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A Small Molecule Inhibitor of the BLM Helicase Modulates Chromosome Stability in Human Cells

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SUMMARY

The Bloom's syndrome protein, BLM, is a member of the conserved RecQ helicase family. Although cell lines lacking BLM exist, these exhibit progressive genomic instability that makes distinguishing primary from secondary effects of BLM loss problematic. In order to be able to acutely disable BLM function in cells, we undertook a high throughput screen of a chemical compound library for small molecule inhibitors of BLM. We present ML216, a potent inhibitor of the DNA unwinding activity of BLM. ML216 shows cell-based activity and can induce sister chromatid exchanges, enhance the toxicity of aphidicolin, and exert antiproliferative activity in cells expressing BLM, but not those lacking BLM. These data indicate that ML216 shows strong selectivity for BLM in cultured cells. We discuss the potential utility of such a BLM-targeting compound as an anticancer agent.

INTRODUCTION

DNA helicases are ATP-dependent enzymes that separate the complementary strands of duplex DNA (Soultanas and Wigley, 2001). Several families of helicases have been identified, including the RecQ helicases, which are conserved between bacteria, yeasts, and all higher eukaryotes (Bachrati and Hickson, 2003). In human cells, there are five RecQ family members, designated RECQ1, RECQ4, RECQ5, BLM, and WRN, all of which contain a centrally located domain with seven signature motifs found in the so-called Superfamily II helicases (Chu and Hickson, 2009). These motifs include the Walker A and B box sequences required for the binding and hydrolysis of ATP that drives DNA strand separation. Mutation of any of the three genes encoding human RecQ proteins (*BLM*, *WRN*, and *RECQ4*) gives rise to a defined genetic disorder associated with premature aging and/or cancer predisposition (Chu and Hickson, 2009). Cells from affected individuals in all cases show excessive chromosomal instability. One of these conditions, Bloom's syndrome (BS) is caused by mutation of the *BLM* gene, which is located on chromosome 15q26.1 in humans (Ellis et al., 1995).

BS is characterized by predisposition to the development of cancer, growth retardation, immunodeficiency, sunlight sensitivity, and fertility defects. Leukemia and lymphoma are the most commonly observed cancer types, but the full spectrum of cancers in the normal population is evident in BS (German, 1997). Although the cells from many BS individuals lack expression of the BLM protein, it is clear that BLM function is essential for viability in mice, with homozygous deletion of the gene leading to embryonic lethality by day 13.5 (Chester et al., 1998). Cells from BS patients exhibit extensive chromosome instability. This is manifested as increased numbers of chromatid gaps and breaks, as well as chromosome structural rearrangements, including symmetrical quadriradials, micronuclei, anaphase bridges, and lagging chromosomes (Chu and Hickson, 2009). The characteristic cellular feature, used in the molecular diagnosis of BS, is an increase in the frequency of sister chromatid exchanges (SCEs) (Chaganti et al., 1974).

BLM protein displays an ATP-dependent, 3′-5′ DNA helicase activity that can unwind a variety of DNA substrates that can arise during DNA replication and repair. These include various types of partial duplex DNA substrates, which are unwound in a DNA structure-specific manner (Mohaghegh et al., 2001). BLM also exhibits an activity that can branch migrate three- and four-way DNA junctions that model a DNA displacement loop (D-loop) and a Holliday junction recombination

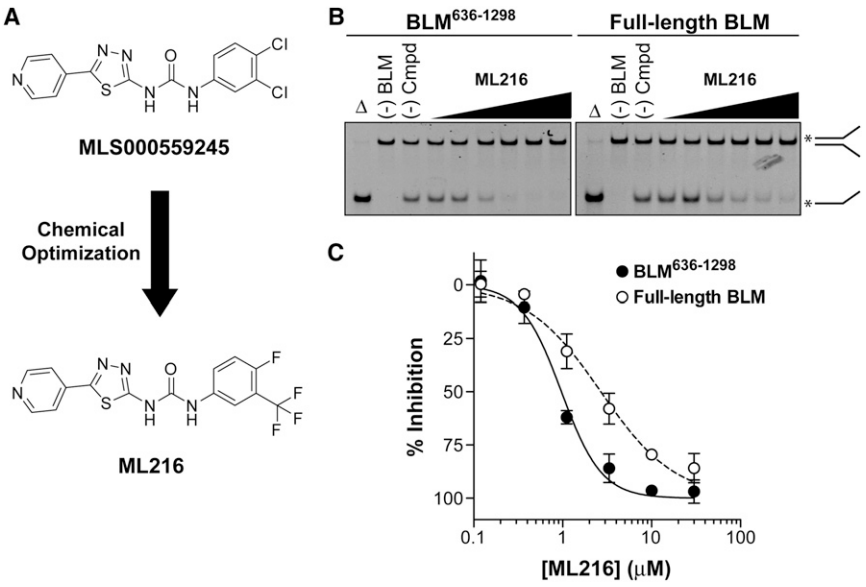

Figure 1. ML216 Is an Inhibitor of BLM Activity In Vitro

(A) Structure of MLS000559245 that was identified during the high-throughput screen and ML216, which was developed through subsequent medicinal chemistry optimization of this chemotype.

(B) Effects on ML216 on the helicase activity of $BLM^{636-1298}$ and full-length BLM. In the absence of ML216, BLM unwinds the forked duplex into ssDNA (depicted diagrammatically on the right). The open triangle above the left lane in each case depicts heat-denatured DNA. The asterisk denotes fluorescent end label.

(C) Quantification of the extent of BLM inhibition by ML216. Data represent the average of three independent experiments. Error bars: SD.

See also Figure S1.

intermediate, respectively (Bachrati et al., 2006; van Brabant et al., 2000). Finally, BLM can disrupt some non-B-form DNAs, such as G-quadruplexes (Sun et al., 1998). To perform these functions, BLM associates with conserved partner proteins, including topoisomerase IIIa and RMI1 and 2 (Brosh et al., 2000; Chang et al., 2005; Meetei et al., 2003; Singh et al., 2008; Wu et al., 2000, 2001; Yin et al., 2005).

Although it is possible to study BLM function using BS cell lines, these cells exhibit ongoing genomic instability making it difficult to distinguish primary from secondary consequences of BLM loss. Attempts to overcome this problem have focused on the use of small interfering RNA (siRNA)/small hairpin RNA (shRNA)-mediated depletion of BLM, which suffers from an inability to acutely disable BLM and requires a timescale of several days to be effective. We reasoned, therefore, that a small molecule inhibitor of BLM would permit a number of studies to be undertaken that cannot be conducted with existing cell lines. Moreover, it was our intention to identify "molecular probes"' of BLM function that could be used not only to conduct synthetic lethality type screens in human cells, but also to serve as lead compounds in the search for potential anticancer agents.

In this study, we report the characterization of a small molecule inhibitor of BLM, identified through high-throughput screening (HTS) and medicinal chemistry optimization. This compound, designated ML216, is potent and acts through competitive inhibition of the DNA binding activity of BLM. In cell-based assays, ML216 induces SCEs in cells containing BLM, but not in otherwise isogenic, BLM-deficient cells. We discuss potential therapeutic uses of BLM-targeting compounds derived from ML216 in the treatment of certain tumor types.

RESULTS

A High-Throughput Screen for Inhibitors of BLM

We developed a fluorescence-quenching assay (Figure S1 available online) to monitor DNA strand separation catalyzed by BLM that was adapted for quantitative, high-throughput screening (qHTS) for inhibitors of BLM (see Experimental Procedures). The assay utilized a dual-labeled, partial duplex DNA substrate ("forked" duplex) in which the fluorescence of a rhodamine-type moiety is normally suppressed by a nonemitting dark quencher (BHQ-2) present on the opposite strand. This quenching is lost following the ATP-dependent unwinding of the two DNA strands by the BLM helicase and hence a fluorescent signal is produced (Figures S1A and S1B). This assay was miniaturized to a 1,536-well format and used to screen the Molecular Libraries Small Molecule Repository library of approximately 350,000 compounds (Figure S1C). The inhibitory activity of the compounds was monitored as a diminution in the fluorescent signal compared to that from the negative control sample containing DMSO alone. Dose-response curves of potential BLM inhibitors from the primary screen are shown in Table S1. Primary screening hits were analyzed in a more traditional helicase assay, where separation of a radiolabeled, forked duplex substrate into single-stranded DNA (ssDNA) products is measured using PAGE. Of the screening hits that showed activity in both the fluorescence- and radioactivity-based assays, compound MLS000559245 was selected for further medicinal chemistry optimization directed primarily at improving physicochemical properties such as solubility and cell permeability. An optimized analog, ML216 (Figure 1A), emerged as the lead molecule for the present study. Further details of the optimization process will be published elsewhere.

ML216 Is a Potent Small Molecule Inhibitor of BLM

In the qHTS assay, we used a truncated variant of BLM that lacks the N-terminal 635 residues and the C-terminal 119 residues of BLM (hereafter called $BLM^{636-1298}$) in order to meet the requirement for milligram quantities of recombinant BLM in the large-scale screen, which cannot be generated for the full-length BLM protein. Although the truncated protein is fully functional as a helicase, we wanted to confirm that ML216 is also able to inhibit the activity of full length BLM, and to do so in a concentration-dependent manner. We found that ML216 is a potent inhibitor of both full-length BLM and $BLM^{636-1298}$, showing similar IC_{50} values of 3.0 and 0.97 μM, respectively (Figures 1B and 1C; Table 1).

Table 1. Inhibition Selectivity of ML216 against Related Helicases

Helicase	IC_{50} (μM)[a]	95% Confidence Interval
$BLM^{636–1298}$	0.97	0.8–1.2
$BLM^{full-length}$	2.98	2.2–4.0
WRN^{short}	12.60	10.7–14.8
WRN^{long}	5.00	4.6–5.5
RecQ1	>50	NA
RecQ5	>50	NA
UvrD	>50	NA

NA, not applicable.
[a]Determined by gel-based DNA unwinding assay.

BLM has a broad substrate range in addition to its ability to unwind the forked duplex DNA used in the initial screen. Additional substrates include DNA displacement loops (D-loops), Holliday junctions and G-quadruplexes (Bachrati et al., 2006; Ellis et al., 1995; Mohaghegh et al., 2001; Sun et al., 1998). In at least the case of the Holliday junction, BLM exerts its effect on the DNA via promoting branch migration of the four-way DNA junction, and not by catalyzing DNA strand separation. Moreover, we have provided evidence previously that BLM might disrupt the mobile D-loop substrate used here via migration of the three-way junction (Bachrati et al., 2006). We asked, therefore, whether ML216 is able to inhibit BLM's action on a D-loop, a G-quadruplex, or a synthetic Holliday junction. We found that ML216 could inhibit disruption of the D-loop and Holliday junction substrates, although only at high concentrations (50 μM) (Figures S1D and S1F). Moreover, only very modest inhibition of BLM's G-quadruplex disrupting activity was observed (Figure S1E). These data suggest that ML216 is much more potent as an inhibitor of BLM's strand separation activity on a forked duplex substrate than it is as an inhibitor of either branch migration of DNA junctions or G-quadruplex DNA disruption.

Next, we asked whether ML216 could inhibit other human RecQ family helicases, which are closely homologous to BLM in their helicase domains. In parallel, we also tested a putative negative control, *Escherichia coli* UvrD helicase, which is a very distantly related enzyme belonging to helicase Superfamily I. ML216 showed only very modest inhibition of the helicase activity of *E. coli* UvrD or human RECQ1 and RECQ5, even at a concentration of 50 μM (Table 1). ML216 did, however, show inhibition of the WRN helicase. ML216 inhibited both the full length WRN and a truncated WRN derivative ($WRN^{500-946}$), with the former being 2.5-fold more sensitive to inhibition (based on IC_{50} values). BLM was a little more sensitive than WRN to inhibition by ML216 (1.7-fold based on IC_{50} values; Table 1). Despite the detectable inhibition of WRN by ML216, this compound appears selective for BLM in human cells, as we discuss below.

Mechanism of Action of ML216

ML216 could, in principle, inhibit BLM via a variety of different mechanisms such as by competing with ATP binding, the driving force behind its DNA unwinding, or by preventing BLM from binding to DNA. To investigate the mechanism of action of ML216, we analyzed its effects on DNA binding by BLM using an electrophoretic mobility shift assay (EMSA). This analysis provided clear evidence of displacement of either ssDNA or forked duplex DNA from a complex with BLM with increasing concentrations of ML216 (Figures 2A, S2A, and S2B). To confirm this finding, fluorescence polarization analysis was undertaken, where the binding of BLM to a fluorescently labeled oligodeoxynucleotide corresponding to the upper strand of the forked duplex substrate (Table S2; A1) was monitored in the presence of a dilution series of ML216. This test also showed that ML216 inhibits the ability of BLM to bind DNA (Figure 2B). Taken together, these data strongly suggest that ML216 competes with the DNA for binding to BLM.

BLM requires ssDNA binding for its ATPase activity. Consistent with ML216 being able to disrupt BLM's binding to DNA, rather than being an inhibitor of ATP binding by BLM, the inhibition of ssDNA-dependent ATPase activity by ML216 (K_i of 1.76 ± 0.26 μM) was noncompetitive with respect to ATP (Figure S2C).

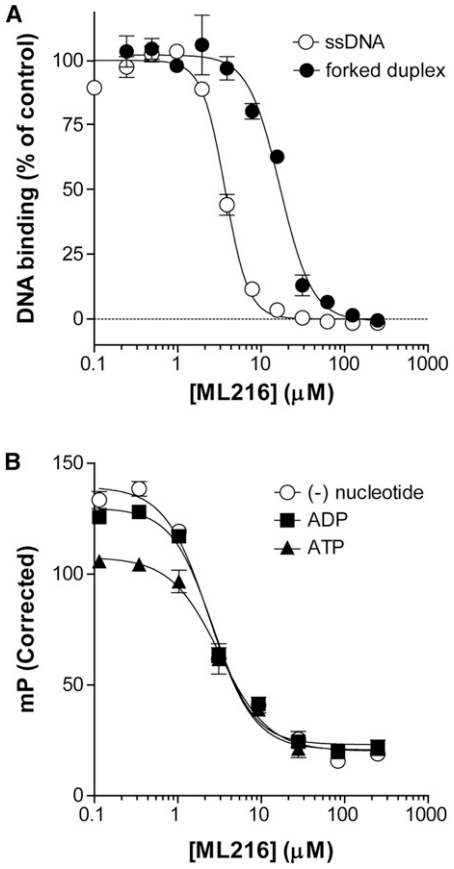

Figure 2. ML216 Affects the DNA Binding Activity of BLM

(A) Effect of ML216 on binding BLM to ssDNA or a forked duplex using EMSA. Data were derived from two independent gel retardation experiments. Error bars: SE.

(B) As in (A), except effects on DNA binding measured using fluorescence polarization (FP). Measurements were performed in the absence of nucleotide or in the presence of ADP or ATP, as indicated. Data were derived from three independent experiments.

Error bars: SD. See also Figure S2.

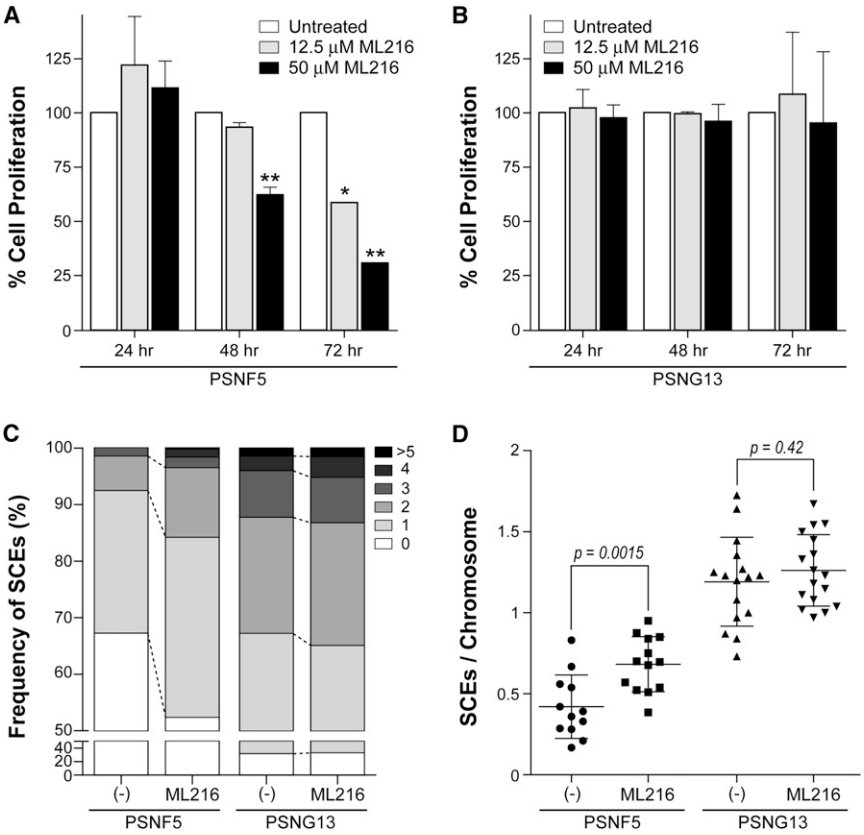

Figure 3. ML216 Has Activity in Human Cells that Depends on BLM

(A and B) Effects of different concentrations of ML216, as indicated, on the rate of cell proliferation in PSNF5 (BLM^+; A) and PSNG13 (BLM^-; B) cells. Data represent the means of three independent experiments. Error bars: SD. $^{**}p < 0.01$; $^{*}p < 0.05$.

(C and D) Effects of ML216 on SCE levels. PSNF5 or PSNG13 cells were untreated or treated with ML216, as indicated below the bars, and the frequency of SCEs per chromosome (C) and the number of SCEs per chromosome (D) were determined.

See also Figure S3.

ML216 Is Active in Cultured Human Cells

For ML216 to be an inhibitor with broad utility, it would have to be cell membrane permeable and capable of inhibiting the function of BLM in living cells. To investigate this, we compared the effects of ML216 in two cell lines that were derived from GM08505, a fibroblast cell line isolated from a patient with BS. One cell line, PSNG13, has been transfected with an empty vector alone, and therefore lacks BLM. The other, PSNF5, is isogenic to PSNG13, but has been transfected with the *BLM* cDNA. Previous studies showed that such re-expression of BLM in the PSNF5 cells corrects the hyper-SCE phenotype of the parental GM08505 cells (Gaymes et al., 2002). Using this isogenic pair of cells, we sought to identify whether ML216 could influence cell growth and SCE induction in a BLM-specific manner. We found that ML216 was an inhibitor of the proliferation of PSNF5 cells, but not of PSNG13 cells, and did so in a concentration-dependent manner (Figures 3A and 3B). Moreover, ML216 treatment led to a statistically significant increase in the frequency of sister chromatid exchanges (SCEs) in PSNF5 cells, but not in PSNG13 cells (Figures 3C and 3D).

In each experiment conducted thus far, the exposure time of cells to ML216 was 24–72 hr. However, for ML216 to have broad utility in cellular studies, it would ideally have to inhibit BLM during only a short treatment period. Hence, we asked whether ML216 could induce SCEs in PSNF5 cells if present only during the last 8 hr of the experiment prior to collection of cells in metaphase. That way, BLM would be targeted by ML216 only during the second S-phase of exposure to bromodeoxyuridine (BrdU), because SCE analysis requires that BrdU be present during two consecutive S-phases before cells are arrested with colcemid and metaphases are harvested. We found that this shorter exposure led to a statistically significant increase in the frequency of SCEs in PSNF5 cells expressing BLM (Figure S3A).

PSNG13 cells are hypersensitive compared to PSNF5 cells to inhibitors of DNA replication, such as aphidicolin, an inhibitor of replicative DNA polymerases (Davies et al., 2004). We asked, therefore, whether ML216 might selectively sensitize PSNF5 cells to aphidicolin, presumably by inhibiting BLM. As shown in Figure 4A, ML216 increased the sensitivity of PSNF5 cells to aphidicolin ($p < 0.01$) but had no sensitizing effect on isogenic PSNG13 cells devoid of BLM.

Previous studies (Chu et al., 2010) using mouse embryonic stem (ES) cells have indicated that BLM is required to convert mitomycin-C-induced DNA damage (likely DNA interstrand crosslinks) into double-strand breaks (DSBs), which can be detected by immunofluorescent staining of cells for the phosphorylated version of histone H2AX (termed γ-H2AX). We found that PSNF5 cells showed a substantially higher frequency of γ-H2AX nuclear foci in response to mitomycin C (MMC) exposure than did PSNG13 cells, in agreement with the previous study of mouse ES cells (Chu et al., 2010). Consistent with ML216 targeting BLM in living cells, we observed a statistically significant reduction in γ-H2AX focus formation in PSNF5 cells treated with the combination of ML216 and MMC compared to PSNF5 cells treated with MMC alone (Figures 4B and S3B; $p < 0.05$). As expected, ML216 showed no significant effect on the control PSNG13 cells, with or without MMC treatment (Figures 4B and S3B; $p > 0.05$) because these cells display no γ-H2AX foci following this MMC treatment regimen.

The cellular data presented thus far indicate that ML216 can target BLM in human cells, but do not rule out the possibility that it also targets WRN, given that WRN protein is inhibited by ML216 in vitro. To investigate this, we tested whether fibroblasts lacking WRN were differentially sensitive to ML216 compared to wild-type control fibroblasts. We found that ML216 inhibited proliferation of WRN^+ and WRN^- cells equally well, and similarly sensitized both cell types to aphidicolin (Figure S4). Taken

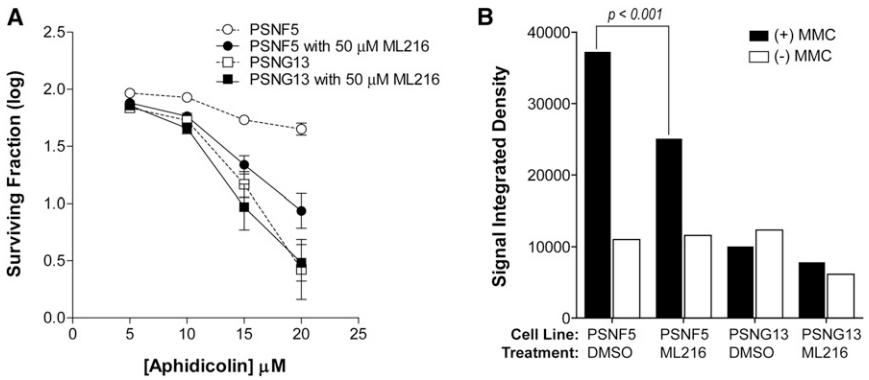

Figure 4. ML216 Affects the Cellular Response to DNA Damage Stress in a BLM-Dependent Manner

(A) ML216 sensitizes PSNF5, but not PSNG13, cells to aphidicolin. Survival curves derived from clonogenic assays are shown from each cell line in the absence or presence of ML216. Cells were exposed to ML216 for 24 hr before aphidicolin was added at the concentrations indicated and the cells were incubated for a further 24 hr. Cells were then washed and incubated in drug-free medium for up to 12 days to allow individual colonies to form. Points represent the average of three independent experiments. Error bars: SD.

(B) ML216 suppresses γ-H2AX focus formation in MMC-treated PSNF5 cells, but not similarly treated PSNG13 cells. Integrated fluorescence density on a minimum of 75 cells in each case was determined using ImageJ software (NIH).

See also Figure S4.

together, these data indicate that ML216 can specifically inhibit the function of BLM in human cells.

DISCUSSION

In this study, we report the identification and characterization of a small molecule inhibitor of the human BLM helicase. This compound, designated ML216, resulted from medicinal chemistry-based optimization of compound MLS000559245, which was identified via high throughput screen of a library of "drug-like" molecules. Based on our analysis of ML216 in human cell lines, we conclude that the compound is membrane permeable and can target BLM in living cells. The general lack of any effects of ML216 on a BS cell line, which does not express BLM, is strongly suggestive that the primary target for ML216 in human cells is BLM.

The initial screen was conducted using a truncated variant of BLM, $BLM^{636-1298}$, which was prepared from bacteria. Nevertheless, we confirmed that full-length recombinant BLM (isolated from yeast cells) was inhibited to a similar degree based on IC_{50} values. This strongly suggests that ML216 targets the central "catalytic core" of BLM contained in both recombinant BLM preparations comprising the helicase, RQC, and HRDC domains. Given the strong selectivity of ML216 for BLM compared to human RECQ1 and RECQ5, we believe that it must target a structural feature of BLM that is poorly conserved, even among many human RecQ family helicases. Although the inhibition of WRN seen in helicase assays indicates that ML216 can target this closely related helicase (albeit, with a somewhat lower potency), the cell-based assay results probing many different functions of BLM indicate strongly that ML216 does not interfere significantly with WRN function in vivo. Despite this contention, it is still possible that ML216 targets WRN in vivo, yet does not have any effect on the cellular endpoints analyzed here, including proliferation and aphidicolin sensitivity. However, the inhibition of WRN did also potentially reveal an interesting feature of the mode of action of ML216. We found that a truncated (but catalytically active) WRN was inhibited weakly by ML216, while full length WRN was more sensitive. In contrast, both truncated BLM (residues 636–1298) and full length BLM were inhibited with similar IC_{50} values. Given that the truncated WRN retains the helicase domain, these data suggest that residues outside of the helicase core of WRN might participate in DNA binding/unwinding and be targeted by ML216. A likely candidate is the RQC domain. Unfortunately, we currently only have access to the high resolution structure of human RECQ1 (Pike et al., 2009), and not of BLM, WRN, or RECQ5, and therefore more detailed analysis of the binding region for ML216 on BLM will have to await new structural studies.

We have provided mechanistic insight into how ML216 targets BLM. ML216 could inhibit the DNA-dependent ATPase activity of BLM, but did so in a noncompetitive manner with respect to ATP. The use of both gel retardation and fluorescence polarization assays revealed that ML216 inhibits DNA binding by BLM, which would be sufficient to block its helicase activity. DNA binding, particularly of partial duplex DNA structures such as the forked duplex studied extensively here, seems to require both the helicase and RQC domains of RecQ helicases (Pike et al., 2009). Moreover, the HRDC domain is known to influence DNA substrate specificity of BLM by altering its affinity for different DNA structures (Wu et al., 2005). ML216 showed preferential inhibition of the unwinding of a forked duplex by BLM. Its ability to inhibit disruption of D-loops, three-/four-way junctions, and G-quadruplexes required approximately 10-fold higher drug concentrations. This pronounced DNA substrate-structure dependency of inhibition, combined with the EMSA and fluorescence polarization studies, argue strongly for a selective engagement of ML216 into the BLM-nucleic acid substrate binding site.

During the cellular analyses of the effects of ML216, we analyzed whether this compound, through targeting BLM, might activate cell-cycle checkpoint responses. However, we obtained no evidence to suggest that ML216 did so. Exposure of cells for 24 hr to 50 μM ML216 did not cause cell-cycle arrest in G1/S or G2/M, and did not appear to cause phosphorylation of well-established ATM or ATR targets, including CHK1 and CHK2 (data not shown). This is consistent with a lack of requirement for BLM to activate these checkpoints in human cells.

We have presented evidence that ML216 modulates the role of BLM in genome maintenance in human cells. The most revealing

phenotypic effect of ML216 treatment of cells was an increase in the frequency of SCEs. Crucially, this induction of SCEs was seen only in cells expressing BLM and not in otherwise isogenic cells lacking BLM. Given the importance of BLM in suppression of SCEs in human cells (Chaganti et al., 1974; Chu and Hickson, 2009), this increase in SCE argues strongly for on-target effects of ML216. An interesting feature of the action of ML216 is its ability to partially inhibit proliferation of cells expressing BLM but not those lacking BLM. It is clear from analysis of cell lines isolated from BS patients that *BLM* is not an essential gene in humans (unlike in mice) (Chester et al., 1998). Nevertheless, as has been observed in numerous other cases, acute inactivation of enzyme function often has more deleterious effects than does removing the protein altogether. This may be due to secondary effects on protein complexes in which the target protein participates. Our in vitro biochemical data indicated that ML216 could inhibit WRN nearly as efficiently as it does BLM. Nevertheless, our cellular studies using cell lines lacking either BLM or WRN indicated that the effects of ML216 on cell proliferation, aphidicolin sensitivity, and SCE induction were consistent with BLM being the primary target of the compound, and not WRN.

Very recently, Aggarwal et al. (2011) characterized a set of small molecule inhibitors of WRN. The compound analyzed in most detail in that study (NSC 19630) was highly toxic to human cells in culture at low micromolar concentrations. We currently do not know why we did not see any antiproliferative effects of ML216 on cells lacking BLM, even though ML216 inhibits WRN with a similar potency to that of NSC 19630. Of note, NSC 19630 is a maleimide derivative and therefore likely to be chemically reactive to the free cysteine thiol groups present on several proteins, as well as on nonprotein cellular components, such as intracellular glutathione, a recognized mediator of cellular response to oxidative stress. It is possible, therefore, that these effects combined could conspire to produce the toxicity observed with NSC 19630.

SIGNIFICANCE

We have identified a small molecule inhibitor of the BLM helicase, the protein defective in the cancer predisposition disorder, Bloom's syndrome. This inhibitor, ML216, is cell membrane permeable and appears to be highly specific for inhibition of BLM in human cells. We suggest that ML216 is a suitable "molecular probe" for detailed analysis of the role of BLM in the maintenance of genome stability, including the identification of factors that are required to compensate for the loss of BLM function. A major future use of BLM-targeting compounds lies in the testing of the hypothesis that tumor cells that depend on the so-called ALT (alternative lengthening of telomeres) mechanism for maintenance of chromosome ends (telomeres), rather than on telomerase, might be susceptible to BLM inhibition. Work in yeast has revealed that the BLM ortholog, Sgs1, is required for telomere maintenance in the absence of telomerase, at least under certain circumstances (Cohen and Sinclair, 2001; Huang et al., 2001; Johnson et al., 2001). Moreover, depletion of the BLM partner protein, Topoisomerase IIIa, from human ALT cells strongly inhibits proliferation (Temime-Smaali et al., 2008). The availability of ML216 now allows the testing of BLM inactivation in ALT cells. A successful proof of principle would open up the potential for analogs of ML216, or newly developed BLM inhibitors, to be used clinically to target the 5%–10% of tumors that depend for their continued proliferation on the ALT pathway. Given that several of the ALT tumor types, such as osteosarcomas, are refractory to conventional treatments, this could open up a new therapeutic strategy for targeting these treatment-resistant tumors.

EXPERIMENTAL PROCEDURES

Proteins and Oligonucleotides

Recombinant human BLM, WRN, and RECQ1 helicases were expressed and purified to near homogeneity as described previously (Mohaghegh et al., 2001; Popuri et al., 2008). *E. coli* UvrD, the human RECQ5, and truncated WRN (residues 500–946 fused to GST) helicases were obtained from Drs. S. Matson, P. Janscak, and R. Brosh, respectively. Oligonucleotides were synthesized by Sigma-Aldrich (Haverhill, UK), Biosearch Technologies (Novato, CA), and the oligonucleotide synthesis service of Cancer Research UK (CRUK) (see Table S2 for sequences).

BLM High-Throughput Screening Assay

The high-throughput screening for inhibitors of BLM activity was conducted using a forked duplex DNA substrate, which harbored a tetramethylrhodamine fluorophore at the 3′ end of one strand (Table S2; A1) and a nonfluorescent, Black Hole Quencher-2 at the 5′ end of the complementary strand (Table S2; A2). An increase in the fluorescence intensity as a result of the ATP-dependent unwinding of the duplex region by BLM was used to measure helicase activity. For details, see Supplemental Experimental Procedures.

Preparation of Radiolabeled Substrates

For each substrate, a single oligonucleotide was 5′ end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The forked duplex, X12 four-way junction (Holliday junction), D-loop, and G4 DNA substrates were generated as described previously (Bachrati et al., 2006; Mohaghegh et al., 2001; Sun et al., 1998; van Brabant et al., 2000). Prior to use, each substrate was gel-purified and dialyzed against the buffers described in Supplemental Experimental Procedures. The concentration of the labeled substrate was determined using scintillation counting. For details, see Supplemental Experimental Procedures.

DNA Helicase Activity Measured by Gel Electrophoresis of Radiolabeled DNA

Helicase assays were carried out in a 100 μl reaction volume containing no more than 1% DMSO, with the inhibitors being added in a 1 μl volume. Results were expressed as percent unwinding normalized to the untreated (taken as 0%) and denatured DNA (100%) control samples. Enzyme concentrations required for 50% substrate unwinding, and inhibitor concentrations required for 50% enzyme inhibition (IC_{50} values) were determined using a nonlinear regression model using GraphPad software. For detailed information, see Supplemental Experimental Procedures.

DNA Helicase Activity Measured by Gel Electrophoresis of Fluorescently Labeled DNA

The forked duplex substrate was prepared by annealing the 3′-TAMRA-labeled A1 oligonucleotide (Table S2) with the unlabeled complementary strand, A2 (Table S2). A standard 20 μl reaction contained 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.1% Tween-20, and 200 nM TAMRA-labeled forked duplex substrate. Following electrophoresis, DNA was visualized using the Bio-Rad ChemiDocTM XRS Gel Imager.

Fluorescence Polarization Assays

Equilibrium binding of DNA helicases to TAMRA-labeled oligonucleotides was monitored by fluorescence polarization. Serial dilutions of selected inhibitors were mixed with the indicated amounts of DNA helicase and the TAMRA-labeled oligonucleotide (Table S2; A1). Following incubation at room

temperature for~2 hr, plates were read on a ViewLux high-throughput CCD imager. For a detailed protocol, see Supplemental Experimental Procedures.

Cell Lines

GM08505 is an SV40-transformed skin fibroblast cell line established from a patient with BS. PSNF5 is a stable transfectant of GM08505 expressing Flag epitope-tagged BLM protein, while PSNG13 is an empty vector control transfectant of the same cell line. These transfected clones have been described previously (Gaymes et al., 2002). GM00637 and AG11395 are SV40-transformed fibroblasts from a normal individual and a patient with WS, respectively. Details of culture conditions for these lines are provided in Supplemental Experimental Procedures.

Proliferation Assays

Cells were treated with BLM inhibitors at the indicated concentrations for up to 72 hr. At each time point, the WST-1 reagent (a pale tetrazolium derivative converted to an intensely colored formazan product by the action of mitochondrial dehydrogenases) was added, and after a 4 hr incubation the plates were analyzed optically at 450 nm with a reference wavelength of 690 nm. For details, see Supplemental Experimental Procedures.

Clonogenic Survival and SCE Analysis

Clonogenic survival assays and SCE analysis were performed as described previously (Davies et al., 2004).

Fluorescence Microscopy

PSNG13 and PSNF5 cells were treated with either DMSO or ML216 at the concentrations described for 48 hr before exposure to mitomycin C for a further 12 hr. Cells were then fixed and permeabilized using 4% paraformaldehyde and ice-cold 70% ethanol. Fixed cells were stained with a primary antibody for γ-H2AX (Upstate anti-mouse; 05-636). DAPI staining was used to define the location of cell nuclei.

Chemical Synthesis of ML216

Synthetic schemes and protocols describing the preparation of ML216 and other compounds, as well as supporting analytical data, are provided in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.10.016.

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REFERENCES

Aggarwal, M., Sommers, J.A., Shoemaker, R.H., and Brosh, R.M., Jr. (2011). Inhibition of helicase activity by a small molecule impairs Werner syndrome helicase (WRN) function in the cellular response to DNA damage or replication stress. Proc. Natl. Acad. Sci. USA *108*, 1525–1530.

Bachrati, C.Z., and Hickson, I.D. (2003). RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem. J. *374*, 577–606.

Bachrati, C.Z., Borts, R.H., and Hickson, I.D. (2006). Mobile D-loops are a preferred substrate for the Bloom's syndrome helicase. Nucleic Acids Res. *34*, 2269–2279.

Brosh, R.M., Jr., Li, J.L., Kenny, M.K., Karow, J.K., Cooper, M.P., Kureekattil, R.P., Hickson, I.D., and Bohr, V.A. (2000). Replication protein A physically interacts with the Bloom's syndrome protein and stimulates its helicase activity. J. Biol. Chem. *275*, 23500–23508.

Chaganti, R.S., Schonberg, S., and German, J. (1974). A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc. Natl. Acad. Sci. USA *71*, 4508–4512.

Chang, M., Bellaoui, M., Zhang, C., Desai, R., Morozov, P., Delgado-Cruzata, L., Rothstein, R., Freyer, G.A., Boone, C., and Brown, G.W. (2005). RMI1/NCE4, a suppressor of genome instability, encodes a member of the RecQ helicase/Topo III complex. EMBO J. *24*, 2024–2033.

Chester, N., Kuo, F., Kozak, C., O'Hara, C.D., and Leder, P. (1998). Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom's syndrome gene. Genes Dev. *12*, 3382–3393.

Chu, W.K., and Hickson, I.D. (2009). RecQ helicases: multifunctional genome caretakers. Nat. Rev. Cancer *9*, 644–654.

Chu, W.K., Hanada, K., Kanaar, R., and Hickson, I.D. (2010). BLM has early and late functions in homologous recombination repair in mouse embryonic stem cells. Oncogene *29*, 4705–4714.

Cohen, H., and Sinclair, D.A. (2001). Recombination-mediated lengthening of terminal telomeric repeats requires the Sgs1 DNA helicase. Proc. Natl. Acad. Sci. USA *98*, 3174–3179.

Davies, S.L., North, P.S., Dart, A., Lakin, N.D., and Hickson, I.D. (2004). Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. Mol. Cell. Biol. *24*, 1279–1291.

Ellis, N.A., Groden, J., Ye, T.Z., Straughen, J., Lennon, D.J., Ciocci, S., Proytcheva, M., and German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. Cell *83*, 655–666.

Gaymes, T.J., North, P.S., Brady, N., Hickson, I.D., Mufti, G.J., and Rassool, F.V. (2002). Increased error-prone non homologous DNA end-joining—a proposed mechanism of chromosomal instability in Bloom's syndrome. Oncogene *21*, 2525–2533.

German, J. (1997). Bloom's syndrome. XX. The first 100 cancers. Cancer Genet. Cytogenet. *93*, 100–106.

Huang, P., Pryde, F.E., Lester, D., Maddison, R.L., Borts, R.H., Hickson, I.D., and Louis, E.J. (2001). SGS1 is required for telomere elongation in the absence of telomerase. Curr. Biol. *11*, 125–129.

Johnson, F.B., Marciniak, R.A., McVey, M., Stewart, S.A., Hahn, W.C., and Guarente, L. (2001). The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase. EMBO J. *20*, 905–913.

Meetei, A.R., Sechi, S., Wallisch, M., Yang, D., Young, M.K., Joenje, H., Hoatlin, M.E., and Wang, W. (2003). A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol. Cell. Biol. *23*, 3417–3426.

Mohaghegh, P., Karow, J.K., Brosh, R.M., Jr., Bohr, V.A., and Hickson, I.D. (2001). The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. Nucleic Acids Res. *29*, 2843–2849.

Pike, A.C., Shrestha, B., Popuri, V., Burgess-Brown, N., Muzzolini, L., Costantini, S., Vindigni, A., and Gileadi, O. (2009). Structure of the human RECQ1 helicase reveals a putative strand-separation pin. Proc. Natl. Acad. Sci. USA *106*, 1039–1044.

Popuri, V., Bachrati, C.Z., Muzzolini, L., Mosedale, G., Costantini, S., Giacomini, E., Hickson, I.D., and Vindigni, A. (2008). The Human RecQ helicases, BLM and RECQ1, display distinct DNA substrate specificities. J. Biol. Chem. *283*, 17766–17776.

Singh, T.R., Ali, A.M., Busygina, V., Raynard, S., Fan, Q., Du, C.H., Andreassen, P.R., Sung, P., and Meetei, A.R. (2008). BLAP18/RMI2, a novel OB-fold-containing protein, is an essential component of the Bloom helicase-double Holliday junction dissolvasome. Genes Dev. *22*, 2856–2868.

Soultanas, P., and Wigley, D.B. (2001). Unwinding the 'Gordian knot' of helicase action. Trends Biochem. Sci. *26*, 47–54.

Sun, H., Karow, J.K., Hickson, I.D., and Maizels, N. (1998). The Bloom's syndrome helicase unwinds G4 DNA. J. Biol. Chem. *273*, 27587–27592.

Temime-Smaali, N., Guittat, L., Wenner, T., Bayart, E., Douarre, C., Gomez, D., Giraud-Panis, M.J., Londono-Vallejo, A., Gilson, E., Amor-Guéret, M., and Riou, J.F. (2008). Topoisomerase IIIalpha is required for normal proliferation and telomere stability in alternative lengthening of telomeres. EMBO J. *27*, 1513–1524.

van Brabant, A.J., Ye, T., Sanz, M., German, J.L., III, Ellis, N.A., and Holloman, W.K. (2000). Binding and melting of D-loops by the Bloom syndrome helicase. Biochemistry *39*, 14617–14625.

Wu, L., Davies, S.L., North, P.S., Goulaouic, H., Riou, J.F., Turley, H., Gatter, K.C., and Hickson, I.D. (2000). The Bloom's syndrome gene product interacts with topoisomerase III. J. Biol. Chem. *275*, 9636–9644.

Wu, L., Davies, S.L., Levitt, N.C., and Hickson, I.D. (2001). Potential role for the BLM helicase in recombinational repair via a conserved interaction with RAD51. J. Biol. Chem. *276*, 19375–19381.

Wu, L., Chan, K.L., Ralf, C., Bernstein, D.A., Garcia, P.L., Bohr, V.A., Vindigni, A., Janscak, P., Keck, J.L., and Hickson, I.D. (2005). The HRDC domain of BLM is required for the dissolution of double Holliday junctions. EMBO J. *24*, 2679–2687.

Yin, J., Sobeck, A., Xu, C., Meetei, A.R., Hoatlin, M., Li, L., and Wang, W. (2005). BLAP75, an essential component of Bloom's syndrome protein complexes that maintain genome integrity. EMBO J. *24*, 1465–1476.

