The Novel Anticancer Agent JNJ-26854165 Induces Cell Death through Inhibition of Cholesterol Transport and Degradation of ABCA1^S

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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 IC_{50} values from 0.25 to 3 μ M/l, in association with an S phase cell cycle arrest. Caspase-3 activation was primarily seen in *wtp53*-bearing cells but also occurred in *mutp53*-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 subfamily 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 depends 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 or high-density lipoprotein (Phillips et al., 1987). Low-density lipoprotein-derived

cholesterol (LDL-c) is transported into cells via the LDL receptor (LDL-R) (Goldstein and Brown, 1984; Yamamoto et al., 1984), whereas high-density lipoprotein-derived cholesterol (HDL-c) enters via scavenger receptor class B member-1 (SR-BI) and is exported by the ATP-binding cassette subfamily A member-1 (ABCA1) (Yancey et al., 2003) and ATP-binding cassette subfamily 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 (Vitolis 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.

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ABBREVIATIONS: 165, JNJ-26854165 (serdemetan); 165R, cells resistant to JNJ-26854165; ABCA1, ATP-binding cassette subfamily A member-1; ABCG1, ATP-binding cassette subfamily G member-1; ERK, extracellular signal-regulated kinase; HDL-c, high-density lipoprotein-derived cholesterol; HDM-2, human double minute 2; LDL-c, low-density lipoprotein-derived cholesterol; LDL-R, low-density lipoprotein receptor; LW, lipid whorl; LXR, liver X receptor; MCL, mantle cell lymphoma; MEFs, mouse embryonic fibroblasts; MM, multiple myeloma; *mutp53*, mutant *p53*; NPC 1/2, Niemann-Pick disease, type C1/2; NBD, 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol; PCR, polymerase chain reaction; RPMIdFBS, RPMI containing 10% delipidated fetal bovine serum; shRNA, short hairpin RNA; SREBF-1/2, sterol regulatory element binding transcription factor 1/2; STR, short tandem repeat; TD, Tangiers disease; U18666A, 3 β -(2-diethylaminoethoxy)androst-5-en-17-one, HCl; WT, wild-type.

Hematologic malignancies overexpress growth-promoting genes found in lipid rafts and cholesterol-rich environments, making them attractive targets for an anticholesterol 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- κ B 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), whereas 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 (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 [β -(2-diethylaminoethoxy)androst-5-en-17-one, HCl] blockades cholesterol transport in melanoma cells, resulting in cell death (Di Stasi et al., 2005), whereas antipsychotic drugs such as pimozide and olanzapine have a profound effect on the growth of lymphoma, neuroblastoma, and 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 (Beerse, Belgium). Pimozide, U18666A, doxycycline, and cycloheximide were from Sigma-Aldrich (St. Louis, MO). Stock solutions of JNJ-26854165 and pimozide were prepared in dimethylsulfoxide, whereas 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), whereas SP-53 was a gift of Masanori Daibata (Kochi University, Kochi, Japan). MEFs containing homozygous p53 deletions, or 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 μ M/l. HeLa ABCA1-tet-off regulatable cells were generated as previously published (Neufeld et al., 2001). All cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich),

100 U/ml penicillin, and 100 μ g/ml streptomycin, except 293T and MEFs, which were grown in Dulbecco's modified Eagle's medium. Cell lines were validated in the M. D. Anderson Cell Line Validation Core Facility by short tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifier Kit (Applied Biosystems, Foster City, CA). The STR profiles were compared with known American Type Culture Collection fingerprints, the Cell Line Integrated Molecular Authentication database version 0.1.200808 (Nucleic Acids Research 37:D925-D932 PMID: PMC2686526), and the MD Anderson fingerprint database.

Real-Time Polymerase Chain Reaction. Total RNA was isolated using an RNeasy Plus kit (Qiagen, Gaithersburg, MD), and cDNA was synthesized using Superscript II (Invitrogen). Real-time polymerase chain reaction (PCR) for ABCA1, sterol regulatory element-binding transcription factors (SREBF)-1 and -2, Niemann-Pick disease, type C1 (NPC1) and NPC2, and liver X receptors- α and - β was performed on a StepOne PCR analyzer (Applied Biosystems) using inventoried real-time Taqman-6-FAM phosphoramidite (FAM) and glyceraldehyde 3-phosphate dehydrogenase-VIC probes. Relative transcript expression 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, α 4 integrin, p53 were from Santa Cruz Biotechnologies, whereas antibodies to ABCA1, ABCG1, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoR), MLN64, NPC1, and NPC2 were from Abcam (Cambridge, MA). Antibodies to phospho-extracellular signal-regulated kinase (ERK)1/2^{Thr202/Tyr204}, Akt^{Ser473}, total ERK, and Akt were purchased from Cell Signaling Technology (Danvers, MA). Additional antibodies included anti- β -actin from Sigma-Aldrich; anti-HDM-2 and p21 antibodies were EMD4Biosciences (Darmstadt, Germany). Cytoplasmic and membrane fractions of U266 cells were prepared after 24 hours of treatment with JNJ-26854165 using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Rockville, MD) as per the manufacturer's instructions. Densitometry was performed on immunoblots after image scanning and analyzed using ImageJ software (NIH, Bethesda, MD); 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 Dickinson, Franklin Lakes, NJ) using FlowJo v.7.6.1 (Tree Star, Inc., Ashland, OR).

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, Milpitas, CA). All flow cytometry was performed using a DAKO CYAN flow cytometer (Beckman-Coulter, Sykesville, MD).

Cell Proliferation Assay. Water-soluble tetrazolium salts-1 (WST-1; Roche Diagnostics, Nutley, NJ) was used to determine median inhibitory concentrations (IC₅₀ values) as previously published (Jones et al., 2008) and the effects of chemotherapeutics (Kuhn et al., 2007). IC₅₀ dose response curves and IC₅₀ values were plotted and calculated using GraphPad Prism 6 (GraphPad, San Diego, CA) using a one site log fit algorithm.

ABCA1 Half-Life Studies. Cycloheximide was added to JeKo-1 cells at 100 μ g/ml, time point samples were harvested, and protein lysates were subjected to Western blotting. Bands were analyzed using ImageJ software.

Gene Silencing. JeKo-1, MAVER-1, U266, and OPM-2 cells with ABCA1 knockdown were created using Mission short hairpin RNA (shRNA) Lentiviral particles (Sigma-Aldrich; TRCN0000029089NM_005502.2-406s1c1). Briefly, cells were plated in 96-V-well plates with 8 μ g/ml PolyBrene (Sigma-Aldrich) and infected for 24 hours. They were then fed with fresh medium, selected with 2 μ g/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 μ M JNJ-26854165, 10 μ M U18666A, or 10 μ M pimozide for 24 hours. Brightfield images were captured in live cultured cells at 40 $\times$ 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), and visualized by ultraviolet fluorescence, and images were captured at 40 $\times$. MM, MCL, and HeLa cells were imaged using an Image-Stream^X Mark II Imaging Flow Cytometer (EMD Millipore, Danvers, MA), with 1000 cells counted per treatment, and visualized at 60 $\times$ magnification.

Cholesterol Efflux Assay. Cells were treated with JNJ-26854165 for 24 hours, washed in phosphate-buffered saline, and loaded with 10 μ g/ml 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (NBD) (Invitrogen) in RPMI containing 10% delipidated fetal bovine serum (RPMIdFBS; EquiTech-Bio, Kerrville, TX) for 2 hours. Cells were washed twice in RPMIdFBS, and cholesterol efflux was initiated by addition of RPMIdFBS containing 20 μ g/ml apolipoprotein A-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 and 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 Wetzlar, Germany), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA). Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp., Woburn, MA) through the MD Anderson High Resolution Electron Microscopy Facility.

Results

JNJ-26854165 Acts Independently of HDM-2. JNJ-26854165 (serdemetan) (Fig. 1A) was previously reported to inhibit HDM-2 function and activate p53 (Arts et al., 2008; Patel and Player, 2008; Kojima et al., 2010). To evaluate its

Fig. 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 dimethylsulfoxide 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 S.E.M. is shown for each point. An unpaired *t* test was used to determine statistical significance of p53 and HDM-2 $-/-$ cells compared with the WT MEF control. **P* values < 0.05.

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 IC₅₀ values (calculated using a one site log fit algorithm) in the 0.25–2 μ M/l range, whereas *wt*p53 MM cells had IC₅₀ values from 1.43 to 2.22 μ M/l (Fig. 1, B and C; Supplemental Table 1). Mutant (*mut*) p53 MCL cells had IC₅₀ values from 0.83 to 2.23 μ M/l and *mut*p53 MM cells from 2.37 to 2.48 μ M/l, whereas the epithelial cell line 293T had an IC₅₀ of 1.59 μ M/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 (Fig. 1D, upper panel). In contrast, in *mut*p53 cell lines, although 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 (Fig. 1D, lower panel). 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. Although p53^{-/-} MEFs were more resistant to JNJ-26854165 than their *wt* counterparts (IC₅₀ 19.95 versus 3.87 μ M/l; $P < 0.05$), the HDM-2 and p53 knockout MEFs showed an IC₅₀ of 19.62 μ M/l ($P < 0.05$) (Fig. 1E). Thus, although 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 *wt*p53-bearing cells to JNJ-26854165 for 48 hours induced an S-phase cell cycle arrest compared with the vehicle control (Fig. 2A, left panel). MAVER-1 *mut*p53 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 (Fig. 2A, right panel). To determine the degree of cell death induced by JNJ-26854165, we used a fluorescent caspase-3 substrate and performed Annexin-V staining in combination with TO-PRO-3 to discriminate

Fig. 2. JNJ-26854165 induces an S-phase arrest and cell death. MM and MCL cells with *wt*p53 and *mut*p53 were treated with IC₅₀ concentrations (determined from the WST-1 assay in Fig. 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. DMSO, dimethylsulfoxide. * $P < 0.05$ relative to the vehicle control.

between viable and dead cells. In *utp53*-bearing cells, JNJ-26854165 induced 60–90% cell death in MM1.S and GRANTA-519 cells, which coincided with caspase-3 activation seen in 55–65% of cells (Fig. 2B, left panel). H929 cells also had caspase-3 activation and increased cell death, albeit at a lower level. The *mutp53* cell models showed 25–60% cell death, which strongly correlated with caspase-3 activity in JeKo-1, U266, and RPMI-8226 (Fig. 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 perinuclear vacuoles not seen in drug-naïve MEFs (Fig. 3A). This phenotype was reminiscent of the cholesterol-loaded endosomes found in the inherited cholesterol transport disorders TD (Assmann and Brewer, 1995) and Niemann-Pick disease (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, whereas the 165R MEFs displayed intense perinuclear staining of cholesterol localized to vesicles within the cytoplasm (Fig. 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 with the membranous distribution seen in the controls (Fig. 3C). Treatment with the cholesterol transport inhibitor, U18666A, or pimozone also resulted in accumulation of cholesterol in vesicles within the

cytoplasm similar to that of the JNJ-26854165-treated cells (Fig. 3C). Filipin staining of the JeKo-1, MAVER-1, OPM-2, and U266 indicated that JNJ-26854165 induced strong perinuclear accumulation of cholesterol (Fig. 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 having a 10–25% decrease in cholesterol efflux, whereas the 293T cells had a 17% decrease in cholesterol efflux (Fig. 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 (Supplemental Fig. 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. 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 (Fig. 5) (Parkinson-Lawrence et al., 2010). Similar LW structures were also observed in JVM-2 and JeKo-1 drug-treated cells (Supplemental Fig. 2). These data support the possibility that serdemetan is mimicking the effects of cholesterol transport inhibitors such as pimozone (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

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

Fig. 4. Decreased cholesterol efflux and transport induced by JNJ-26854165 in MM and MCL cells. (A) MM and MCL cell lines were treated with 5 μ M JNJ-26854165 for 24 hours stained with Filipin and cholesterol localization determined using an ImageStream^X Mark II (60 $\times$ magnification). (B) MCL, MM, and 293T cell lines were treated with 5 μ M JNJ-26854165 for 24 hours and loaded with NBD-cholesterol, and cholesterol efflux was monitored using a fluorescent plate reader for 24 hours using apolipoprotein A-I as an acceptor. DMSO, dimethylsulfoxide. * P < 0.05 relative to vehicle.

regulatory genes SREBF-1 and -2 and the liver X receptor (LXR)- α and - β . JNJ-26854165 induced a 1.5-fold increase in SREBF-1 and -2 in U266 at 24 hours, whereas LXR- α / β was induced by over 1.5-fold at 24 hours and remained so compared with the baseline at 72 hours (Fig. 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, whereas 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 (Fig. 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 (Fig. 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 (Fig. 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 quantitative PCR assay in JeKo-1, with increases in NPC-1 (3-fold) and -2 (4-fold) occurring at 48 hours post-treatment with JNJ-26854165 (Fig. 6B; Supplemental Table 2), whereas 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 after 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, whereas in JeKo-1 cells, HMGCoR

Fig. 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 IC_{50} concentrations of JNJ-26854165 and then analyzed by transmission electron microscopy (7,500 and 25,000 $\times$ magnification). Black arrows indicate LW, MITO indicates mitochondria, and CNT indicates centriole.

increased 2-fold only at 72 hours (Fig. 6B; Supplemental 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 with 66.3 minutes in treated cells ($P < 0.05$) (Fig. 6C). We also evaluated the effect of JNJ-26854165 at 24 hours on the wild-type (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 MEFs treated with JNJ-26854165 (Fig. 6D). ABCA1 was strongly induced by JNJ-26854165 treatment in the WT MEF; interestingly the 165R MEF had strong expression of ABCA1 compared with the WT counterparts and no changes in expression of NPC1/2, and ABCG1 (Fig. 6D). The 293T cells had low level expression of ABCA1 compared with 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 were detectable in the MLN64 cholesterol transporter with JNJ-26854165 treatment (Fig. 6D).

We also evaluated if JNJ-26854165 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 (Supplemental Fig. 3A). Treatment with JNJ-26854165 for 24 hours actually increased the cytoplasmic ABCA1 pool and decreased the plasma membrane pool (Supplemental Fig. 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 downregulated phosphorylated Akt

and ERK in MAVER-1, JeKo-1, OPM-2, and U266 cells (note U266 had no detectable basal phosphorylated ERK) (Supplemental Fig. 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 with 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 cells lines tested) (Supplemental Fig. 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 with the other cholesterol transporters (Supplemental Fig. 3C). Overall these data suggest JNJ-26854165 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 (Supplemental Fig. 4A), and when cells were treated with JNJ-26854165, the IC_{50} of the ABCA1-depleted cells fell to 0.50 μ M, compared with 2.42 μ M in the control cells ($P < 0.05$) (Fig. 7A). Evaluation of the cells by fluorescence microscopy further supported the enhanced sensitivity of

Fig. 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 relative transcript expression. * $P < 0.05$ compared with 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 Supplemental Table 2. (C) JeKo-1 cells were treated with 2 μ 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 were determined using densitometry. Relative ABCA1 half-life normalized to the NT (no treatment) control is shown. * $P < 0.05$ comparing vehicle to JNJ-26854165-treated cells using an unpaired t test. (D) 293T, WT, and 165R MEF cells were treated with 5 μ 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 $\pm$ standard error from three independent experiments. CHX, cycloheximide; DMSO, dimethylsulfoxide; RQ, relative quantification.

the ABCA1-depleted cells (Fig. 7B). Filipin staining of HeLa ABCA1-green fluorescent protein cells treated with JNJ-26854165 for 24 hours demonstrated accumulation of cholesterol in the cytoplasm versus membrane staining in the vehicle-treated cells (Fig. 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 with 20% efflux in the ABCA1-depleted cells (Fig. 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 (Supplemental Fig. 4B). JNJ-26854165 treatment of 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 (Fig. 7E). The ABCA1-depleted cells had no expression of ABCA1 and 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 Fig. 5)

Fig. 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 μg/ml). (B) HeLa ABCA1-GFP cells were treated for 3 days with JNJ-26854165 in the presence or absence of 1 μg/ml DOXY, and fluorescence microscopy images were captured at 40× magnification. (C) doxycycline (DOXY)-treated HeLa ABCA1-GFP cells were treated 24 hours with 165 stained with Filipin, cholesterol localization was determined using an ImageStream[®] Mark II (60× magnification). (D) Cholesterol efflux was measured in ABCA1-GFP-depleted HeLa cells and compared with the control cells treated with 165 using NBD-cholesterol. **P* < 0.05 relative to vehicle. (E) ABCA1-GFP-depleted HeLa cells treated with 165 for 24 hours and immunoblotted for ABCA1, NPC1, and NPC2. DMSO, dimethylsulfoxide; TET, tetracycline.

substantially enhanced the cytotoxic effects of JNJ-26854165 in cells such as MAVER-1, JeKo-1, and U266, reducing the IC₅₀ 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 with their controls (Fig. 8).

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 overexpressed in cancer. One particular example of this is the *cis*-imidazole 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 (Kojima et al., 2010; Chargari et al., 2011). 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

Fig. 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 with the control, and *P* values < 0.05 are indicated with *.

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 with *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 effect on 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 that, when treated for 24 hours, accumulated vesicles that were found to contain cholesterol. Furthermore, electron microscopy 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-26854165 down-regulates ABCA1 expression, resulting in cholesterol accumulation within the cells; when cells sense this, they attempt to compensate by upregulating *SREBF-1/2* and *LXR-α/β* and, as a result, also ABCA1 transcription. However, this is insufficient to reverse the downregulation 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 upregulation 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 are 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 values.

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 antipsychotic agents such as pimozide (Wiklund et al., 2010), imipramine (Rodriguez-Lafraze et al., 1990), and olanzopine (Kristiana et al., 2010). These antipsychotic 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 *wtp53*-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 (Vitols et al., 1985; Tatidis et al., 2001; Pugliese et al., 2006), 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 with 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 show 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 upregulated 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 enhanced cell death in vitro in SUP-T1 lymphoma cells (Pugliese et al., 2010). Also, in a phase I trial, pravastatin augmented idarubicin/cytarabine cytotoxicity in acute myeloid leukemia (Kornblau et al., 2007), suggesting disruption of cholesterol metabolism augments the efficacy of chemotherapeutic regimens.

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Authorship Contributions

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