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Sudemycins, Novel Small Molecule Analogues of FR901464, Induce Alternative Gene Splicing

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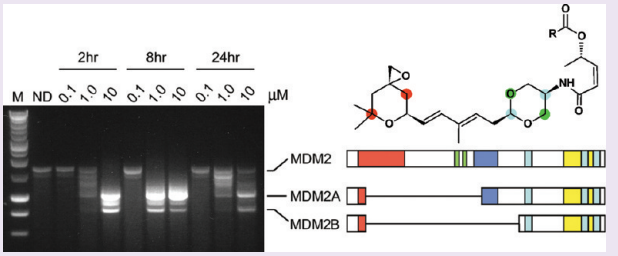
Supporting Information

ABSTRACT: Two unrelated bacterial natural products, FR901464 and pladienolide B, have previously been shown to have significant antitumor activity *in vivo*. These compounds target the SF3b subunit of the spliceosome, with a derivative of pladienolide (E7107) entering clinical trials for cancer. However, due to the structural complexity of these molecules, their research and development has been significantly constrained. We have generated a set of novel analogues (Sudemycins) that possess the pharmacophore that is common to FR901464 and pladienolide, *via* a flexible enantioselective route, which allows for the production of gram quantities of drug. These compounds demonstrate cytotoxicity toward human tumor cell lines in culture and exhibit antitumor activity in a xenograft model. Here, we present evidence that Sudemycins are potent modulators of alternative splicing in human cells, both of endogenous genes and from minigene constructs. Furthermore, levels of alternative splicing are increased in tumor cells relative to normal cells, and these modifications can be observed in human tumor xenografts *in vivo* following exposure of animals to the drug. In addition, the change in the splicing pattern observed with the Sudemycins are similar to that observed with Spliceostatin A, a molecule known to interact with the SF3b subunit of the spliceosome. Hence, we conclude that Sudemycins can regulate the production of alternatively spliced RNA transcripts and these alterations are more prevalent in tumors, as compared to normal cells, following drug exposure. These studies suggest that modulation of alternative splicing may play a role in the antitumor activity of this class of agents.



RNA splicing in mammalian cells is an exceedingly complex process that requires over 150 proteins, a panel of small nuclear ribonucleoprotein particles, and a series of small nuclear RNAs (snRNA). These molecules act in concert to achieve high fidelity editing of pre-mRNAs to yield *bona fide* mRNAs that are then subject to translation. Hence, the specificity and reproducibility of the events involved in the generation of these mature RNAs is likely to be highly regulated. Furthermore, due to the critical nature of these processes and their necessity to achieve accurate protein production, potentially the spliceosome represents a valid target for cytotoxic molecules. To this end, several natural products (pladienolide B and FR901464;[1,2] Figure 1) have been identified that interact with, and disrupt the function of, the spliceosome. These compounds are both potent cytotoxins with IC_{50} values in the low nanomolar range, and the latter arrests cells in the G1 and G2/M phases of the cell cycle. The specific biological target for these drugs has been identified as the SF3b subunit of the spliceosome, a complex of at least six proteins and small nuclear RNAs (snRNA;[1,2]). It is thought that the entire SF3 subunit (including SF3a and SF3b) prevents inappropriate nucleophilic attack by other components of the spliceosome, prior to the initial transesterification reaction that must occur to achieve RNA splicing.[3]

Both pladienolide B and FR901464 are chemically complex and contain 9 and 10 stereocenters, respectively, such that they cannot be readily synthesized (the total synthesis of the latter molecule has recently been reported and requires greater than 40 independent reaction steps[4]). In addition, these molecules have limited chemical stability and are readily degraded in biological fluids. Although the derivative E7107 (Figure 1) has been used in preclinical studies,[2] it is unclear whether the problems and liabilities associated with these natural products are predisposed for facile and effective use as therapeutic agents.[5,6] Therefore, we have developed a series of analogues (Sudemycins; Figure 1[7,8]), based upon the consensus pharmacophore obtained from pladienolide B and FR901464. The Sudemycins are structurally much less complex (demonstrating 6 stereocenters fewer than the natural products), show much better chemical stability,[9] are not degraded in human plasma, exhibit IC_{50} values of ~80−500 nM in a panel of human tumor cell lines, and have demonstrated activity in human lymphoma xenograft models.[7,8] We have also demonstrated that Sudemycin derivatives show the same effects as have been reported for the natural product spliceosome modulators, including inhibition of spicing in an *in vitro* cell-free splicing assay, inhibition of splicing in a cell-based dual reporter assay, cell cycle arrest, and alteration of the cellular localization of

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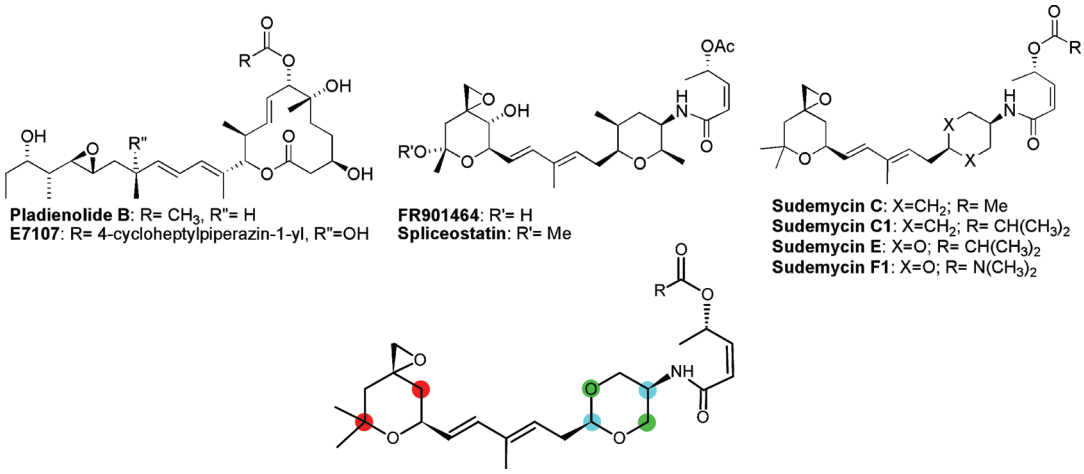

Figure 1. Structures of pladienolide B, E7107, FR901464, Spliceostatin A, and the Sudemycins. The lower structure indicates a summary of the design of concise analogues of FR901464, showing Sudemycin E with color-coded highlighting of the atoms that have been modified in order to remove chirality and to give a less complex and more chemically stable structure when compared to FR901464. Red, gain of symmetry and loss of a chemically destabilizing OH group; green, loss of a methyl group and/or atom type change to enhance solubility; cyan, gain of symmetry.

SF3b splicing factors.[7,8] Furthermore, a considerable amount of SAR analyses have been performed to identify key functionalities and chemotypes within the scaffold that are required for biological activity. On the basis of these results, 3 analogues have been further developed, namely, Sudemycin C1, E, and F, respectively (Figure 1).

Whereas Sudemycins and the associated natural products demonstrate significant potency toward tumor cells, in general, these molecules are much less toxic to normal human cells.[1,2,7,8] The mechanism for this selective toxicity is not understood, since it would be anticipated that inhibition of the spliceosome would likely be a lethal event. We have therefore sought to evaluate the role of the Sudemycins in the alteration of RNA splicing in tumor cells, since this phenomenon has been found to be present in numerous tumor lines.[10] Our results indicate that these compounds are potent modulators of alternative splicing using both endogenous genes and in minigene constructs in tumor and normal cell lines. In addition, these effects can be demonstrated both *in vitro* and *in vivo*. We conclude that modulation of mRNA splicing is a key event in the toxicity of the Sudemycins.

RESULTS AND DISCUSSION

The identification of two antitumor natural products that target the spliceosome has indicated this macromolecular complex may be considered as a potential therapeutic target for cancer.[1,2,9] However, due to the chemical complexity of these bacterially derived products, it is not clear whether practical clinically efficacious agents could be developed from these compounds. Recently, we have developed structurally related but synthetically tractable analogues of these compounds (Sudemycins) and have demonstrated that these molecules are cytotoxic in culture and induce tumor growth inhibition in human tumor xenograft models.[7,8] However, in our initial studies with these agents, we observed that potent modulation of alternative gene splicing occurred in cells exposed to Sudemycin. On the basis of these observations, we conducted detailed analysis of the changes that were present following drug treatment.

Sudemycins Modulate Splicing of *MDM2*. Because MDM2 is a critical regulator of p53 and known to be subject to alternative splicing in tumors, we used this as a marker for *in vitro* studies. Experiments were therefore performed using Rh18 cells, a cell line known to express low levels of *MDM2* splice variants.[11,12] To determine whether Sudemycin C1 could regulate the pattern of transcripts obtained from this gene in tumor cells, we exposed Rh18 cells to different concentrations of drug (0.1, 1, and 10 μM), for time intervals ranging from 2 to 24 h. RNA was then prepared and subjected to RT-PCR using oligonucleotides at the 5′ and 3′ends of the mRNA. As indicated in Figure 2A, transcripts of reduced size were observed following drug treatment, consistent with alternate splicing of the *MDM2* pre-mRNA to yield *MDM2A* and *MDM2B*. Furthermore, the yield of these smaller isoforms was dependent upon the dose of drug and the time of exposure. No change was seen in the expression of ubiquitin (Figure 2B) since this gene lacks introns, consistent with the hypothesis that Sudemycins interacts with the spliceosome, rather than modulating levels of RNA by affecting transcription.

To determine whether these transcripts were translated, we performed Western analysis using antibodies directed toward Mdm2. As indicated in Figure 2D, the presence of Mdm2A protein could be detected at 8 h following drug treatment, with significant levels observed after 24 h. It is uncertain whether this antibody definitively recognizes the Mdm2B isoform; however, we did identify a signal that would correspond to this protein (Figure 2D). Interestingly, no change was seen in the levels or splicing pattern of the control protein β-tubulin. Similar studies performed using A549 and BT-3 cells also confirmed that alterations in *MDM2* splicing could be observed following drug treatment (see Supplementary Figure S1). Hence, these data are consistent with the hypothesis that Sudemycins modulate the levels of alternatively spliced transcripts in these cells, resulting in the formation of truncated proteins.

Sudemycin-Induced Aberrant Splicing of *MDM2* Can Be Assessed Using Minigenes. To develop a system that could be used to evaluate *MDM2* splicing in essentially any cell line, we obtained two different *MDM2* minigenes (called 3−4 and 3−10; Figure 3A) and assessed the changes in RNA splicing following transfection into Rh18 cells. Following exposure to 10 μM Sudemycin C1 for 24 h (Figure 3B), the vast majority of the minigene-encoded RNA present within the cells consisted of just

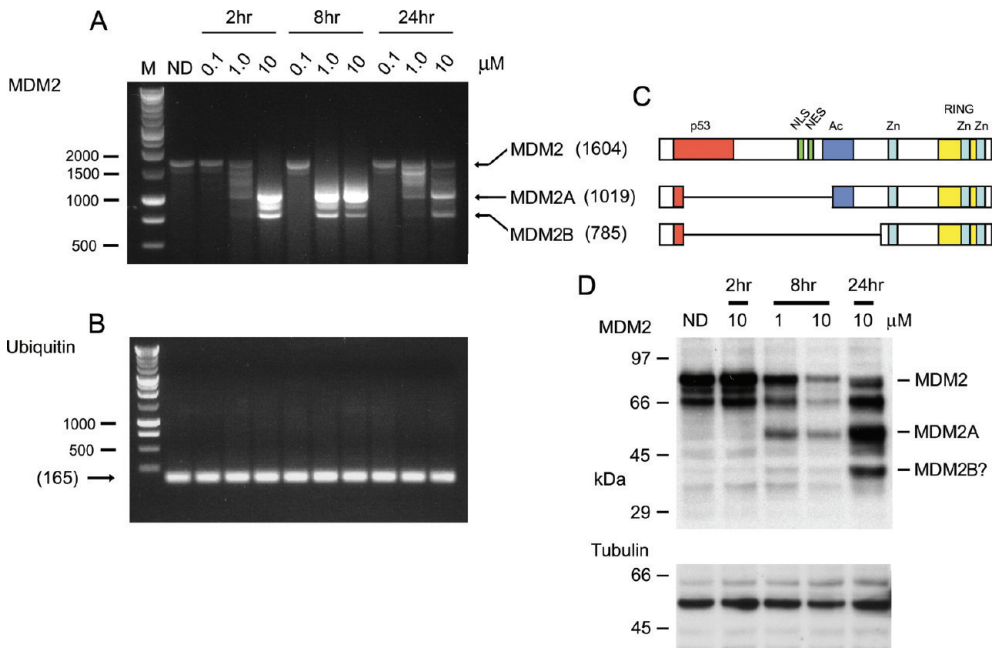

Figure 2. Sudemycin C1 modulates alternate splicing of *MDM2* in Rh18 cells. (A) PCR analysis of *MDM2* transcripts present in Rh18 cells following exposure to drug. Cells were exposed to 0.1, 1, or 10 μM Sudemycin C1 for 2, 8, or 24 h prior to analysis. The PCR products are indicated by the arrows and their sizes (in bp) are included in parentheses. M, markers (1 kb ladder); ND, no drug. (B) Analysis of ubiquitin transcripts present in the same cells as analyzed in panel A. M, markers (100 bp ladder). (C) Schematics of the truncated Mdm2 proteins that would be encoded by the alternatively spliced transcripts identified in panel A. The different regions with the protein are identified as p53—p53 binding domain (red); NLS, nuclear localization signal (green); NES, nuclear export signal (green); Ac, acidic domain (dark blue); Zn, zinc finger domains (pale blue); and RING, ring finger domain (yellow). (D) Western analysis of Mdm2 and β-tubulin expression in Rh18 cells following exposure to Sudemycin C1. It is presumed that the signal at ~40 kDa in the upper panel represents Mdm2B protein; however, no definitive evidence has been obtained to confirm that this antibody cross-reacts with this isoform.

exons 3 and 12 of *MDM2*. This was true for both minigenes and again supports the hypothesis that this drug directly interacts with the spliceosome. An examination of the time course of these events (Figure 3C) indicated that alternatively spliced transcripts were detectable at 8 h and were maximal at the later time points. Experiments were not conducted for longer time periods due to the fact that after 24 h cell death occurs, and transcripts would likely be generated by mechanisms other than by direct interaction with the splicing machinery.

While initial studies used the endogenous MDM2 pre-mRNA for analysis (Figure 2), we reasoned that since the levels of this transcript expressed in different cells is unlikely to be comparable, the evaluation of the effects of a drug on transcript splicing would be complex. Hence, we chose to use minigene plasmids to monitor alternative splicing of this gene in different cell lines. The advantage of this latter system is that direct effects on the spliceosome in unrelated cells can be evaluated, regardless of the status of the *MDM2* gene.

Sudemycin-Induced Splicing of *MDM2* Is More Apparent in Tumor Cells. Having established that *MDM2* minigene constructs could be used to assess drug-induced splicing, we sought to examine whether these events were specific to Rh18. Hence, we transfected either Rh18 or LHCN-M2 cells with the plasmid containing the 3—4 MDM2 minigene and following exposure to Sudemycin evaluated the presence of transcripts arising from this construct by RT-PCR. In these studies, three different drug analogues were used (Sudemycin C1, E, and F), to ensure that any observed changes were due to drug treatment. As indicated in Figure 4, the levels of splicing observed were consistently reduced in normal cells (LHCN-M2) as compared to the tumor line. For example, a direct comparison of both cell types treated with Sudemycin C1, demonstrates the presence of the full length unspliced product (containing exons 3—4—10—11—12) in LHCN-M2 cells, whereas this transcript is not present in drug-treated Rh18 cells.

Furthermore, the overall levels of minigene splicing in cells exposed to Sudemycin E and F were reduced as compared to cells with the C1 analogue. These data correlate with the IC_{50} values for these drugs in these cell lines (Table 1), which indicates that the latter two analogues are less potent agents, especially toward the normal myoblast line. Again, these studies indicate the validity of using the *MDM2* minigenes, since although *MDM2* RNA can be detected in LHCN-M2 cells, the levels of expression are very low in comparison to Rh18 cells. Hence, subtle changes in splicing would be difficult to evaluate. However, when the *MDM2* minigene plasmids are used (Figures 3 and 4), the analysis of the splicing events that occur following drug treatment is facile. This system therefore represents a more reproducible and effective technique in which to evaluate alternative splicing by novel compounds. Overall, these studies indicate that alterations in *MDM2* splicing can be used as a marker for drug exposure and that potentially these events may initiate key cellular changes that result in apoptosis.

Sudemycin-Mediated Alteration of MDM2 Splicing Is Similar to That Observed with Spliceostatin A. Having determined that Sudemycins could modulate *MDM2* splicing in both Rh18 and LHCN-M2 cells, we evaluated whether similar observations would be obtained when using the known SF3b inhibitor Spliceostatin A, although to our knowledge, this effect on the modification of the alternate splicing pattern of

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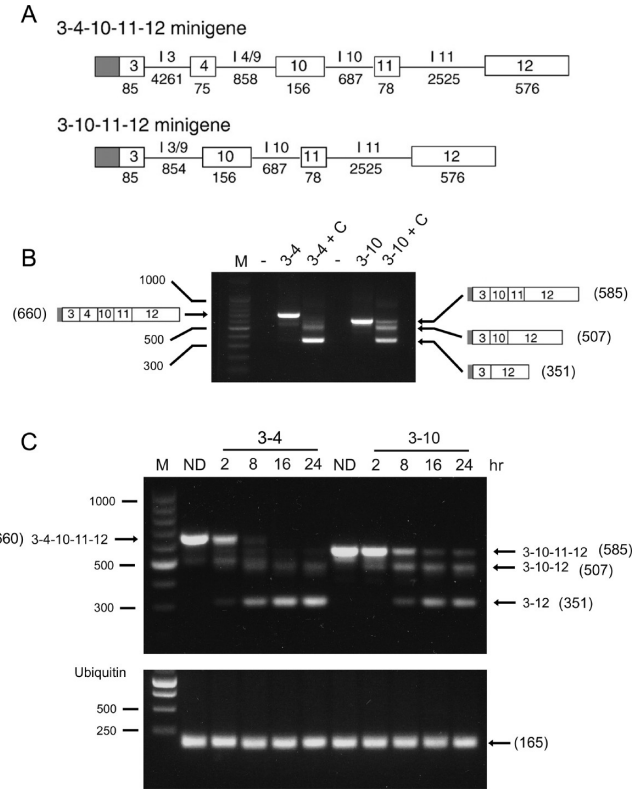

Figure 3. Sudemycin modulates alternative splicing from *MDM2* minigenes. (A) The structure of the introns and exons from the *MDM2* gene present within the two minigene plasmids. (B) PCR analysis of *MDM2* transcripts observed in Sudemycin C1 treated Rh18 cells following transfection with either the 3−4 or 3−12 minigene plasmid. The identity of the PCR products corresponding to the alternatively spliced transcripts is indicated at the side of the figure. These bands are identified by the arrows and their sizes (in bp) are included in parentheses. M, markers (100 bp ladder); 3−4, cells transfected with the 3−4 minigene; 3−4 + C, cells transfected with the 3−4 minigene and subsequently treated with 10 μM Sudemycin C1; 3−10, cells transfected with the 3−10 minigene; 3−10 + C, cells transfected with the 3−4 minigene and subsequently treated with 10 μM Sudemycin C1. (C) A time course of *MDM2* minigene splicing in Rh18 cells. Cells were transfected with either the 3−4 or the 3−10 minigene, and at various times after exposure to 10 μM Sudemycin C1, RNA was prepared and the presence of the different minigene transcripts was assessed (upper panel). The identity of the transcripts, indicated at the side of the figure, was validated by DNA sequencing. The lower panel indicates the analysis of the ubiquitin transcripts in the same samples. As above, products are identified by arrows and sizes are indicated in parentheses. M, markers (upper panel, 100 bp ladder; lower panel, 1 kb ladder); ND, no drug.

genes in cells has not been reported for this compound or for other natural product spliceosome modulators. Therefore, we undertook experiments identical to those described previously; however, in these studies we used Spliceostatin A at a concentration of 100 nM. This concentration was chosen because previous reports have indicated that this dose results in modulation of the presence of unspliced RNA molecules in cells.[1] Figure 5A demonstrates that following exposure of Rh18 cells to Spliceostatin A, essentially the same changes in the splicing of endogenous *MDM2* pre-mRNA were observed as those seen following treatment with Sudemycin. On the basis of these experiments, we transfected the 3−4 *MDM2* minigene

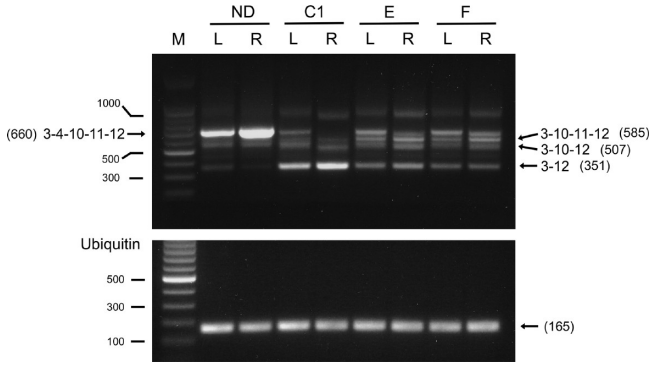

Figure 4. Modulation of *MDM2* minigene splicing in LHCN-M2 and Rh18 cells following exposure to the different Sudemycin analogues. Following transfection, cells were exposed to 10 μM drug for 24 h, and the presence of the different transcripts was determined by PCR and DNA sequencing. The identity of the products is indicated at the sides of the figure. The lower panel indicates the analysis of the ubiquitin transcripts in the same samples. The PCR products are indicated by the arrows and their sizes (in bp) are included in parentheses. M, markers (100 bp ladder); ND, no drug; C1, Sudemycin C1; E, Sudemycin E; F, Sudemycin F; L, LHCN-M2 cells; R, Rh18 cells.

into Rh18 or LHCN-M2 and assessed modulation of splicing following treatment of cells with Spliceostatin A. As can be seen in Figure 5B, this drug induces alternative splicing to yield the 3−12 product in Rh18 cells, whereas little if any changes are observed in the normal myoblast line. As expected, no changes in the levels of ubiquitin expression were observed in either cell line, suggesting that these results arise from direct effects on the spliceosome, rather than altering RNA transcription.

In our previous *in vivo* studies, we observed no deleterious effects to the mice following treatment with Sudemycins,[7] suggesting that these molecules may have limited toxicity toward normal cells and tissues. While in part this was limited by the solubility of the drug, the studies presented here clearly demonstrate that both Sudemycins and Spliceostatin A are less effective at inducing changes in RNA splicing in LHCN-M2 (normal) versus Rh18 (tumor) cells. In addition, the former agent is less cytotoxic to these normal cells (Table 1). It would appear therefore that there is inherent selectivity of these compounds toward tumor cells, although at present the reason for this is unclear. Potentially, normal cells may respond to the inhibition of the spliceosome and modulate associated processes to mitigate the toxic effects of these compounds. For example, enhanced nonsense mediated decay may eliminate inappropriately generated RNAs with unknown biological consequences. Under similar conditions, as a result of the presence of genome instability, the likelihood of specific mutations in genes involved in cell growth and survival, and other genetic defects, tumor cells may be unable to respond to changes in the activity of this complex. This would then result in alternative splicing and the generation of truncated and mutant proteins that have deleterious effects on the cell. Alternately, uptake of these agents into tumor cells might be enhanced, and/or drug efflux may be more efficient in normal cells. While speculation, since our data clearly demonstrate that at equivalent drug concentrations normal cells respond differently to the damage induced by Sudemycin derivative and Spliceostatin A, as compared to tumor cells, it is likely that these events play a role in the cytotoxic activity of these agents.

Table 1. IC_{50} Values for Mammalian Cells Treated with Sudemycin Analogues

		IC_{50} (μM)[a] Sudemycin analogue			IC_{50} (nM)		
cell line	time of drug exposure (h)	C1	E	F	Sudemycin C	Pladienolide B	FR901464
Rh18	2	1.00	>30[b]	>30[b]	ND[c]	ND[c]	ND[c]
Rh18	8	0.50	4.96	2.48	ND[c]	ND[c]	ND[c]
Rh18	72	0.36	0.78	0.62	ND[c]	ND[c]	ND[c]
LHCN-M2	2	1.24	>30[b]	>30[b]	ND[c]	ND[c]	ND[c]
LHCN-M2	8	1.39	>30[b]	>30[b]	ND[c]	ND[c]	ND[c]
LHCN-M2	72	0.90	2.40	3.13	ND[c]	ND[c]	ND[c]
WiDr	72	ND[c]	ND[c]	ND[c]	2030[d]	0.86[e]	ND[c]
MCF-7	72	ND[c]	ND[c]	ND[c]	2290[d]	0.40[e]	1.80[f]
A549	72	ND[c]	ND[c]	ND[c]	4650[d]	1.40[e]	1.30[f]

[a] Results represent a typical experiment using 5 determinations per data point using 8 concentrations of drug. [b] Greater than 50% cell viability was seen at the maximum drug concentration (30 μM). [c] Not determined. [d] Data taken from Lagisetti *et al.*[8] [e] Data taken from Kotake *et al.*[2] [f] Data taken from Nakajima *et al.*[6]

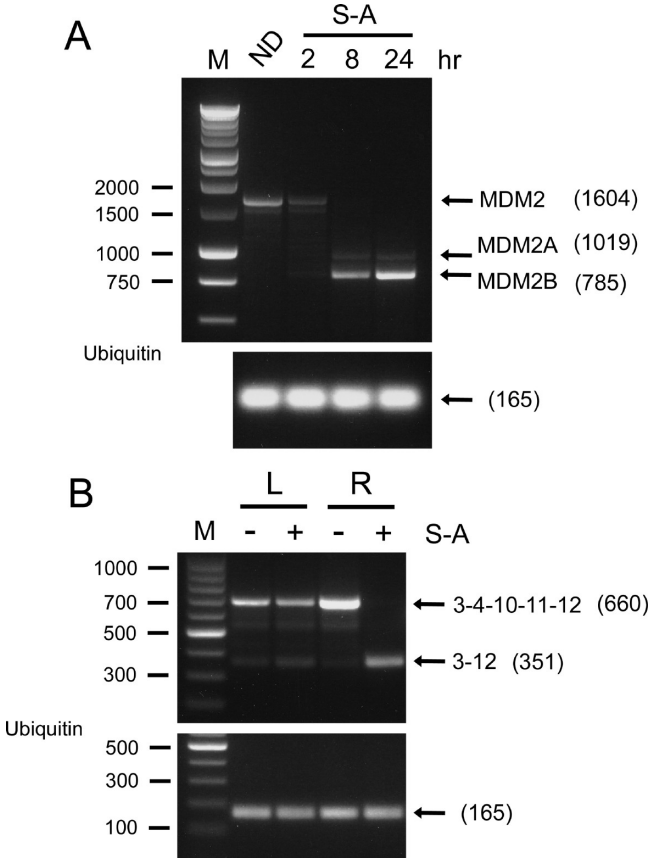

Figure 5. Spliceostatin modulates *MDM2* splicing in an identical fashion to that observed with Sudemycin. (A) Rh18 cells were treated with 100 nM Spliceostatin A for up to 24 h, samples were harvested, and *MDM2* RNA splicing was evaluated by PCR. The lower panel indicates the analysis of the ubiquitin transcripts in the same samples. The PCR products are indicated by the arrows and their sizes (in bp) are included in parentheses. M, markers (1 kb ladder); ND, no drug; S-A, Spliceostatin A. (B) LHCN-M2 and Rh18 cells were transfected with the 3−4 *MDM2* minigene, and the effects of Spliceostatin A on RNA splicing were assessed by PCR. The lower panel indicates the analysis of the ubiquitin transcripts in the same samples. As above, products are identified by arrows, and sizes are indicated in parentheses. M, markers (100 bp ladder); "−", no drug; "+", cells treated with 100 nM Spliceostatin A; L, LHCN-M2 cells; R, Rh18 cells.

Drug-Induced Changes in Gene Splicing Is Not Specific for *MDM2*. Having established that specific changes in *MDM2* splicing could be observed in both tumor and normal cells following exposure to Sudemycin, we sought to examine whether this was specific for this pre-mRNA transcript or whether this was a more global phenomenon. Therefore, we assessed alterations in the RNA splicing pattern for *caspase* 2 and 9, as well as *BCL-X*, in Rh18 following exposure to Sudemycin. As depicted in Figure 6, the disappearance of the S form of caspase 2 was observed over time. Since the ratio of the S to L forms has been correlated with induction of apoptosis,[13−15] potentially these changes represent one mechanism of cytotoxicity afforded by these agents.

Changes were also observed in *caspase 9* splicing with the loss of the 742bp signal being observed in response to drug treatment. However, no alteration in *BCL-X* splicing was seen, suggesting that either the cellular response to targeting of the spliceosome has selectivity or that potentially the half-life of these mRNAs is sufficiently long that such changes would not be observed over the time course of these studies.

While inhibition of splicing has been reported for Spliceostatin A[1] and pladieonlide B,[2] in target genes such as *p27* and *DNAJB1*, we are unaware of any articles that detail the effects of these compounds on the alternative splicing pattern of different genes. Our studies clearly demonstrate that both the Sudemycins and Spliceostatin A can regulate this phenomenon, potentially providing a key to the mechanism of cytotoxicity afforded by these agents. For example, we demonstrated that treatment of cells with Sudemycin C1 reduces the levels of the S form of caspase 2 (Figure 6). Since expression of this protein can reduce the levels of apoptosis induced by chemotherapeutic agents such as etoposide,[16] it is possible that the splicing inhibitors induce cells death by changing the ratios of the proteins involved (caspase 2S and 2L) in this process. While we realize that we have surveyed only a small fraction of the genes that might be involved in the response to drug treatment, the profound effects that these compounds have on both gene splicing and the generation of truncated proteins is likely to impact the cellular response to these agents. More detailed studies to definitively identify the mechanism by which Sudemycin induces cytotoxicity are currently underway.

Alteration of Splicing in Human Tumor Xenografts Following Sudemycin Exposure. To verify that the effects that we had observed were not limited to tissue culture-based experiments,

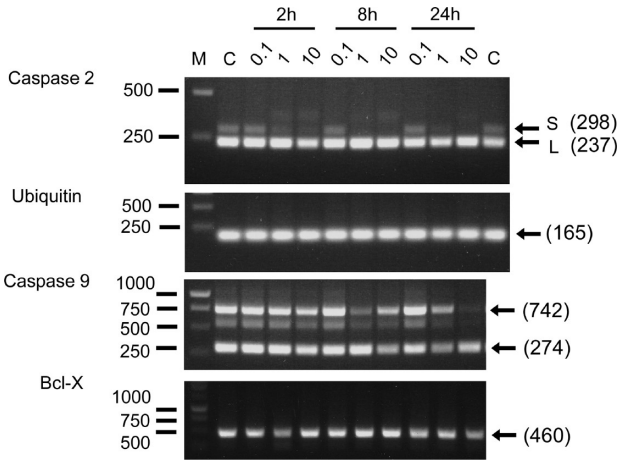

Figure 6. Effects of Sudemycin on the splicing of other genes in Rh18 cells. Cells were treated with 0.1, 1, or 10 μM Sudemycin C1 for 2, 8, or 24 h, and splicing of transcripts for *caspase 2*, *caspase 9*, and *BCL-X* were assessed by PCR. The validity of all *Caspase 2S* and *2L* transcripts was determined by DNA sequencing. *Ubiquitin* was used as a control for these studies. In all panels, the PCR products are indicated by the arrows and their sizes (in bp) are included in parentheses. M, Markers (100 bp ladder); C, no drug.

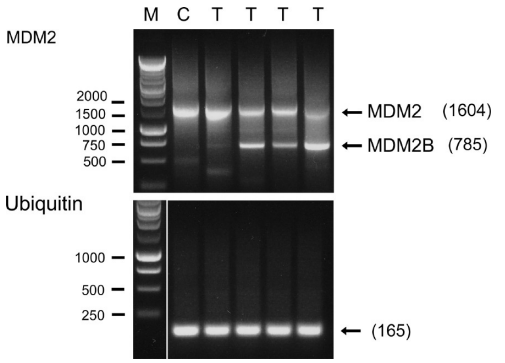

Figure 7. Sudemycin modulates *MDM2* splicing in Rh18 xenografts. Mice bearing subcutaneous Rh18 xenografts were dosed with 50 mg/kg Sudemycin E, and 24 h later tumors were harvested and *MDM2* RNA splicing was determined by PCR. The lower panel indicates the analysis of the ubiquitin transcripts in the same samples. The PCR products are indicated by the arrows and their sizes (in bp) are included in parentheses. M, markers (upper panel, 1 kb ladder; lower panel 100 bp ladder); C, no drug; T, Rh18 tumor treated with Sudemycin.

we evaluated whether altered *MDM2* splicing could be observed in Rh18 xenografts following treatment with Sudemycin. In these studies, Sudemycin E was employed because it is considerably more water-soluble than the C1 analogue, and it was used at a concentration that we have previously determined to be nontoxic to animals.[7] As indicated in Figure 7, following exposure to drug, the presence of *MDM2B* could be readily detected in the tumors, whereas this transcript was not apparent in the untreated sample. As expected, no changes were observed with the ubiquitin RNA. Similar studies were also performed using the liver and kidney obtained from these animals; however, due to the very low levels of Mdm2 expression in these tissues, it was not possible to assess alternative RNA splicing in these samples (see Supplementary Figure S2). Overall, these results suggest that Sudemycin can modulate RNA splicing in tumor cells *in vivo*, at nontoxic concentrations.

Although numerous agents have been identified that can alter gene splicing,[17] it is likely that the majority of the effects observed for these compounds are *via* mechanisms other than direct interaction with the RNA splicing machinery. For example, drugs that target histone deacetylase and topoisomerase I have been demonstrated to change splice site selection in *in vitro* assays (see ref 17 and articles cited therein). Since the processes by which agents that primarily target DNA structure would be involved in RNA processing is unclear, it is difficult to envisage how these agents would specifically modulate the activity of the spliceosome. More likely, these compounds would alter the global rate of RNA transcription, leading to changes in the levels of the input sequences for the spliceosome. In addition, these drugs may also alter the levels of the snRNAs, components that are essential for high fidelity RNA splicing. With Sudemycin and Spliceostatin A, it is likely that both molecules directly interact with SF3b, resulting in very specific changes in the function of the spliceosome. In the biological system that we used to evaluate this process, *MDM2* was found to be an excellent marker of drug exposure and potentially may play a role in the induction of apoptosis in tumor cells. As indicated above, studies to further document the specific roles of these, and other proteins, in the mechanism of cytotoxicity induced by these agents are currently underway.

Overall, we have demonstrated that Sudemycin is a potent modulator of alternative splicing of several different genes in tumor cells. These effects are less pronounced in normal cells, although the functional relevance of these alternatively spliced RNAs and the proteins that they encode is unclear. Future studies will involve the use of high resolution genomic approaches (*e.g.*, exon junction splice arrays and transcriptome sequencing) to validate the effects of Sudemycin in both tumor and normal cells, and to define the mechanism of cytotoxicity induced by these compounds.

METHODS

Cells and Plasmids. The pediatric rhabdomyosarcoma cell line Rh18 was obtained from Dr. P. Houghton (Nationwide Children's Hospital, Columbus, OH) and grown in DMEM containing 10% fetal calf serum under an atmosphere of 10% CO_2 at 37 °C.[11] The telomerase immortalized human myoblast line (LHCN-M2) was provided by Dr. W. Wright (University of Texas Southwestern Medical Center, Dallas, TX). These latter cells are non-tumorigenic and can be induced to undergo myogenic differentiation under the appropriate conditions. They were grown in 80% DMEM/20% Medium 199 containing 15% fetal calf serum, supplemented with 0.03 mg/L $ZnSO_4$, 1.4 mg/L vitamin B12, 55 μg/L dexamethasone and 2.5 μg/L human growth hormone.[18] Cell growth was conducted under an atmosphere of 5% CO_2 at 37 °C.

Plasmids containing *MDM2* minigenes were obtained from Dr. D. Chandler (Nationwide Children's Hospital, Columbus, OH).[19] These contained either exons 3−4−10−11−12 or 3−10−11−12 of this gene.

Sudemycin and Spliceostatin A. Sudemycins were synthesized as previously described.[7,8] All compounds were purified by SFC and were greater than 98% pure as determined by HPLC and chiral SFC. Spliceostatin A was kindly provided by Dr. Yoshida (RIKEN). Both drugs were dissolved in DMSO prior to use.

RT-PCR. Following exposure of exponentially growing cells to drug for determined periods of time, total RNA was prepared using an RNEasy Mini kit (Qiagen). After conversion to cDNA using Superscript III reverse transcriptase (Invitrogen), PCR was performed using specific oligonucleotides using standard protocols. Products were analyzed using agarose gel electrophoresis and ethidium bromide staining and

Table 2. Oligonucleotides Used for Assessing RNA Splicing

gene	forward (F) or reverse (R)	sequence	isoform; expected product size (bp)
MDM2	F	CTGGGGAGTCTTGAGGGACC	MDM2; 1604
			MDM2A; 1019
	R	CAGGTTGTCTAAATTCCTAG	MDM2B; 785
MDM2 minigene	F	AACAAAAGCTGGAGCTCCAC	3−4−10−11−12; 660
			3−10−11−12; 585
	R	CAATCAGGAACATCAAAGCC	3−10−12; 507
			3−12; 351
Ubiquitin	F	ACCTGACCAGCAGCGTCTGATATT	165
	R	TCGCAGTTGTATTTCTGGGCAAGC	
Caspase 2	F	TTGCACAGTTACCTGCACACC	Caspase 2S; 298
	R	GGTTCTTTCCATCTTGTTGGTC	Caspase 2 L; 237
Caspase 9	F	GCTCTTCCTTTGTTCATCTCC	Caspase 9 long;742
	R	CATCTGGCTCGGGGTTACTGC	Caspase 9 short; 274
BCL-X	F	GAGGCAGGCGACGAGTTTGAA	460
	R	TGGGAGGGTAGAGTGGATGGT	

compared to either 1 kb or 100 bp DNA ladders (Promega). The identity of specific transcripts was verified using sequencing.

Oligonucleotides Used for Assessing RNA Splicing. The sequences of the oligonucleotides used for these studies are presented in Table 2. Controls for all experiments utilized primers targeting ubiquitin since this gene lacks introns.

Cell Viability Assays. Assessment of cell viability following drug treatment was performed using Alamar Blue.[20] Briefly, 2,500 cells were plated in each well of a 96-well plate, and after allowing 24 h to attach to the plastic drug was added in fresh media. At requisite time intervals, drug was removed and fresh media applied. Cells were then allowed to grow for an additional 72 h (approximately 3 cell doublings), and viability was determined using Alamar Blue (Invitrogen), using conditions recommended by the supplier. The presence of the fluorescent product, resorufin (indicative of cell metabolism), was determined spectrofluorometrically using wavelengths of 530 and 590 nm for λ_{ex} and λ_{em}, respectively. Routinely, all assays were performed in quintuplicate using 8 concentrations of drug (0.01 to 30 μM), and data were analyzed using GraphPad Prism software to generate an IC_{50} value.

Western Analyses. Whole cell extracts were separated by SDS-PAGE, and following transfer to an Immobilon-P membrane (Millipore) the presence of Mdm2 was determined using an anti Mdm2 antibody (R & D Systems, catalogue no. AF1244). This antibody recognizes both full length protein and several splice variants including Mdm2A. Equal gel loading and protein transfer was assessed using an antibody directed toward β-tubulin (Tub 2.1, Sigma Aldrich).

Cell Transfection. Cells were transfected using 1.6 μg of plasmid DNA with Lipofectamine 2000 (Invitrogen). The next day, drug was added, and at various time periods later, cells were harvested and RNA was analyzed as described above.

Human Tumor Xenografts. Scid mice were implanted with subcutaneous Rh18 xenografts using previously established protocols.[21] After tumors reached a volume of ~250 mm^3, 50 mg/kg Sudemycin E was administered i.v., and samples were harvested 24 h later by flash freezing in liquid nitrogen. Tumors were then processed to assess alteration in gene splicing as described above. All animal studies were performed in accordance with the St. Jude Children's Research Hospital Animal Care and Use Committee.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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