Cancer Therapy: Preclinical

Candidate Biomarkers of Response to an Experimental Cancer Drug Identified through a Large-scale RNA Interference Genetic Screen

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Abstract  Purpose: A major impediment in the optimal selection of cancer patients for the most effective therapy is the lack of suitable biomarkers that foretell the response of a patient to a given drug. In the present study, we have used large-scale RNA interference–based genetic screens to find candidate biomarkers of resistance to a new acyl sulfonamide derivative, R3200. This compound inhibits the proliferation of tumor cells in vitro and in vivo, but its mechanism of action is unknown.  Experimental Design: We used a large-scale RNA interference genetic screen to identify modulators of the efficacy of R3200. We searched for genes whose suppression in an in vitro cell system could cause resistance to the anticancer effects of R3200.  Results: We report here that knockdown of either RBX1 or DDB1 causes resistance to the anticancer effects of R3200, raising the possibility that these two genes may have utility as biomarkers of response to this drug in a clinical setting. Interestingly, both RBX1 and DDB1 are part of an E3 ubiquitin ligase complex.  Conclusions: We propose that suppression of the activity of a RBX1 and DDB1-containing E3 ligase complex leads to the stabilization of certain proteins, the increased abundance of which is in turn responsible for resistance to R3200. Moreover, our data suggest that RBX1 and DDB1 could potentially be developed into biomarkers of resistance to acyl sulfonamide–based cancer drugs. This will require clinical validation in a series of patients treated with R3200. (Clin Cancer Res 2009;15(18):5811–9)

Effective cancer therapy has been hampered by the absence of drugs that selectively target cancer cells. The identification and characterization of new compounds that potentially specifically kill cancer cells is therefore of major interest. Recently, a new class of compounds was shown to be effective in inhibiting the growth of cancer cells in vitro and in vivo: the acyl sulfonamides (1–4). However, the efficacy of these acyl sulfonamides varies greatly, depending on the cell models used. This is in line with clinical observations that patients with comparable diagnosis can often respond very differently to the same treatment. Knowledge of whether a patient will respond to a drug is obviously of major importance for the selection of the most appropriate therapy for the individual patient. Responses to some drugs can be predicted using biomarkers (5, 6). Identifying such biomarkers is not a trivial process and it would be greatly facilitated when the mechanism of action of the drug is well-understood.

Many different approaches can be taken to establish the mode of action of a drug and to identify drug response biomarkers. Recently, RNA interference (RNAi)–based genetic screens have shown utility in the identification of drug response biomarkers (7–9). RNAi screens can be done in several different ways (10). Single-well siRNA screens often make use of synthetic siRNAs. This usually requires complex automation, executed in high throughput. Due to the short half-life of siRNAs, this approach does not allow the selection of phenotypes in long-term assays. These drawbacks can be bypassed by using short hairpin RNAs (shRNA) to mediate stable gene knockdown. shRNA screening is efficient and requires minimal automation. Because shRNA screening makes use of retroviral shRNA vectors, both long-term and short-term assays can be used for screening.

We have developed an shRNA library and have used this library to show that loss of PTEN confers resistance of HER2-amplified breast cancer cells to treatment with trastuzumab (9). This observation suggested that the phosphoinositide-3-kinase pathway, which is negatively regulated by PTEN, is a major determinant of trastuzumab efficacy, a notion that was validated inaccordancewith18U.S.C.Section1734solelytoindicatethisfact.

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Translational Relevance

One of the most significant problems in the treatment of cancer is the heterogeneity of the responses to therapy. This can be explained in part by the diverse nature of the genetic lesions that give rise to apparently similar tumors. At the same time, this mixed response underscores the need to stratify patients with suitable biomarkers that predict response to therapy. In this article, we describe the use of large-scale genetic screens to identify genes whose suppression causes resistance to an experimental cancer drug of unknown mechanism of action. We identify a protein complex whose abundance determines the response to this drug. The components of this complex may have clinical utility to identify those patients that are most likely to respond to this compound.

Materials and Methods

Cell lines and culture conditions. Cell lines were derived from American Type Culture Collection, National Cancer Institute or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The MTLn3 cell line is a mouse cell line, all other cell lines were human cell lines. All cells were cultivated in RPMI 1640 supplemented with 10% FCS, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO2. For the RNAi barcode screen, HCT116 and Phoenix cells were cultured at 37°C in 5% CO2 in DMEM supplemented with 10% FCS, penicillin, and glutamine.

Reagents. R3200 (2-phenyl-ethenesulfonic acid 2,4-dichlorobenzoylamide sodium salt) was dissolved in DMSO to get a stock solution of unknown mechanism of action. We identify a protein complex whose abundance determines the response to this drug. The components of this complex may have clinical utility to identify those patients that are most likely to respond to this compound.

Western blotting. The following antibodies were used for Western blotting. Anti–c-Jun (9162), c-Jun-pS63 (9262), and c-Jun-pS73 (9164) were obtained from Cell Signaling Technologies. Anti–cyclin A (611269), cyclin B (610220), and MAD2 (610679) were obtained from BD Transduction Laboratories.

HCT116 xenograft study. Severe combined immunodeficiency (SCID) beige mice were purchased from Charles River Germany; HCT116 tumor cells were obtained from the NCI. To generate the primary tumors, 5 × 10^6 HCT116 cells were injected s.c. into the flank of each mouse in a volume of 100 μL of PBS. Body weight and tumor volume were measured thrice weekly. Tumors were measured in two dimensions (length and width), and the corresponding tumor volume was estimated using the formula: \( V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{width} / 2 \). Randomization (six groups containing 10 animals each), and start of treatment was on day 12, when the tumors had reached an average volume of ~150 mm^3. Treatment was once daily for 3 days (on days 12, 13, and 14) by i.v. injection of vehicle or different doses of R3200. On day 31, the final day of the experiment, blood samples were taken from the animals and analyzed for hematology (RBC count, hemoglobin, packed cell volume, reticulocyte count, platelet count) and clinical chemistry (alanine aminotransferase, creatinine, and urea) variables.

Netherlands Cancer Institute (NKI) shRNA library. The construction of the library was described previously (11). Briefly, the NKI shRNA library was designed to target 7,914 human genes, using three shRNA vectors for every targeted gene, cumulating in a total of 23,742 shRNA vectors. The shRNAs are cloned into a retroviral vector (pRetroSUPER, pRS) to enable infection of target cells.

Retroviral infection. Phoenix cells were transfected using the calcium phosphate method. Viral supernatant was cleared through a 0.45-μm filter. Cells were infected with the viral supernatant in the presence of polybrene (8 μg/mL). The infection was repeated twice.

shRNA screen. To screen the NKI shRNA library, we reasoned that we would need 100-fold coverage of the library to get a good representation of shRNA vectors present in the library. The screen was initiated by transduction of HCT116 cells with the shRNA library. After infection, the cells were selected with puromycin (2 μg/mL). When the selection was completed, 2 × 10^6 HCT116 cells (100-fold library coverage) were seeded at a density of 150,000 cells/15 cm dish. Approximately 24 h after seeding, R3200 was added at a final concentration of 20 μmol/L. Colonies of resistant cells appeared in the plates containing cells infected with the shRNA library, no colonies were observed in control infected cells treated with R3200. After 21 days of exposure to 20 μmol/L of R3200, the colonies were harvested. All colonies were pooled and the genomic DNA was isolated using DNAzol (Invitrogen). The shRNA cassettes were amplified by PCR, and recloned into the pBS shRNA vector. This plasmid pool of shRNA vectors was infected into HCT116 cells at a low multiplicity of infection. The colony formation was repeated and 16 colonies were isolated. From these colonies, genomic DNA was isolated and the shRNA cassettes were amplified by PCR. The PCR product was cloned into the pCR plasmid. To identify the shRNA cassette, five recloned plasmids were sequenced for every recloned PCR product. The sequences of the shRNAs isolated from the colonies are listed in Table S3.

Generation of additional shRNA vectors targeting RBX1 and DDB1. The following 19-mer sequences were used to construct shRNA plasmids targeting RBX1 and DDB1 in this study.

<table>
<thead>
<tr>
<th>RBX1 (NM_014248)</th>
<th>DDB1 (NM_001923)</th>
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<tbody>
<tr>
<td>Lib GACCTITCCATCCAGGCTT</td>
<td>Lib CAGGCAAGGCTTCTTGAAG</td>
</tr>
<tr>
<td>1 GCACTGAGCCCTAGTGAACCT</td>
<td>1 GACAGCTGTCGCTGCTGTTAG</td>
</tr>
<tr>
<td>2 CCGATGAAATGTCAAGCTAA</td>
<td>2 GCAAGAAGCCCTTGAAGT</td>
</tr>
<tr>
<td>3 GCATAGAATGTCAAGCTAA</td>
<td>3 CCAAGAGAGGTCGGGATTC</td>
</tr>
<tr>
<td>4 GAAGCGCTTTGAAGTGAAA</td>
<td>4 CACAAGGAGAAGGGATTC</td>
</tr>
<tr>
<td>5 GCATAGAATGTCAAGCTAA</td>
<td>5 CCAAGAGAGGTCGGGATTC</td>
</tr>
<tr>
<td>6 Lib GAACCCAAAAGCTCTGGTCA</td>
<td>Lib CAGGCAAGGCTTCTTGAAG</td>
</tr>
<tr>
<td>1 GCACTGAGCCCTAGTGAACCT</td>
<td>1 GACAGCTGTCGCTGCTGTTAG</td>
</tr>
<tr>
<td>2 CCGATGAAATGTCAAGCTAA</td>
<td>2 GCAAGAAGCCCTTGAAGT</td>
</tr>
<tr>
<td>3 GCATAGAATGTCAAGCTAA</td>
<td>3 CCAAGAGAGGTCGGGATTC</td>
</tr>
<tr>
<td>4 GAAGCGCTTTGAAGTGAAA</td>
<td>4 CACAAGGAGAAGGGATTC</td>
</tr>
<tr>
<td>5 GCATAGAATGTCAAGCTAA</td>
<td>5 CCAAGAGAGGTCGGGATTC</td>
</tr>
<tr>
<td>6 Lib GAACCCAAAAGCTCTGGTCA</td>
<td>Lib CAGGCAAGGCTTCTTGAAG</td>
</tr>
<tr>
<td>1 GCACTGAGCCCTAGTGAACCT</td>
<td>1 GACAGCTGTCGCTGCTGTTAG</td>
</tr>
<tr>
<td>2 CCGATGAAATGTCAAGCTAA</td>
<td>2 GCAAGAAGCCCTTGAAGT</td>
</tr>
<tr>
<td>3 GCATAGAATGTCAAGCTAA</td>
<td>3 CCAAGAGAGGTCGGGATTC</td>
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<tr>
<td>4 GAAGCGCTTTGAAGTGAAA</td>
<td>4 CACAAGGAGAAGGGATTC</td>
</tr>
<tr>
<td>5 GCATAGAATGTCAAGCTAA</td>
<td>5 CCAAGAGAGGTCGGGATTC</td>
</tr>
<tr>
<td>6 Lib GAACCCAAAAGCTCTGGTCA</td>
<td>Lib CAGGCAAGGCTTCTTGAAG</td>
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**Colony formation assay.** Cells were infected with retroviral supernatant and selected with puromycin. When the selection was completed, cells were seeded at 150,000/15 cm dish, 50,000/10 cm dish, or 10,000/well in a six-well plate. The following day, the R3200 was added. Cells were cultured in the presence of R3200 for ~10 days. When colonies appeared, cells were fixed in methanol/glacial acetic acid (3:1) and subsequently stained in staining solution (50% methanol/10% glacial acetic acid/0.1% Coomassie).

**Quantitative real-time PCR.** Total RNA was isolated using TRIzol (Invitrogen) from cells that were infected with pRS plasmids and selected with puromycin. From the total RNA, cDNA was generated using Superscript II (Invitrogen) with random primers (Invitrogen). cDNA was diluted and quantitative real-time (QRT) reaction was done using SYBR green PCR master mix (Applied Biosystems). All QRT reactions were run in parallel for actin B to control for amount of input cDNA. The QRT reaction was followed by a melting curve to confirm the formation of a single PCR product. The QRT reactions were run at an AB7500 Fast Real-time PCR system (Applied Biosystems). The PCR primer sequences were obtained from the qPrimerDepot (12).

**FACS cell cycle analysis.** Cells were infected with retroviral supernatant and selected with puromycin. When the selection was completed, cells were seeded at 500,000/10 cm dish. One day after seeding, R3200 (30 μmol/L) was added for 48 h. Cells were harvested and fixed in 70% ethanol and subsequently stained with PI. FACS analysis was done by gating out the doublet cells.

**Results**

**In vitro analysis of antiproliferative effects of R3200.** It was shown previously that acyl sulfonamides have prominent antiproliferative effects in cancer cell lines (1–4). We have synthesized a novel acyl sulfonamide, R3200 (Fig. 1A). To assess if R3200 can inhibit proliferation in cell lines derived from human cancers, we used 29 cell lines to determine the IC50 (Table S1). Included in this panel were cell lines generated from breast, colon, prostate, and pancreas tumors. FCS can significantly modulate the response to drug treatment of cells in vitro due to binding of acyl sulfonamides to (bovine) serum albumin. Therefore, we determined the IC50 value both in cells cultured in regular conditions (10% FCS) and in low concentrations of FCS (2.5%). In both conditions, we observed a wide range of responses, which translated into IC50 values ranging from 2.3 to >100 μmol/L in assays in medium with 2.5% FCS and 10.4 to >100 μmol/L in assays in medium with 10% FCS. For further in vitro studies, we decided to use two colon cancer–derived cell lines: a very sensitive cell line, HCT116 (IC50 = 4.9 μmol/L in 2.5% FCS) and a relatively resistant cell line, HT-29 (IC50 = 71.6 μmol/L in 2.5% FCS).

The antiproliferative activity of the acyl sulfonamide R3200 is due to cell cycle arrest followed by apoptosis. The antiproliferative effects described above could be caused by various effects.
of the drug, most notably apoptosis and cell cycle arrest. To address these issues, a cell cycle analysis was done following treatment of cells with R3200. When HCT116 cells were exposed to 30 μmol/L of R3200 for 48 hours, we observed an increase in the G2-M cell cycle phase (Fig. 1B). Quantification of the results from cell cycle profiles show that >80% of the HCT116 cells have accumulated in the G2-M phase of the cell cycle (Supplementary Fig. S1). The FACS profile obtained from the HCT116 cells suggests that the cells have arrested either in the G2 or M phase of the cell cycle, we cannot exclude that the cells have undergone endoreduplication of their genome and are arrested in the G1 phase of the next cell cycle with 4n DNA content. In the resistant HT-29 cell line, no cell cycle arrest was detected after treatment with R3200 (Fig. 1B), as expected.

R3200 induces apoptosis in human cancer cell lines. Many anticancer drugs induce a cell cycle arrest in short-term assays, but could also induce apoptosis after prolonged drug exposure. We subjected HCT116 and HT-29 cells to R3200 for 48 and 72 hours and stained the cells with Annexin V/PI to identify dead cells. Maximal effect was observed in HCT116 cells after 72 hours of R3200 treatment (Fig. 1C). The amount of cells that were killed by the R3200 treatment was 64%. In HT-29 cells, only a mild cell death was observed after 72 hours of treatment. The IC50 of apoptosis induction in HCT116 cells determined after 72 hours of drug exposure was 35 μmol/L, whereas for HT-29 cells, no IC50 could be determined.

Inhibitory activity in a drug-resistant cell line: influence of P-glycoprotein. Many drugs have limited efficacy because they are substrates for P-glycoprotein (P-gp) drug resistance pumps (13, 14). We therefore tested if R3200 is a substrate of P-gp ATP binding cassette (ABC) transporters. A mouse leukemia cell line, P388 (parental, non/low expression of P-gp), and a derivative P388/ADR overexpressing the multidrug drug resistance pump P-gp encoded by the ABCC1 gene were used to examine the influence of compounds on cell viability and proliferation. In these cells, we determined the IC50 for R3200, taxol, etoposide, nocodazole, and cisplatin. In contrast to taxol and etoposide, which are P-gp substrates, R3200 showed nearly no difference in cell viability in both cell lines (Table 1). This suggests that R3200 is not transported by this P-gp drug pump.

R3200-induced changes in cell cycle proteins. Next, we tested if the observed G2-M cell cycle arrest was also accompanied by altered levels of cell cycle–regulated proteins. We treated HT-29 and HCT116 cells with R3200 (30 μmol/L) for 24 and 48 hours, and after lysis of the cells, we detected the protein levels of cell cycle regulators by Western blot analysis (Fig. 2A). In HCT116, but not in HT-29 cells, we observed a decrease in protein levels of cyclin A, cyclin B, and MAD2. This indicates that HCT116 cells are indeed inhibited in their proliferation whereas HT-29 cells are unaffected by R3200 treatment.

To investigate if R3200 treatment activates cellular stress response pathways, we measured c-Jun protein levels (Fig. 2B). A similar increase in levels of phosphorylated c-Jun (pS63 and pS73) was seen, suggesting that c-Jun protein levels are induced rather than specific phosphorylation of the protein. This indicates that stress response pathways are activated by prolonged treatment of R3200, and might explain some of the effects on cell cycle proteins seen after longer exposure to drug. In the HT-29 cells, we did not observe any effect of R3200 on c-Jun or phosphorylated c-Jun protein levels (Fig. 2B).

Table 1. R3200 is not a substrate for P-gp drug transporters

<table>
<thead>
<tr>
<th></th>
<th>P388 IC50</th>
<th>P388/ADR IC50</th>
<th>Difference n-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3200</td>
<td>27.60</td>
<td>31.70</td>
<td>1.10</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.08</td>
<td>4.40</td>
<td>55.00</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.14</td>
<td>&gt;10</td>
<td>&gt;71</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>12.10</td>
<td>46.20</td>
<td>3.80</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>0.01</td>
<td>0.03</td>
<td>2.80</td>
</tr>
</tbody>
</table>

NOTE: IC50 values for wt P388 and P-gp overexpressing P388/ADR cells (as indicated) treated with R3200.

In vivo antiproliferative effects of R3200. To test whether the in vitro properties of R3200 translate into inhibition of tumor growth in vivo, the effect of R3200 on xenografts was assessed. R3200 induced acute toxicity in mice at doses exceeding 120 mg/kg (data not shown). Therefore, doses between 5 and 80 mg/kg were chosen to assess the in vivo anticancer effects of R3200 in xenograft experiments using subcutaneously growing HCT116 tumors in immunodeficient SCID beige mice.
The animals were randomized into six study groups containing 10 tumor-bearing mice each; treatment was started on day 12 after tumor cell injection, when the tumors had reached an average volume of \( \sim 150 \text{ mm}^3 \). Treatment consisted of three i.v. administrations on 3 consecutive days (i.e., days 12, 13, and 14 after tumor cell implantation). Tumor volume (Fig. 3A) and body weight (Fig. 3B) were observed until day 31 after tumor cell implantation.

Treatment with R3200 caused a marked, and clearly dose-dependent, tumor growth inhibition which seemed to reach a plateau for the maximal antitumor effect at 40 mg/kg (Fig. 3A). In fact, R3200 treatment initially even led to tumor shrinkage, with the maximum occurring \( \sim 7 \) days after the first injection. At the same time, R3200 was tolerated well at all five doses and did not have a significant effect on body weight (Fig. 3B). Similarly, on day 31, the final day of the study, blood samples were taken from the animals and analyzed for hematologic (RBC count, hemoglobin, packed cell volume, reticulocyte count, and platelet count) and clinical chemistry (alanine aminotransferase, creatinine, and urea) variables. In brief, no differences were found for any of the variables when compared with the vehicle control animals; all values were in the range expected for SCID/beige mice (data not shown). To confirm the results obtained in the HCT116 model, the xenograft experiments were extended using four additional human xenograft models. Thus, in total, three of the models were originally derived from human colon carcinomas (HCT116, Colo-205, and SW620), and one each from an ovarian (IGROV-1) and a prostate (PC3) tumor. In all, five significant xenograft tumor growth inhibitions were observed in the mice treated with R3200 (Table S2). In addition, HCT116 tumors were also tested in a rat xenograft model. In this experiment, similar to the mouse studies, a significant inhibition of tumor growth was observed (Table S2).

**Biomarkers of R3200 resistance.** To find biomarkers of resistance to R3200, we infected HCT116 cells with the retroviral shRNA library targeting \( \sim 8,000 \) human genes for suppression...
by RNAi (11). We have shown previously that this library can be used to identify biomarkers of drug resistance (9, 15). To identify genes whose suppression allows cells to escape the cytotoxic effects of R3200, we treated infected cells with R3200. After 2 weeks, colonies of R3200-resistant cells were observed in cells infected with the library, but not in control cells treated with R3200 (Fig. 4A).

The screen was done with a high multiplicity of infection, which leads to multiple shRNAs being present per cell. Consequently, each R3200-resistant cell colony has several “passenger” shRNA vectors that do not contribute to the resistance phenotype. To identify the active shRNA vectors in the population of resistant cells, we first recovered the shRNAs from the R3200-resistant colonies by isolating the genomic DNA. On this genomic DNA, we did a PCR reaction with primers that specifically amplify the shRNA cassette. These shRNA cassettes were cloned into the original retroviral shRNA vectors in a plasmidic format.

Next, we infected the recovered shRNA vectors from the initial screen into fresh HCT116 cells at a low multiplicity of infection, leading to a single retroviral insertion per cell. Cells were again treated with R3200, and again (in this case more), colonies of resistant cells were observed. Sixteen colonies were picked and the shRNA cassettes were identified by sequencing, which revealed that 11 out of 16 R3200-resistant colonies contained a shRNA vector targeting RBX1 (also known as ROC1). The five other colonies all contained a different shRNA vector, among which was a shRNA vector targeting DDB1 (a list of all shRNAs identified in the colonies can be found in Supplementary Table S3). This is particularly interesting as it has been reported that RBX1 can bind DDB1 (16). Together with CUL4A, RBX1 and DDB1 can form a ubiquitin E3 ligase complex. We therefore decided to further study the possible role of RBX1 and DDB1 in R3200-induced cytotoxicity.

**Validation of the role of RBX1 and DDB1 in R3200-induced cytotoxicity.** Next, we set out to validate that knockdown of RBX1 and DDB1 could indeed give resistance to R3200 treatment. To address this, we infected HCT116 cells with the shRNA vectors targeting RBX1 and DDB1 which were identified in the rescued colonies from the screen (these vectors were named RBX1-Lib and DDB1-Lib). When the HCT116 cells with knockdown of RBX1 or DDB1 were exposed to R3200, these cells proved to be resistant to R3200 treatment (Fig. 4B). In addition, we tested if RBX1 and DDB1 knockdown can also protect cells from higher concentrations of R3200. A clear rescue was observed up to 35 μmol/L in cells with knockdown of RBX1 and DDB1 (Fig. 4C).

**Multiple shRNA vectors targeting RBX1 and DDB1 can produce resistance to R3200 treatment.** To find support for the notion that the knockdown vectors targeting RBX1 and DDB1 give rescue due to an “on-target” effect, we designed four new shRNA vectors targeting both genes (for sequences, see Materials and Methods, vectors 1-4) and used these to infect HCT116 cells. We first assessed if the new shRNAs could suppress DDB1 and RBX1 mRNA. A clear reduction in mRNA levels as measured by QRT-PCR was observed in cells that were infected with shRNA vectors targeting RBX1 and DDB1 compared with control-infected cells (Fig. 5A and B). Next, we set out to investigate if we could find a correlation between the degree of knockdown and resistance to R3200. The first observation we made was that strong knockdown of RBX1 and DDB1 had an adverse effect on cell proliferation, even in the absence of R3200 (Fig. 5C), a phenotype that has been reported previously (17, 18). We also observed that cells infected with different shRNA vectors targeting DDB1 (pRS-DDB1-2 and -3) and RBX1 (pRS-RBX1-3 and -4) produced resistance to R3200 treatment. In addition, these cells were also resistant to R3200 treatment in culture conditions with low levels of serum (2.5% FCS; Supplementary Fig. S2). Furthermore, we decided to determine the IC50 of R3200-treated HCT116 cells with knockdown of RBX1 or DDB1. We observed an IC50 for RBX1-Lib–infected cells of 75 μmol/L and for DDB1-Lib–infected cells of 68 μmol/L, compared with an IC50 of 18 μmol/L for control-infected cells (Supplementary Fig. S3). From these data, we conclude that suppression of both RBX1 and DDB1 could confer resistance to R3200 treatment in vitro.

**Suppression of RBX1 and DDB1 can abrogate the R3200-induced cell cycle arrest.** As shown above, treatment with R3200 leads to a G2-M arrest in cancer cell lines. Therefore, we tested if knockdown of RBX1 and DDB1 can also lead to bypass of the G2-M arrest in HCT116 cells. Cells with a stable knockdown of RBX1 or DDB1 were treated with R3200 and subjected to cell cycle analysis (Fig. 5D). For RBX1, we used pRS-Lib, -3, and -4 and for DDB1 we used pRS-Lib, -2, and -3. These vectors induced no antiproliferative effects in untreated cells, and in addition, produced the best rescue after treatment with R3200.
The G2-M arrest in control cells was very efficient, meaning that 80% of treated cells were arrested. When we tested cells with knockdown of RBX1 or DDB1, we observed that the G2-M peak was reduced, accompanied by an increased G1 fraction (Fig. 5D).

We therefore conclude that knockdown of RBX1 and DDB1 can also alleviate the cell cycle arrest in cells treated with R3200.

**RBX1 and DDB1 knockdown do not cause general drug resistance.** Next, we did some experiments to ensure that the

![Graphs and images showing knockdown of RBX1 and DDB1](Figures 5A-D)
observed effects were not specific for both the cell type and drug used. To address the drug specificity issue, we treated HCT116 cells with an unrelated drug, cisplatin, in the presence or absence of RBX1 or DDB1 knockdown. As HCT116 cells are quite sensitive to low concentrations (5 μmol/L) of cisplatin, a strong decrease in proliferation can be observed (Supplementary Fig. S4). The antiproliferative effects of cisplatin could not be overcome by knockdown of either RBX1 or DDB1. This experiment shows that suppression of RBX1 or DDB1 confers resistance to R3200 treatment but not to antiproliferative drugs in general.

**Suppression of RBX1 and DDB1 also confers resistance to R3200 treatment in U2OS cells.** To exclude that the observations we made are specific for HCT116 cells, we decided to repeat some of the experiments in an unrelated cell type. For this, we used U2OS cells, a cell line isolated from a human osteosarcoma. U2OS cells are equally sensitive to R3200 as HCT116 (U2OS, IC₅₀ = 5.3 μmol/L; HCT116, IC₅₀ = 4.9 μmol/L). We used the active shRNA vectors to knock down RBX1 and DDB1 in U2OS cells. When the mRNA levels of RBX1 and DDB1 were assessed, we observed comparable results as in HCT116 cells (Supplementary Fig. S5A and B). Subsequently, we treated the U2OS cells in which we knocked down RBX1 and DDB1 with various concentrations of R3200. In cells that were suppressed for either RBX1 or DDB1, we saw a clear rescue from the antiproliferative actions of R3200 as measured by colony formation assays (Supplementary Fig. S5C).

**Discussion and Conclusion**

In this study, we report biochemical and genetic studies using the new anticancer acyl sulfonamide, R3200. In previous studies, it was shown that acyl sulfonamides could inhibit cell proliferation in vitro and in vivo. However, no analysis was done on the possible mode of action of acyl sulfonamides (1–4). Here, we show that R3200 has a profound antiproliferative action in a series of cancer cell lines including a P-gp–overexpressing cell line. This antiproliferative effect can be attributed to two different physiologic responses. First, when we performed a cell cycle analysis on R3200-treated cells, we observed a cell cycle arrest. Second, R3200 treatment leads to substantial apoptosis when cells are exposed to a drug for a longer time. After these promising in vitro results, we tested the effects of R3200 in mouse and rat xenografts. In these experiments, we observed a strong antiproliferative effect towards the grafted human cancer cells resulting in strong tumor growth inhibition.

To identify the mode of action of R3200 and to find biomarkers of resistance to this drug, we have done a large-scale RNAi genetic screen. Two genes were identified that, when suppressed, could bypass the cytostatic actions of R3200. Interestingly, these two genes, RBX1 and DDB1, can form a complex with CUL4A, leading to the formation of an active ubiquitin E3 ligase complex (19). Suppression of components of this E3 ligase complex potentially leads to the stabilization of the target proteins. It is therefore possible that R3200 inhibits a protein, which is targeted for destruction by this ubiquitin ligase complex containing RBX1 and DDB1, as inhibition of this destruction complex may lead to increased concentrations of the putative protein target(s) of R3200, thereby conferring resistance to the drug. Many targets have been reported for the ubiquitin ligase complex containing RBX1 and DDB1, the most striking targets are CDT1, c-Jun, and histone H3 and H4 (16, 20–22). CDT1 is required for the formation of the pre-RC complex and therefore is essential for replication of the genome (23). It has been described that CDT1, histone H3 and H4 can be ubiquitinated by the complex containing RBX1 and DDB1 after DNA damage (7, 22).

Whatever the mechanism may be, our data indicate that the level of RBX1 and DDB1 may have clinical utility to predict the response of cancer cells to R3200. Therefore, RBX1 and DDB1 should be evaluated as biomarkers for clinical response in the treatment of patients with R3200 and potentially also in clinical studies involving other acyl sulfonamides. If a tumor contains very low levels of transcripts encoding for either RBX1 or DDB1, it might be advisable not to use R3200 in this setting. One of these examples would be patients that suffer from a form of xeroderma pigmentosum. Patients with xeroderma pigmentosum complementation group E suffer from a defect in either the DDB1 or DDB2 gene (24, 25). Therefore, treatment of xeroderma pigmentosum patients with a defect in DDB1 or DDB2 using R3200 might be less effective. Clinical studies involving these candidate biomarkers may substantiate these predictions in the future.

**Disclosure of Potential Conflicts of Interest**

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**References**


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