryonic fibroblasts. MITO, Mitochondria. MM, multiple myeloma. Mut, mutant. mutp53, mutant p53. n, number of replicates. NPC 1/2, Niemann-Pick disease, type C1/2. NPD, Niemann-Pick disease. RQ, relative transcript expression. SEM, standard error of the mean. SR-B1, scavenger receptor class-B1. SREBF-1/2, sterol regulatory element binding transcription factor ½. TD, Tangiers disease. WT, wild-type. wtp53, wild-type p53

Chemotherapy, Antibiotics, and Gene Therapy
Abstract

JNJ-26854165 (serdemetan) has previously been reported to inhibit the function of the E3 ligase human double minute-2, and we initially sought to characterize its activity in models of mantle cell lymphoma (MCL) and multiple myeloma (MM). Serdemetan induced a dose dependent inhibition of proliferation in both wild-type (wt) and mutant (mut) p53 cell lines, with IC50’s from 0.25 μM/L to 3 μM/L, in association with an S phase cell cycle arrest. Caspase-3 activation was primarily seen in wt p53 bearing cells, but also occurred in mut p53 bearing cells, albeit to a lesser extent. 293T cells treated with JNJ-26854165, and serdemetan-resistant fibroblasts displayed accumulation of cholesterol within endosomes, a phenotype reminiscent of that seen in the ATP-binding cassette sub-family A member-1 (ABCA1) cholesterol transport disorder, Tangiers disease. MM and MCL cells had decreased cholesterol efflux and electron microscopy demonstrated the accumulation of lipid whorls, confirming the lysosomal storage disease phenotype. JNJ-26854165 induced induction of cholesterol regulatory genes sterol regulatory element-binding transcription factor-1 and -2, liver X receptors α and β, along with increased expression of Niemann-Pick disease type-C1 and -C2. However, JNJ-26854165 induced enhanced ABCA1 turnover despite enhancing transcription. Finally, ABCA1 depletion resulted in enhanced sensitivity to JNJ-26854165. Overall, these findings support the hypothesis that serdemetan functions in part by inhibiting cholesterol transport, and that this pathway is a potential new target for the treatment of MCL and MM.
Introduction

A wide array of cellular functions depend on cholesterol, from formation of the plasma membrane and lipid rafts, to steroid hormone production. Cholesterol is transported into cells with either low density lipoprotein (LDL) or high density lipoprotein (HDL) (Phillips et al., 1987). LDL-derived cholesterol (LDL-c) is transported into cells via the LDL receptor (LDL-R) (Goldstein and Brown, 1984; Yamamoto et al., 1984), while HDL-cholesterol (HDL-c) enters via scavenger receptor class B member-1 (SR-BI) and is exported by the ATP-binding cassette sub-family A member-1 (ABCA1) (Yancey et al., 2003) and ATP-binding cassette sub-family G member-1 (ABCG1) (Prosser et al., 2012). Cancer patients often present with elevated or depleted levels of HDL-c and LDL-c (Cantafora and Blotta, 1996; Tomiki et al., 2004), and malignant cells exhibit abnormal regulation of cholesterol-regulated genes. The deregulation of LDL-R (Tatidis et al., 1997), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Vitols et al., 1994), and cholesterol transporters such as ABCA1 (Yancey et al., 2003), suggests malignant cells require more cholesterol than normal cells, which may be linked to their enhanced proliferation.

Hematological malignancies overexpress growth promoting genes found in lipid rafts and cholesterol-rich environments, making them attractive targets for an anti-cholesterol therapeutic approach. These genes include protein kinase C-β protein (Mahshid et al., 2009), part of the B cell receptor signaling complex (Shinohara and Kurosaki, 2009) which plays an important role in nuclear factor-κB activation (Shinohara and Kurosaki, 2009), and is involved in angiogenesis (Yoshiji et al., 1999), and 5-lipoxygenase, which enhances leukotriene release (Claesson and Dahlen, 1999), while the CD70/CD27 complex results in autocrine stimulation in mantle cell lymphoma (MCL) (Boyd et al., 2009). Anaplastic large cell lymphomas overexpress nucleophosmin and the anaplastic large cell lymphoma kinase,
resulting in induction of emopamil binding protein, which is involved in cholesterol biosynthesis (Villalva et al., 2002). Multiple myeloma (MM) cells overexpress LDL-R, allowing the use of LDL-c as a growth factor resulting in low serum LDL-c (Hungria et al., 2004).

Cholesterol localization within cancer cells has been exploited as a potential therapeutic strategy by induction of a Niemann-Pick disease (NPD) (Liscum and Klansek, 1998) or Tangiers disease (TD) (Neufeld et al., 2004) phenotype, whereby cholesterol localized within endolysosomes prevents its trafficking to the plasma membrane. The cholesterol transport inhibitor U18666A, blockades cholesterol transport in melanoma cells, resulting in cell death (Di Stasi et al., 2005), while anti-psychotic drugs such as pimozide and olanzopine have a profound effect on the growth of lymphoma, neuroblastoma, breast and lung carcinoma cells through inhibition of cholesterol transport (Kristiana et al., 2010; Wiklund et al., 2010).

JNJ-26854165 is a novel chemotherapeutic with p53 activating properties reported to act as a Human double minute protein (HDM)-2 inhibitor with preclinical efficacy in acute myeloid and lymphoid leukemias (Kojima et al., 2010; Smith et al., 2012), as well as various solid tumors (Chargari et al., 2011), and has completed phase I trials (Tabernero et al., 2011). Here we show JNJ-26854165 has an alternative mechanism of action whereby it inhibits cholesterol transport, inducing a TD phenotype and turnover of ABCA1, along with cell death. These data suggest that inhibition of cholesterol transport is a novel and effective strategy to inhibit cholesterol and lipid raft signaling pathways in MCL and MM.
Materials and Methods

Reagents. JNJ-26854165 was provided by Janssen Research & Development, a Division of Janssen Pharmaceutica NV. Pimozide, U18666A, doxycycline and cycloheximide were from Sigma-Aldrich. Stock solutions of JNJ-26854165 and pimozide were prepared in DMSO, whilst doxycycline and cycloheximide were dissolved in ethanol, and U18666A in H₂O.

Cell culture. All cell lines, with the exception of SP-53 and mouse embryonic fibroblasts (MEFs), were from the American Type Culture Collection (Manassas, VA), while SP-53 was a gift of Masanori Daibata (Kochi University, Japan). MEFs containing homozygous p53 deletions, or of both p53 and HDM-2, were described previously (Barboza et al., 2008). SV40-transformed MEFs resistant to JNJ-26854165 (165R) were generated by growing the cells in increasing drug concentrations until they were resistant at 5 μM/L. HeLa ABCA1-tet off regulatable cells were generated as previously published (Neufeld et al., 2001). All cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin, except 293T and MEFs, which were grown in DMEM. Cell lines were validated in the M. D. Anderson Cell Line Validation Core Facility by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifiler Kit (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints, the Cell Line Integrated Molecular Authentication database version 0.1.200808 (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and the M. D. Anderson fingerprint database.

Real-time PCR. Total RNA was isolated using an RNeasy Plus kit (Qiagen), and cDNA was synthesized using Superscript II (Invitrogen). Real-time PCR for ABCA1, sterol regulatory element-binding transcription factors (SREBF)-1 and -2, NPC1 and NPC2, and liver X
receptors-α and -β was performed on a Stepone PCR analyzer (Applied Biosystems) using inventoried real time Taqman-FAM and GAPDH-VIC probes. Relative transcript expression (RQ) was determined using vehicle-treated cells as a calibrator and the \( \Delta \Delta CT \) method.

**Immunoblot.** Protein expression in drug-treated cells was measured by immunoblot analysis performed as previously described (Jones et al., 2008). Antibodies to HSP90, α4 Integrin, p53 were from Santa Cruz Biotechnologies, while antibodies to ABCA1, ABCG1, HMGcoR, MLN64, NPC1, NPC2, were from Abcam. Antibodies to phospho ERK1/2\(^{Thr202/Tyr204}\), Akt\(^{Ser473}\), total ERK and Akt were purchased from Cell Signaling. Additional antibodies included anti-β-actin from Sigma-Aldrich; anti-HDM-2 and p21 antibodies were EMD4Biosciences. Cytoplasmic and membrane fractions of U266 cells were prepared after 24 hours treatment with JNJ-26854165 using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) as per the manufacturer’s instructions. Densitometry was performed on immunoblots after image scanning and analyzed using Image J software (National Institutes for Health), acquired values were normalized to the β-actin loading control for each lane.

**Cell cycle analysis.** Cells were treated with drug for 48 hours, fixed in 70% ethanol, and stained with propidium iodide (Sigma-Aldrich). Cell cycle data were analyzed on a CANTO II flow cytometer (Becton-Dickson) using FlowJo v.7.6.1 (Tree Star, Inc).

**Flow cytometry.** Cell death was measured using Annexin-V Pacific Blue (Invitrogen) and TO-PRO-3 (Invitrogen) staining. Caspase-3 activity was measured using the CaspGLOW Red Staining Kit (Biovision). All flow cytometry was performed using a DAKO CYAN flow cytometer (Beckman-Coulter).
Cell proliferation assay. WST-1 (Roche Diagnostics) was used to determine median inhibitory concentrations (IC$_{50}$'s) as previously published (Jones et al., 2008), and the effects of chemotherapeutics (Kuhn et al., 2007). IC$_{50}$ dose response curves and IC$_{50}$ values were plotted and calculated using GraphPad Prism 6 (GraphPad) using a one site log fit algorithm.

ABCA1 half-life studies. Cycloheximide was added to JeKo-1 cells at 100 μg/mL, time point samples harvested, and protein lysates were subjected to Western blotting. Bands were analyzed using Image J software (National Institutes of Health).

Gene silencing. JeKo-1, MAVER-1, U266 and OPM-2 cells with ABCA1 knockdown were created using Mission shRNA Lentiviral particles (Sigma-Aldrich; TRCN0000029089NM_005502.2-406s1c1). Briefly, cells were plated in 96-V-well plates with 8 μg/mL PolyBrene (Sigma-Aldrich), and infected for 24 hours. They were then fed with fresh medium, selected with 2 μg/mL puromycin (Invitrogen) and colonies were screened for ABCA1 expression.

Fluorescence microscopy and fluorescent staining. Wild-type MEF, 165R, and 293T cells were grown on glass coverslips and treated with 5 μM/L JNJ-26854165, 10 μM/L U18666A or 10 μM/L pimozide for 24 hours. Brightfield images were captured in live cultured cells at 40x magnification. Cellular cholesterol distribution was determined by fixing cells in 3% paraformaldehyde, and then staining them in 0.05 mg/mL Filipin solution (Sigma-Aldrich). Cells were then washed, mounted in ProLong anti-fade reagent (Invitrogen), visualized by ultraviolet fluorescence, and images were captured at 40x. MM, MCL and HeLa cells were imaged using an ImageStream$^X$ Mark II Imaging Flow Cytometer (EMD Millipore), with 1000 cells counted per treatment and visualized at 60x magnification.
**Cholesterol Efflux Assay.** Cells were treated with JNJ-26854165 for 24 hours, washed in PBS and loaded with 10 μg/ml NBD-Cholesterol (Invitrogen) in RPMI containing 10% delipidated fetal bovine serum (RPMIdFBS)(EquiTec-Bio) for 2 hours. Cells were washed twice in RPMIdFBS and cholesterol efflux initiated by addition of RPMIdFBS containing 20 μg/mL ApoA-I (EMD4Biosciences) as an acceptor for 24 hours. Fractions containing medium and cells were collected. NBD-Cholesterol fluorescence was measured in the collected media samples using a fluorescent plate reader at 490 nM excitation and emission at 520 nM. The percentage cholesterol efflux was calculated by dividing the fluorescence in the media fraction by the media and cell fraction and expressed as percentage efflux.

**Electron Microscopy.** Samples were fixed with a solution containing 3% glutaraldehyde/2% paraformaldehyde in cacodylate buffer, then washed and treated with Millipore-filtered cacodylate-buffered tannic acid, post-fixed with 1% buffered osmium tetroxide, and stained en bloc with Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, embedded in Spurr’s low viscosity medium, and polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc). Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp) through the M. D. Anderson High Resolution Electron Microscopy Facility.
Results

**JNJ-26854165 acts independently of HDM-2.** JNJ-26854165 (serdemetan) (Figure 1A) was previously reported to inhibit HDM-2 function and activate p53 (Arts et al., 2008; Kojima et al., 2010; Patel and Player, 2008). To evaluate its activity against MM and MCL models, we studied a panel of cell lines with varying p53 and HDM-2 status to determine sensitivity to JNJ-26854165. Wild-type (wt) p53 MCL cells exhibited IC_{50}'s (calculated using a one site log fit algorithm) in the 0.25-2 μM/L range, while wt p53 MM cells had IC_{50} values from 1.43-2.22 μM/L (Figure 1B and C, Supplementary Table 1). Mutant (mut) p53 MCL cells had IC50 values from 0.83-2.23 μM/L, and mut p53 MM cells from 2.37-2.48 μM/L, while the epithelial cell line 293T had an IC_{50} of 1.59 μM/L. These data suggested that the presence of wt p53 only had a limited effect on sensitivity to JNJ-26854165. We next examined the effects of JNJ-26854165 on the expression levels of p53 and HDM-2. Treatment of wt p53 MCL and MM cells induced p53 in all the cells tested, and a corresponding increase in HDM-2 and p21 in most cell lines (Figure 1D, upper panel). In contrast, in mut p53 cell lines, while serdemetan increased p53 in MAVER-1, U266, and OPM-2 cells, it had no relative effect on RPMI 8226 and 293T cells. HDM-2 was only readily detectable in 293T cells, which showed an increase in HDM-2 and some increase in p21 (Figure 1D, lower panel). In order to define the requirement of p53 or HDM-2 for the activity of JNJ-26854165, we used MEFs with homozygous deletions of p53, or both p53 and HDM-2. While p53^-/- MEFs were more resistant to JNJ-26854165 than their wt counterparts (IC_{50} 19.95 vs. 3.87 μM/L; p<0.05), the HDM-2 and p53 knockout MEFs showed an IC_{50} of 19.62 μM/L (p<0.05)(Figure 1E). Thus, while functional p53 had some impact on sensitivity to JNJ-26854165, HDM-2 appeared to be dispensable for its action.

**JNJ-26854165 induces S phase cell cycle arrest with caspase-3 mediated cell death.**

We next investigated the cell cycle and cell death effects induced by JNJ-26854165.
Exposure of \textit{wtp53} bearing cells to JNJ-26854165 for 48 hours induced an S-phase cell cycle arrest compared to the vehicle control (Figure 2A, left panel). MAVER-1 \textit{mutp53} cells demonstrated increased S-phase accumulation in response to JNJ-26854165, whereas JeKo-1 cells had a 2-fold increase in the G₂M fraction and a slight increase in the S-phase fraction. Similarly, an increase in the G₂M fraction was observed in U266 cells, and in OPM-2 cells no discernible cell cycle was detectable (Figure 2A, right panel). To determine the degree of cell death induced by JNJ-26854165, we utilized a fluorescent caspase-3 substrate and performed Annexin-V staining in combination with TO-PRO-3 to discriminate between viable and dead cells. In \textit{wtp53} bearing cells, JNJ-26854165 induced 60-90\% cell death in MM1.S and GRANTA-519 cells, which coincided with capase-3 activation seen in 55-65\% of cells (Figure 2B, left panel). H929 cells also had capase-3 activation and increased cell death, albeit at a lower level. The \textit{mutp53} cell models showed 25-60\% cell death, which strongly correlated with capase-3 activity in JeKo-1, U266 and RPMI-8226 (Figure 2B, right panel). This was not the case in MAVER-1 and OPM-2 cells, however, despite having a significant amount of cell death, possibly suggesting another pathway of cell death was activated in selected cells.

**Inhibition of cholesterol transport by JNJ-26854165.** To further define a mechanism of action for JNJ-26854165, we developed a resistant MEF cell line. Initial evaluation of the resistant MEFs (165R) by microscopy indicated that they contained multiple peri-nuclear vacuoles not seen in drug-naïve MEFs (Figure 3A). This phenotype was reminiscent of the cholesterol-loaded endosomes found in the inherited cholesterol transport disorders TD (Assmann G, 1995) and NPD (Peake and Vance, 2010). We therefore stained the drug-naïve and 165R MEFs with the cholesterol stain filipin (Bornig and Geyer, 1974). Drug-naïve MEFs had low levels of cholesterol and staining was limited to the cell membrane, while the 165R MEFs displayed intense peri-nuclear staining of cholesterol localized to vesicles within the cytoplasm (Figure 3B). In addition, 293T cells exposed for 24 hours to JNJ-26854165
displayed the same staining, suggesting that cholesterol was locked within the cytoplasm compared to the membranous distribution seen in the controls (Figure 3C). Treatment with the cholesterol transport inhibitor, U18666A or pimozide also resulted in accumulation of cholesterol in vesicles within the cytoplasm similar to that of the JNJ-26854165-treated cells (Figure 3C). Filipin staining of the JeKo-1, MAVER-1, OPM-2 and U266 indicated that JNJ-26854165 induced strong peri-nuclear accumulation of cholesterol (Figure 4A). Accumulation of cholesterol within the cytoplasm would indicate a block in cholesterol efflux, and we therefore performed a cholesterol efflux assay in both the lymphoid cells and 293T cells treated 24 hours with JNJ-26854165. All cells showed a decrease in cholesterol efflux with the lymphoid cells on average have a 10-25% decrease in cholesterol efflux, while the 293T cells had a 17% decrease in cholesterol efflux (Figure 4B). Cell viability as measured at 24 hours showed minimal decrease in cell proliferation, whereas at 48 hours (when efflux was measured), MAVER-1, JeKo-1 and OPM-2 had a 30% decrease in cell proliferation (Supplementary Figure 1). Whereas, U266 had a 50.8% decrease and 293T had a 25% decrease in proliferation.

We further examined the phenotypic effects of JNJ-26854165 on the lymphoid cells using electron microscopy (EM). MM1.S and RPMI-8226 treated with JNJ-26854165 induced the accumulation of vacuoles containing multilayered lamellar structures known as lipid whorls (LW) which are implicated in lysosomal storage disease and cholesterol accumulation in lysosomes (Figure 5)(Parkinson-Lawrence et al., 2010). Similar LW structures were also observed in JVM-2 and JeKo-1 drug-treated cells (Supplemental Figure 2). These data support the possibility that serdemetan is mimicking the effects of cholesterol transport inhibitors such as pimozide (Wiklund et al., 2010), U18666A (Cenedella, 2009), and probucol, which convey a TD phenotype (Tsujita et al., 2000).
Modulation of cholesterol regulatory genes and transporters is induced by JNJ-26854165. We next evaluated the effect of JNJ-26854165 on the cholesterol regulatory genes SREBF-1 and -2, and the LXR-α and -β receptors. JNJ-26854165 induced a 1.5-fold increase in SREBF-1 and -2 in U266 at 24 hours, while LXR-α/β were induced by over 1.5-fold at 24 hours, and remained so compared to the baseline at 72 hours (Figure 6A, left panel). JeKo-1 cells had a 1.7-fold increase in SREBF-1 at 24 hours, which was 2-fold at 72 hours, while SREBF-2 only increased at 72 hours. LXR-α showed a 1.5-fold increase at 24 hours, which increased further to over 2-fold at 72 hours, whereas LXR-β increased close to 1.5-fold at 72 hours (Figure 6A, right panel). The intracellular cholesterol transporters NPC-1, -2, and ABCA1 were induced at 24 hours, with levels increasing to 1.8- and 2-fold over baseline, respectively, whereas ABCA-1 increased 1.5-fold at 72 hours with JNJ-26854165 treatment (Figure 6A, lower left panel). Similarly, JeKo-1 cells showed slower kinetics of NPC-1 induction, with a 1.5-fold increase at 48 hours declining to baseline at 72 hours. In contrast, NPC-2 had a 3-fold increase at 72 hours after addition of JNJ-26854165 and, similarly, transcription of ABCA1 increased 2-fold at 72 hours (Figure 6A, lower right panel).

ABCA1 mRNA levels are often discordant with that of ABCA1 protein (Wellington et al., 2002), this reflecting heavy post-translational modification. To address this we evaluated ABCA1 expression by immunoblot along with other cholesterol transporters. Immunoblotting and densitometry indicated that NPC-2 protein levels increased in line with that predicted by the qPCR assay in JeKo-1, with increases in NPC-1 (3 fold) and -2 (4 fold) occurring at 48 hours post-treatment with JNJ-26854165 (Figure 6B and Supplementary Table 2), while NPC-1 did not change significantly in U266. ABCA1 protein increased 2.5 fold at 6 hours in U266 and remained expressed until 48 hours, after which it was, unexpectedly, rapidly depleted by 6 fold at 72 hours. JeKo-1 showed a similar effect, albeit with a more rapid loss of ABCA1, with a
1.5 fold increase at 6 hours, but was then rapidly depleted at 12 hours and lost at 72 hours representing a 4 fold decrease relative to the 0 hour time point. We also examined the other major cholesterol transporter, ABCG1 (Wang et al., 2004) and HMGcoR, which regulates cholesterol production (Goldstein and Brown, 1990). ABCG1 increased at 12 hours and remained high at 48 hours representing a 2 fold increase, after which its expression returned to baseline in U266. Similarly, in JeKo-1, ABCG1 expression increased 1.5 fold at 6 hours, but returned to baseline at 72 hours following JNJ-26854165 treatment. HMGcoR was induced 2 fold at 24 hours, but the expression levels returned to that of baseline level at 72 hours in U266, while in JeKo-1 cells, HMGcoR increased 2 fold only at 72 hours (Figure 6B and Supplementary Table 2).

As ABCA1 protein was depleted by JNJ-26854165, we sought to determine if ABCA1 turnover was being enhanced. JNJ-26854165 treatment resulted in rapid turnover of ABCA1 protein in a cycloheximide half-life study, with ABCA1 in the vehicle treated cells having a half-life of 106.2 minutes compared to 66.3 minutes in treated cells (p<0.05)(Figure 6C). We also evaluated the effect of JNJ-26854165 at 24 hours on the WT MEF, as well as 293T cells. NPC1, -2 and ABCG1 were barely detectable in WT MEF, but a slight increase was observable in the MEF’s treated with JNJ-26854165 (Figure 6D). ABCA1 was strongly induced by JNJ-26854165 treatment in the WT MEF, interestingly the 165R MEF had strong expression of ABCA1 compared to the WT counterparts and no changes in expression of NPC1, -2 and ABCG1 (Figure 6D). The 293T cells had low level expression of ABCA1 compared to the lymphoid cells and MEF, with JNJ-26854165 depleting the low levels of ABCA1 and no significant change in expression of NPC1, -2 and ABCG1. In the MEF and 293T cells no changes was detectable in the MLN64 cholesterol transporter with JNJ-26854165 treatment (Figure 6D).
We also evaluated if JNJ26854165 had an effect on the balance of ABCA1 pools between the plasma membrane and cytoplasm. Fractionation analysis indicated the majority of ABCA1 is found within the plasma membrane and barely detectable in the cytoplasm of U266 cells (Supplementary Figure 3A). Treatment with JNJ-26854165 for 24 hours actually increased the cytoplasmic ABCA1 pool and decreased the plasma membrane pool (Supplemental Figure 3A), indicating drug treatment is simultaneously enhancing ABCA1 production in the cytoplasm but depleting the plasma membrane pool. ABCA1 expression has been shown to enable HDL-c to activate the Akt/ERK signaling cascade in prostate cancer (Sekine et al., 2010). JNJ-26854165 treatment of both MM and MCL cells for 3 days down regulated both, phosphorylated Akt and ERK in MAVER-1, JeKo-1, OPM-2 and U266 cells (note U266 had no detectable basal phosphorylated ERK) (Supplementary Figure 3B). These results indicate that JNJ-26854165 may also blockade HDL-c signal transduction through inhibiting ABCA1.

As the MM and MCL cells appeared to have a relatively high expression of ABCA1 compared to the 293T cells, we examined the expression of several genes involved in cholesterol transport. Immunoblotting of ABCA1 in the MM and MCL cells showed an abundance of ABCA1, with U266 having very high levels and OPM-2 having barely detectable ABCA1 (relative to the other cell lines tested) (Supplementary Figure 3C). Similarly NPC1 and ABCG1 were strongly expressed in the MM and MCL cell lines, whereas NPC2 expression was expressed mainly in the U266 cells with OPM-2, JeKo-1 and MAVER-1 having lower levels of expression. We also found expression of the Scavenger Receptor B-I/II and MLN64, but at lower levels compared to the other cholesterol transporters (Supplementary Figure 3C). Overall this data suggests JNJ-2684165 may blockade cholesterol transport by degrading ABCA1, resulting in cholesterol accumulation within the cell.

**ABCA1 expression modulates sensitivity to JNJ-26854165.** As JNJ-26854165 induced a blockade of cholesterol transport and affected ABCA1 expression, this suggested the
possibility that at least part of its mechanism of action could relate to turnover of ABCA1. We therefore determined the effects of ABCA1 protein expression in relation to modulation of sensitivity to JNJ-26854165. First, we evaluated the sensitivity of HeLa cells expressing a tetracycline-off regulatable ABCA1 expression cassette. Addition of doxycycline for 1 week suppressed ABCA1 expression (Supplementary Figure 4A), and when cells were treated with JNJ-26854165, the IC$_{50}$ of the ABCA1-depleted cells fell to 0.50 $\mu$M, compared to 2.42 $\mu$M in the control cells (p<0.05)(Figure 7A). Evaluation of the cells by fluorescence microscopy further supported the enhanced sensitivity of the ABCA1-depleted cells (Figure 7B). Filipin staining of HeLa ABCA1-GFP cells treated with JNJ-26854165 for 24 hours, demonstrated accumulation of cholesterol in the cytoplasm versus membrane staining in the vehicle treated cells (Figure 7C). Interestingly, the ABCA1 depleted cells treated with JNJ-26854165 had a greater accumulation of cytoplasmic cholesterol versus that seen in the ABCA1 expressing HeLa cells. Measurement of cholesterol efflux in the ABCA1 depleted cells treated with JNJ-26854165 showed they had stronger suppression of cholesterol efflux, with ABCA1 expressing cells having 35% relative efflux compared to 20% efflux in the ABCA1 depleted cells (Figure 7D). This coincided with a decrease in cell viability at 48 hours, with ABCA1 expressing cells being 87% viable versus 64% in the ABCA1 depleted cells (Supplementary Figure 4B) JNJ-26854165 treatment for 3 days in the ABCA1 expressing HeLa cells depleted ABCA1, this was accompanied with a slight increase in NPC1, whereas NPC2 was not readily detectable (Figure 7E). The ABCA1 depleted cells had no expression of ABCA1, no change in NPC1 but did have a strong increase in NPC2. Finally, stable shRNA knockdown of ABCA1 in both MM and MCL cells (Supplemental Figure 5) substantially enhanced the cytotoxic effects of JNJ-26854165 in cells such as MAVER-1, JeKo-1 and U266, reducing the IC$_{50}$ 1.79-fold for MAVER-1, 1.6-fold for JeKo-1, and 2.71-fold for U266, with a weaker effect.
of 1.29-fold in OPM-2 in the shRNA knockdown cells (p<0.05) compared to their controls (Figure 8C).
Discussion

Small molecule inhibitors for use in cancer treatment have been at the forefront of developmental therapeutics in recent years. This research focus encompasses a wide array of cellular targets, many of which are oncogenes or cell growth pathways over-expressed in cancer. One particular example of this is the cis-imadazoline analog Nutlin-3, which is a site-specific inhibitor of the interaction between the oncogene HDM-2 and the tumor suppressor p53 (Vassilev et al., 2004). JNJ-26854165 was reported to be a novel HDM-2/p53 interaction inhibitor (Arts et al., 2008), and have activity in cancer cell lines (Chargari et al., 2011; Kojima et al., 2010). Its mechanism of action was also initially borne out by a phase I clinical trial in patients with different solid tumors who, after being treated with JNJ-26854165, showed increased p53 accumulation in skin biopsies and induction of the p53 activation marker macrophage inhibitory cytokine-1 (Tabernero et al., 2011).

We evaluated JNJ-26854165 in MCL and MM cell lines with varying p53 status and found that, unlike the Nutlins, which are significantly active only in wt p53 models, serdemetan showed robust activity against cells containing wt or mutp53. However, when p53 was deleted in MEFs, and compared to p53 and HDM-2 deleted MEFs, there was no change in IC50, suggesting that JNJ-26854165 was not a bona fide HDM-2/p53 interaction inhibitor, but instead had some properties that made it appear so. We did observe induction of p53, HDM-2 and p21 expression in wt p53 cells, and some p53 induction in mutp53, but these cells lacked significant HDM-2 and p21 induction. The most notable effects upon cells was an S-phase cell cycle arrest in both wt p53 and mutp53 cell lines, with cell death being linked to caspase-3 activity primarily in wt p53 bearing cells, and to a lesser extent in cells harboring mutp53.

The use of a JNJ-26854165-resistant MEF cell line indicated the cells had a phenotype reminiscent of that seen in diseases such as Tangiers and Niemann-Pick disease type C.
This phenotype could be recapitulated transiently in drug-naïve cells which, when treated for 24 hours, accumulated vesicles that were found to contain cholesterol. Furthermore, EM demonstrated the accumulation of LW within cells along with diminished cholesterol efflux, coinciding with changes in transcription of SREBF and LXR genes, which regulate cholesterol transporters such as the ABCA1 HDL-c transporter, as well as NPC-1 and -2. NPC-1/2 transcript and protein levels increased in response to JNJ-26854165, whereas ABCA1 showed an increase in transcription only. This was not reflected at the protein level, where ABCA1 was ultimately lost, indicating JNJ-26854165 affects post-translational modification of ABCA1 independent of the transcriptional affect. We suspect that JNJ-26851465 down-regulates ABCA1 expression, resulting in cholesterol accumulation within the cells, when cells sense this, they attempt to compensate by up-regulating SREBF-1/2 and LXR-α/β, and as a result also ABCA1 transcription. However, this is insufficient to reverse the down-regulation of ABCA1 protein, leading to further accumulation of cholesterol to the point that these levels become cytotoxic, resulting in cell death. Modulation of ABCA1 expression levels using shRNAs, and a regulated ABCA1 expression vector, did alter sensitivity to JNJ-26854165, in that reduction of ABCA1 levels reduced the amount of ABCA1 available to transport cholesterol, thereby requiring less JNJ-26854165 to induce cell death. Of particular interest was the up regulation of ABCA1 in the resistant MEF cells, in the absence of NPC1,-2 which suggests the cells enhance ABCA1 expression to circumvent the effect of JNJ-26854165 and supports the idea that ABCA1 is involved in sensitizing cells to this novel agent. We also noted that JNJ-26854165 enhanced the cytoplasmic pool of ABCA1, which no doubt reflects the increase in transcription. What was more intriguing was the simultaneous depletion of the plasma membrane ABCA1 fraction, this could suggest that ABCA1 and its cholesterol cargo is being inhibited from trafficking to the plasma membrane and this blockade could explain the enhanced Filipin staining of cholesterol within the cytoplasm. However, the exact mechanism
through which JNJ-26854165 affects ABCA1 remains to be elucidated, and it is possible that these effects are due to a global impact on cholesterol metabolism and transport genes. Furthermore, despite the higher expression of ABCA1 in the control cells, they still remained sensitive to the drug, albeit with higher IC50’s.

A link between disturbance of cholesterol homeostasis and induction of cell death has been reported previously in melanoma cells treated with the cholesterol transport inhibitor U18666A, and this report suggests that JNJ-26854165 shares some of these properties. The accumulation of cholesterol within cytoplasmic vesicles would suggest that JNJ-26854165 is acting as a lysosomotropic agent and inhibiting cholesterol trafficking and transport, an effect reported for anti-psychotic agents such as pimozide (Wiklund et al., 2010), imipramine (Rodriguez-Lafrasse et al., 1990), and olanzopine (Kristiana et al., 2010). These anti-psychotic agents are cationic amphiphiles, and in fibroblasts induce cholesterol accumulation and suppression of sterol regulatory element binding proteins, resulting in reduced cholesterol synthesis (Kristiana et al., 2010). JNJ-26854165 differs in that it appears to stimulate cholesterol regulatory genes, but simultaneously inhibits cholesterol transport. It is clear that JNJ-26854165 has a broad spectrum of action operating at the lysosome resulting in inhibition of cholesterol transport, and induces a p53 response in wt p53 bearing cells. The mechanism of action of JNJ-26854165 remains undefined, but it provides an interesting insight into the importance of cholesterol for cancer cell growth. This is particularly so in patients with lymphoid malignancies, as they frequently have decreased levels of cholesterol (Pugliese et al., 2006; Tatidis et al., 2001; Vitols et al., 1985), which is thought to be due to the ability of the neoplastic cells to use LDL-c as a growth factor, thereby depleting serum cholesterol levels. Myeloma patients have decreased levels of total cholesterol, as well as HDL-c and LDL-c, compared to normal controls, and this is related to disease progression (Yavasoglu et
al., 2008), and expression of the LDL-R enabling myeloma cells to use LDL-c as a growth factor (Tirado-Velez et al., 2012). Our data clearly shows an abundance of cholesterol transporters in both MM and MCL cells and given the ability of cholesterol to act as a growth factor for malignant cells, disruption of cholesterol availability or production using agents such as U18666A, JNJ-26854165, or statins, may be an attractive therapeutic approach to augment chemotherapy regimens. ABCA1 is up-regulated in advanced prostate cancer patient biopsies, and conveys cells the ability to use HDL-c as a growth factor (Sekine et al., 2010). Drugs such as JNJ-26854165, which inhibit cholesterol transport, or probucol, which inhibits ABCA1 expression and activity (Favari et al., 2004), are well placed for use as targeted approaches in combination chemotherapy by disrupting cholesterol-induced Akt/ERK signaling cascades. Indeed, mevastatin in combination with danorubicin synergized and enhance cell death in vitro in SUP-T1 lymphoma cells (Pugliese et al., 2010). Also, in a phase I trial, pravastatin augmented idarubicin/cytarbine cytotoxicity in acute myeloid leukemia (Kornblau et al., 2007), suggesting disruption of cholesterol metabolism augments the efficacy of chemotherapeutic regimens.
Acknowledgments

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Authorship

Participated in research design: Jones, Vreys, Bashir, Remaley, Orlowski.

Conducted experiments: Jones, Gu, Kuiatse,

Performed data analysis: Jones, Orlowski.

Wrote or contributed to the writing of the manuscript: Jones, Vreys, Bashir, Orlowski.
Conflict of Interest Disclosure

Bashir and Vreys are employees of Janssen Research & Development. Orlowski has served on an advisory board for Johnson & Johnson PRDU, and received research funding from this entity.
References


Footnotes

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Figure Legends

Figure 1. JNJ-26854165 acts independent of HDM-2 in MCL and MM cell lines.

(A) Chemical structure of JNJ-26854165. MCL (B) and MM (C) cell lines were seeded in 96-well plates for viability analyses using WST-1 and treated with JNJ-26854165 for 72 hours. Results are expressed as the percentage of cell viability in relation to the vehicle-treated sample for each cell line, which was arbitrarily set at 100%. (D) Immunoblot analysis was performed on MCL and MM cells treated with serdemetan or DMSO for 48 hours. Protein extracts were probed for their content of p53, HDM-2, and β-actin as a loading control. A representative Western blot is shown of triplicate experiments. (E) MEFs were evaluated for sensitivity to serdemetan using WST-1 as indicated above. Experiments were done in triplicate, and the standard error of the mean (SEM) is shown for each point. An unpaired T test was used to determine statistical significance of p53 and HDM-2 -/- cells compared to the WT MEF control. "**" denotes p values <0.05.

Figure 2. JNJ-26854165 induces an S phase arrest and cell death.

MM and MCL cells with wtp53 and mutp53 were treated with IC_{50} concentrations (determined from the WST-1 assay in Figure 1) of JNJ-26854165 for 48 hours, followed by cell cycle analysis along with caspase-3 and Annexin-V cell death assays. (A) Cell cycle analysis of MM and MCL cells. (B) Viable cell numbers were determined using Annexin-V/TO-PRO exclusion, cell death was determined by counting Annexin-V/TO-PRO positive cells and Caspase-3 activity was measured using the substrate Red-DEVD-FMK. All experiments were performed in triplicate and a representative profile is shown. "**" denotes p<0.05 relative to the vehicle control.

Figure 3. Inhibition of cholesterol transport occurs with JNJ-26854165 treatment.
(A) Wild-type (WT) MEFs and JNJ-26854165-resistant MEFs (165R) were visualized by light microscopy (40x magnification). (B) Fluorescent staining of cholesterol using Filipin in WT and 165R MEFs (40x magnification). (C) 293T cells were treated for 24 hours with JNJ-26854165, U18666A, or pimozide, and stained with Filipin to determine cholesterol localization (40x magnification). Representative images are shown in both panels from one of three independent experiments.

Figure 4. Decreased cholesterol efflux and transport induced by JNJ-26854165 in MM and MCL cells. (A) MM and MCL cell lines were treated with 5μM/L JNJ-26854165 for 24 hours stained with Filipin and cholesterol localization determined using an ImageStream X Mark II (60X magnification). (B) MCL, MM and 293T cell lines were treated with 5μM/L JNJ-26854165 for 24 hours and loaded with NBD-cholesterol, and cholesterol efflux was monitored using a fluorescent plate reader for 24 hours using ApoA-I as an acceptor. “*” denotes p<0.05 relative to vehicle.

Figure 5. JNJ-26854165 induces lipid whorl accumulation similar to that seen in lysosomal storage disease. MM1.S and RPMI-8226 cells were treated for 48 hours with IC50 concentrations of JNJ-26854165 and then analyzed by transmission electron microscopy (7,500 and 25,000 x magnification). Black arrows indicate lipid whorls (LW), MITO indicates mitochondria and CNT indicates centriole.

Figure 6. Modulation of cholesterol regulatory genes and cholesterol transporters is induced by JNJ-26854165. U266 and JeKo-1 cells were treated with JNJ-26854165 and cells harvested at the indicated time points. (A) RNA was extracted and cDNA was synthesized. The cholesterol regulatory genes, SREBF-1 and -2, and LXR-α and -β were
measured, along with the cholesterol transporters NPC-1, NPC-2 and ABCA1, by quantitative real-time PCR using the $\Delta\Delta CT$ method, with the vehicle treated cells used as a relative calibrator and values expressed as RQ. "*" denotes p<0.05 compared to the 0 hour time point using an unpaired $T$ test. (B) Immunoblotting of protein lysates from harvested cells at the indicated time points and probed with the indicated sera. Densitometry values for immunoblotting are shown in supplementary Table 2. (C) JeKo-1 cells were treated with 2 $\mu$M/L JNJ-26854165 (165) or DMSO in combination with cycloheximide and harvested at the indicated time points. Protein lysates were immunoblotted for ABCA1, and expression levels determined using densitometry. Relative ABCA1 half-life normalized to the NT (no treatment) control is shown. "**" denotes p<0.05 comparing vehicle to JNJ-26854165 treated cells using an unpaired $T$ test. (D) 293T, WT and 165R MEF cells were treated with 5$\mu$M/L JNJ-26854165 for 24 hours and lysates evaluated for the indicated cholesterol regulatory genes. All experiments were performed in triplicate and a representative profile is shown. Values represent the mean ± standard error from three independent experiments.

**Figure 7. Depletion of ABCA1 expression enhances sensitivity to JNJ-26854165.**

HeLa cells expressing a tetracycline-off regulatable ABCA1-green fluorescent protein (GFP) were maintained in the absence of doxycycline (DOXY) or with DOXY to suppress ABCA1 expression. (A) Cell viability of HeLa ABCA1-GFP cells treated with JNJ-26854165 (165) for 3 days in the presence of vehicle or DOXY (1 $\mu$g/mL). (B) HeLa ABCA1-GFP cells were treated for 3 days with JNJ-26854165 in the presence or absence of 1 $\mu$g/mL DOXY, and fluorescence microscopy images were captured at 40x magnification. (C) DOXY treated HeLa ABCA1-GFP cells were treated 24 hours with 165 stained with Filipin, cholesterol localization was determined using an ImageStreamX Mark II (60X magnification). (D) Cholesterol efflux was measured in ABCA1-GFP depleted HeLa cells and compared to the
control cells treated with 165 using NBD-cholesterol. "**" denotes p<0.05 relative to vehicle.

**ABCA1-GFP depleted HeLa cells treated with 165 for 24 hours and immunoblotted for ABCA1, NPC1 and NPC2.**

**Figure 8. Suppression of ABCA1 expression enhances JNJ-26854165 sensitivity in MCL and MM cell lines.** MCL and MM cells with stable shRNA knockdown of ABCA1 were treated with JNJ-26854165 for 3 days and cell viability was determined. All experiments were performed in triplicate and an unpaired t test was performed on indicated panels compared to the control, and p values <0.05 are indicated with "**".
Figure 1.

(A) Chemical structure of JNJ-26954465.

(B) Cell viability of various cell lines treated with different concentrations of JNJ-26954465. The x-axis represents the concentration of JNJ-26954465 (µM/L) and the y-axis represents cell viability relative to the vehicle control.

(C) Cell viability of various cell lines treated with different concentrations of JNJ-26954465.

(D) Western blot analysis of HDM-2, p53, p21, and β-actin expression in cell lines with wild-type (wtp53) and mutant-type (mutp53) p53.

(E) IC₅₀ values for JNJ-26954465 in cell lines with wild-type and mutant-type p53.
Figure 2.

(A) wtp53

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Figure 4

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(B)

![Graph showing NBD-cholesterol efflux for different cell lines with bar plots and asterisks indicating significance.](graph.png)
Figure 5.
Figure 6.

(A) U266

(B) U266

(C) JeKo-1

(D) WT MEF

ABCA1 Half-Life
106.2 minutes
66.3 minutes

JeKo-1

+ CHX + CHX

-actin

ABCA1

NPC1

NPC2

HMGcoR

β-actin

Vehicle

165R

293T

Vehicle

165
Figure 7.

(A) 

![Graph showing IC50 of JNJ-26854165 (µM/L)]

- **Vehicle**: 2.42 µM/L
- **DOXY**: 0.50 µM/L

(D) 

![Bar graph showing NBD-cholesterol Eflux (%)]

- **Vehicle**: 40%
- **165**: 20%

(B) 

HeLa ABCA1-GFP TET-off

- **DMSO**: 165 µM/L
- **Vehicle**: 1 µM/L
- **DOXY**: 5 µM/L

(E) 

![Western blot showing ABCA1, NPC1, NPC2, β-Actin]

- **Vehicle**: 165 µM/L
- **DOXY**: 165 µM/L