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siRNAs targeting cyclin D1 and cyclin D2 enhance the cytotoxicity of chemotherapeutic agents in mantle cell lymphoma cell lines

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Abstract

Cyclin D1 (CCND1) is a known cell cycle regulator whose overexpression is a hallmark of mantle cell lymphoma (MCL). Although molecular techniques have unified the diagnostic approach to MCL, no therapeutic advances have been made to target this particular pathway. The significance of CCND1 in the pathogenesis and treatment of MCL has yet to be defined. We have taken advantage of RNA interference (RNAi) to downregulate CCND1 expression in two MCL cell lines (Granta-519 and Jeko-1) to investigate the cytotoxic effect of combining RNAi with conventional chemotherapeutic agents. We designed four siRNAs specific to CCND1, one specific to CCND2, and one dual-targeting siRNA that simultaneously downregulates CCND1 and CCND2. Etoposide and doxorubicin were used as chemotherapeutics in combination with the siRNAs. The transfected siRNAs in MCL cell lines triggered 40% to 60% reduction in target mRNA and protein levels. Importantly, the siRNA-mediated reduction in cyclins resulted in decreased IC50 values for both doxorubicin and etoposide. The combination of siRNA-mediated inhibition of the cyclins along with chemotherapeutic agents could potentially be used to lower the effective doses of the chemotherapeutic agents and reduce drug-related toxicities.

INTRODUCTION

Mantle cell lymphoma (MCL) is a distinct subtype of B-cell non-Hodgkin lymphoma (NHL). The clinical evolution of MCL is aggressive, with the lowest 5-year survival rate of any type of lymphoma, and is characterized by poor responses to conventional therapeutic regimens [1–3]. Furthermore, MCL often becomes resistant to conventional therapies and is thus considered to be an incurable malignancy. The genetic hallmark of MCL is the t(11;14) (q13;q32) translocation [4], which occurs in more than 95% of patients [5] and is considered the initial oncogenic event during the development of this malignancy. The proto-oncogene CCND1 is located on chromosome 11 and in MCL it is displaced downstream of the

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enhancer region for the IgH heavy chain region on chromosome 14. This translocation results in constitutive over-expression of CCND1 [6] and cell cycle dysregulation, promoting G1 to S phase progression.

Although the t(11;14)(q13;32) translocation is found in virtually all MCL, the degree of CCND1 overexpression varies among MCL-derived cell lines. Certain MCL cell lines express a truncated CCND1 mRNA isomer, which leads to enhanced overexpression of CCND1 mRNA, protein, and more rapid cell cycle progression [7]. Similarly, patients with MCL have different prognoses and clinical outcomes based on levels of CCND1 overexpression [6]. Currently, there is no therapeutic agent that specifically targets CCND1. The results of clinical trials for this disease have been disappointing and thus far no treatment has been established as a standard therapy. Novel biologic agents with activity against MCL include cell cycle inhibitors like flavopiridol [8], histone deacetylase inhibitors like vorinostat [9](10) and proteasome inhibitors such as bortezomib [10]. Interestingly, both vorinostat and bortezomib have also been shown to decrease CCND1 expression in cell culture. These results further highlight the importance of CCND1 regulation in the treatment of MCL.

RNA interference (RNAi) has shown great promise in elucidating the function of specific genes in disease pathology, including cancer. RNAi is an endogenous cellular regulatory mechanism in which double-stranded RNA (dsRNA) triggers the cleavage or translational repression of complementary single-stranded RNA (ssRNA) levels [11]. In lower eukaryotes long dsRNAs are processed into small interfering RNAs (siRNAs) by the RNase-III endonuclease, Dicer, resulting in 21–23 bp siRNAs with two nucleotide 3' overhangs [12]. One or both strands of the siRNAs can be incorporated into a multi-subunit ribonucleoprotein complex, termed the RNA-induced silencing complex (RISC). One of the two strands is released from the complex following ATP-dependent unwinding [13] or passenger-strand cleavage [14]. The resultant guide strand, when perfectly complementary to a messenger RNA, directs site-specific cleavage and subsequent degradation of the mRNA. In the present study we have taken advantage of siRNAs to downregulate the expression of CCND1 and CCND2 for potential application in the treatment of MCL.

We have synthesized and tested in MCL cell lines, several different siRNAs targeting CCND1 and CCND2, and examined their potencies in directing target downregulation. We have combined the most potent of these siRNAs with the traditional chemotherapy agents etoposide and doxorubicin. Our findings suggest that downregulation of the cyclins lowers the effective drug IC₅₀ values and enhances cytotoxicity of these drugs in MCL cell lines.

DESIGN AND METHODS

Cell culture

The Granta-519 cell line was kindly provided by Dr. Witzig's laboratory (Mayo Clinic) and sustained in 10% fetal bovine serum (FBS), 2mM glutamine and 1mM sodium pyruvate supplemented Dulbecco's Modified Eagle Medium (DMEM) (Mediatech Inc.). The Jeko-1 cells were purchased from ATCC and cultured in 20% FBS and 1% glutamine containing Roswell Park Memorial Institute Medium 1640 (RPMI) (Mediatech Inc.). Z138 cells, a kind gift from Dr. Tucker (British Columbia Cancer Agency) were grown in RPMI containing 10% FBS and 1% glutamine. The same medium was used for JVM-2, Rec-1, SUDHL6, SUDHL4, Raji and Daudi cells that were purchased from ATCC. All cell lines were cultured at 37°C in a humidified, 5% CO₂ atmosphere.

siRNA design and nomenclature

The siRNAs are named after their target (CCND1, D2) and if more than one siRNA targets the same mRNA the name denotes the binding site within the mRNA (224, 391, 778 and trunc). All 25/27mer siRNAs contain two deoxyribonucleotides in the 3' end of the sense strand highlighted by lower case letters. The sequences are as followed:

CCND1 224 antisense strand: 5'-GAUGGUUCCACUUCGCAGCACAGGAG-3'

CCND1 224 sense strand: 5'-CCUGUGCUGCGAAGUGGAAACCA_{tc}-3'

CCND1 391 antisense strand: 5'-CCUCGCAGACCUCCAGCAUCCAGGUGG-3'

CCND1 391 sense strand: 5'-ACCUGGAUGCUGGAGGUCUGCGA_{gg}-3'

CCND1 778 antisense strand: 5'-UUGGAAAUGAACUUCACAUCUGUGGCA-3'

CCND1 778 sense strand: 5'-CCACAGAUGUGAAGUUCAUUUCC_{aa}-3'

CCND1 trunc antisense strand: 5'-UUUUUAAUAUAAAAACGAGUU-3'

CCND1 trunc sense strand: 5'-CUCGUUUUUAUAUUAAAAAAA-3'

siD1/D2 antisense strand 1: 5'-CCGAACUUAGGUUGAGUACCCUAAUUU

siD1/D2 sense strand 2: 5'-AUAAGGGUGAUGAACAUAAAGUUC_{gg}

D2 antisense strand: 5'-GCCAAGAAACGGUCCAGGUAAUUCAUG-3'

D2 sense strand: 5'-UCAAUUACCUGGACCGUUUCUUG_{gc}-3'

Electroporation and Lipofection

Granta-519 cells were transfected with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. The final concentration of siRNA was 50nM.

Jeko-1 cells were transfected via electroporation with the Bio-Rad GenePulser® MXcell at a final concentration of 200nM siRNA. Manufacturer's protocol was followed and the electroporation program previously optimized in our laboratory for Jeko-1 used was: exponential wave form, voltage of 200V, capacitance of 350mF and resistance to 1,000Ω. Mock or control Jeko-1 cells were treated with electroporation. Mock or control Granta-519 cells were treated with lipofection.

RNA extraction and quantitative real time PCR

Total RNA was extracted using RNA-STAT60 (Teltest), following manufacturer's protocol. Residual DNA was digested using the DNA-free kit following the manufacturer's instructions (Ambion).

cDNA was obtained by transcribing 2ug of RNA with Maloney murine leukemia virus (MMLV) reverse transcriptase and random primers according to manufacturer's instructions (Invitrogen). Expression of human mRNAs encoding CCND1 (CCND1-F6 5'-CTCCTCTCCGGAGCATTTTGATA-3' and CCND1-R6 5'-TTAAAGACAGTTTTTGGGTAATCT-3' to measure total CCND1 mRNA), CCND2 (CCND2-F 5'-CGCAAGCATGCTCAGACCTT-3', CCND2-R 5'-TGCGATCATCGACGGTGG-3') and RPLP0 (RPLP0-F 5'-GGCGACCTGGAAGTCCAA-3' and RPLP0-R 5'-CCATCAGCACCACAGCCTTC-3') were determined by quantitative real-time PCR (CFX96, BIO-RAD) using 2X iQ SYBR® Green Mastermix (BIO-RAD) according to manufacturer's protocol. RPLP0 expression was used as internal control.

Protein extraction and Immunoblotting

Protein extraction was performed by adding 50–100µl M-Per® Mammalian Protein Extraction Reagent lysis buffer (Thermo Scientific), containing Complete, Mini Protease Inhibitor Cocktail (Roche). Samples were frozen in an ethanol bath for 30 seconds and thawed for 30 seconds in a 37°C water bath, repeating freeze thaw cycles twice followed by centrifugation at 13,000rpm for 10min at 4°C. The concentration of the samples was determined with the Bio-Rad Protein Assay according to manufacturer's instructions. Samples were stored at –80°C.

Proteins (25µg) were separated by 12% SDS-PAGE gel electrophoresis. Immunoblotting identified CCND1, CCND2 and α-tubulin proteins. Cyclin D1 (36kDa) was detected with mouse anti-cyclinD1 IgG from BD Bioscience Pharmigen, Cyclin D2 (34kDa) with rabbit anti-cyclinD2 IgG from Santa Cruz Biotechnology and the loading control alpha-tubulin (55kDa) was detected with mouse anti-α tubulin IgG from Sigma-Aldrich as primary antibody and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) as secondary antibody.

MTS Proliferation assay

Cell lines were treated with increasing amounts (0.5nM up to 10uM) of chemotherapy agent doxorubicin or etoposide (GD Biosciences) in combination with CCND1 siRNAs. Cell viability was measured with CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to manufacturer's instructions. IC₅₀ values were calculated from the dose response curve. For combination experiments the drugs were mixed in 1.2:1 ratio of IC₅₀ concentration or maximum achievable dosage and diluted to ½ and ¼ concentrations before the addition to the cells. The interaction between drug combinations was analyzed using Calcsyn software program (Biosoft, Cambridge, UK) to determine whether the combination was additive or synergistic. Data from cell viability assay (MTS) were expressed as the fraction of cells killed by the individual drugs or the combination of drugs treated and compared with untreated cells. This program is based on Chou-Talalay method and calculates a combination index (CI), when CI =1 indicates an additive effect and below 1.0 indicates synergism.

RESULTS

Downregulation of CCND1

Mantle cell lymphomas express higher levels of CCND1 than other B-cell lymphomas and their levels also differ within MCL cell lines (e.g. JVM-2, Z-138, Granta-519, Rec-1, Jeko-1) (Figure 1). In our hands JVM-2 showed the lowest level of CCND1 protein expression followed by Granta-519. In contrast Western blot analyses detected very high levels of CCND1 protein in Jeko-1, Z-138, and Rec-1. Raji and Daudi, two Burkitt's lymphoma cell lines, as well as SUDHL-4 and SUDLH-6, two diffuse large B-cell Lymphoma cell lines, showed undetectable or very low CCND1 protein levels (Figure 1).

We evaluated the inhibitory effect of four siRNAs targeting CCND1 at different sites in Granta-519 and Jeko-1 cells using real-time PCR and Western blotting to monitor RNA and protein levels (Figure 2). We employed lipofection for Granta-519 and electroporation for Jeko-1 cells. In both cell lines, CCND1 778 and CCND1 224 demonstrated the highest efficacy in CCND1 mRNA reduction. CCND1 224 gave 45% mRNA reduction in Granta-519 (Figure 2A), and 60% in Jeko-1 (Figure 2B). CCND1 778 demonstrated 40% mRNA reduction in Granta-519 and 80% reduction in Jeko-1. CCND1 391 resulted in 35% mRNA reduction in Granta-519 and 70% in Jeko-1 cell lines. The fourth siRNA, CCND1 trunc, was synthesized to target only the truncated cyclin D1 mRNA isomer by bridging the poly A site and upstream UTR sequences (see methods). This siRNA was the least efficient

in silencing CCND1 mRNA with only an approximately 20% reduction in both cell lines. Overall, the siRNAs CCND1 778 and CCND1 224 showed the highest downregulation of cyclin D1 mRNA and protein levels in both cell lines, with CCND1 778 being the most potent in both Granta-519 (40% mRNA and 26% protein reduction) and Jeko-1 (60% mRNA and 48% protein reduction). Given the difficulties in transfecting and electroporating siRNAs into these cell lines, the inhibitory activities are respectable.

Downregulation of CCND2

Cyclin D2 (CCND2) is upregulated upon downregulation of cyclin D1 [15]. Thus, we examined D2 levels with real-time PCR following treatment with the CCND1 siRNAs in Granta-519 and Jeko-1 cells (Figure 3). In the Granta-519 cells the CCND2 mRNA (Figure 3A) were upregulated post treatment by all siRNAs except the CCND1 trunc. Jeko-1 cells showed high Cyclin D2 mRNA levels post CCND1 224 and CCND1 778 and little or no increase in CCND2 mRNA levels could be detected with CCND1 391 and CCND1 trunc (Figure 3B). We attempted to examine the protein expression of CCND2 with Western Blot analysis. However, the antibody used did not yield sufficient quality western blots for analysis.

To inhibit this compensation by CCND2 upregulation, we chose the two most efficient siRNAs against CCND1 (CCND1 224 and CCND1 778) and co-transfected them with an siRNA targeting CCND2 (D2) maintaining the same final total siRNA concentration as for the individual siRNAs. In Granta-519, the combination of CCND1 778 and D2 downregulated CCND1 mRNA and protein as efficiently as did CCND1 778 (Figure 4A–B) and also suppressed the upregulation of CCND2 mRNA (Figure 4A). The combination of CCND1 224 and D2 also inhibited both mRNA and proteins efficiently (Figure 4A–B). Jeko-1 cells transfected with the combination of siRNAs (CCND1 224+D2, CCND1 778+D2) showed less CCND1 mRNA level reduction than CCND1 778 alone (Figure 5A). Cyclin D1 protein downregulation was slightly better in CCND1 778+D2 than CCND1 778 transfected cells (Figure 5B). Comparison of cyclin D1 protein reduction of CCND1 224+D2 with CCND1 224 alone illustrates a weaker potency when using the siRNAs in combination (Figure 5B). We recently published a novel dual-targeting approach for siRNAs in which each strand of an siRNA duplex independently down-regulates a different targeted mRNA [16]. One of our designs simultaneously targets CCND1 and CCND2 (siD1/D2). This dual-targeting siRNA efficiently inhibited both cyclins in Granta-519 cells (Figure 4A–B) whereas little or no inhibitory effect was observed in Jeko-1 cells (Figure 5A–B). We can best explain this seeming paradox by the different transfection methods used for the two different cell lines. Electroporation, which is the most efficient method of transfection into Jeko-1, generates enough heat to destabilize the imperfectly matched duplex characteristic of the siD1/D2 dual-targeting siRNA. Given the potent dual knockdown of these two targets in Granta-519 cells, the dual-targeting siRNA is a good candidate for applications where lipid based or other non-mechanical delivery is available.

Enhanced cytotoxicity of chemotherapy

To establish the best treatment time point for the combination study of chemotherapy and siRNAs, a time course experiment (24h–96h) was performed in Granta-519 cells with CCND1 778 (Online Supplementary Figure 1). The time point at which mRNA and protein levels were most efficiently reduced was chosen for the treatment with chemotherapeutics. The mRNA and protein levels were lowest after 48 hours. In Jeko-1 the 48 hour time point also indicated the most efficient decrease in CCND1 (data not shown). Both cell lines were transfected with three single siRNAs and treated with doxorubicin and etoposide using concentrations ranging from 0.5nM to 10mM. Granta cell lines were treated with lipofection and Jeko-1 cells lines were treated with electroporation as negative control. CCND1 trunc

was not used in these experiments since it had the least inhibitory effect on the CCND1 mRNA and protein levels in MCL cell lines. MTS cell viability assays were performed 48 hours post treatment. All experiments were done in triplicate. The IC₅₀ values of the drugs alone and combinatorial treatments were calculated with PrismGraph5 (Table 1, Online Supplementary Figure 2).

In Granta-519 cells, all siRNAs tested in combination with doxorubicin showed lower IC₅₀ values than for doxorubicin alone (Table 1) with the exception of CCND1 778. CCND1 224 reduced the IC₅₀ by 32% while CCND1 778 slightly increased the IC₅₀. All of the siRNAs in combination with etoposide resulted in lowered IC₅₀ values and cytotoxicity than etoposide alone, with effects ranging from 28% to 53%. In Jeko-1 cells, all siRNAs in combination with doxorubicin lowered IC₅₀ values versus doxorubicin alone, with effects ranging from 37% to 51%. However, the combination of siRNAs and etoposide increased the IC₅₀. In summary, combined treatment with anti-CCND1 siRNAs and doxorubicin provided a lowering effect of IC₅₀s in both Granta-519 and Jeko-1 cells. In contrast, etoposide in combination with the siRNAs was only effective in Granta-519 cells. We then analyzed whether the siRNA is synergistic with doxorubicin in Jeko-1. The combination of Si-778 and doxorubicin is synergistic with a combination index (CI) of 0.40.

We next examined whether the downregulation of both CCND1 and CCND2 would further enhance the cytotoxicity of doxorubicin and etoposide. We transfected Granta-519 and Jeko-1 cells, with three different combinations of siRNAs: CCND1 778+D2, CCND1 224 +D2 and siD1/D2. The combination of siRNAs targeting CCND1 and D2 and doxorubicin achieved a much higher cytotoxic effect than did the CCND1 siRNAs alone in Granta-519 (Table 1, Online Supplementary Figure 2). CCND1 224+D2 reduced the IC₅₀ of doxorubicin by 57% as compared to a 32% reduction by CCND1 224 alone. CCND1 778+D2 decreased the IC₅₀ by 58% whereas CCND1 778 alone resulted in a 12% increase in the IC₅₀. The dual-targeting siRNA provided an IC₅₀ reduction of approximately 45% for both doxorubicin and etoposide. In Jeko-1 cells CCND1 224+D2 reduced the IC₅₀ of doxorubicin by 60% as compared to 49% by CCND1 224 alone (Table 1, Online Supplementary Figure 3). CCND1 778+D2 did not enhance the drug efficacy. It decreased the IC₅₀ by 42% while CCND1 778 alone provided a 51% reduction in IC₅₀. No consistent effects were observed with the combination of siRNAs and etoposide in Jeko-1 cells. Overall the combinations of cyclin D1 and cyclin D2 siRNAs had the greatest effect on reducing the IC₅₀ values in Granta-519 cells. Furthermore the combination of siRNA and doxorubicin was more effective than siRNA and etoposide.

DISCUSSION

One of the major goals of this study was to investigate the role of inhibiting CCND1 in the treatment of MCL. We utilized siRNA-mediated CCND1 knockdown to inhibit CCND1 protein expression in two MCL cell lines (Jeko-1 and Granta-519). By combining siRNAs against CCND1 with standard MCL chemotherapeutic agents, we were able to enhance the cytotoxicity of doxorubicin in both cell lines, and etoposide in Granta-519. Furthermore, we confirmed the previously reported compensating overexpression of CCND2, when CCND1 is downregulated [15]. By inhibiting both CCND1 and D2 with siRNA, we were able to further enhance the cytotoxicity of doxorubicin in Granta-519 cells.

Our study provides several novel and important findings. CCND1, a well known cell cycle regulator, has been extensively analyzed in MCL. It is also well known that high proliferative genetic signatures in MCL are associated with a poor prognosis [17–21]. Prior to this study, an shRNA was used to inhibit CCND1, causing a modest decrease in cell cycle progression, but having no effect on cell viability or apoptosis. Our approach focused on the

potential clinical application of anti-CCND1 siRNAs. We found that downregulation of CCND1 leads to enhanced cytotoxicity of chemotherapy agents. Zuckerman *et al.* show that siRNA knockout of ribonucleotide reductase is synergistic with the chemotherapeutic agent temozolamide in melanoma cell lines [22]. Our work confirmed that siRNA can be used to augment the cytotoxic effects of chemotherapy, and is the first study showing that manipulation of CCND1 can lead to an enhanced efficacy of chemotherapy agents.

The inhibitory effects of siRNAs on CCND1 depend on the site targeted, the cell line and the method of siRNA delivery. For instance, in our hands CCND1 778 seems to be the optimal siRNA in both cell lines, decreasing CCND1 mRNA expression in Granta-519 by 40% and Jeko-1 by 60%. The mRNA expression was also accompanied by reduced CCND1 protein expression in Granta-519 (24%) and Jeko-1 (48%). The differences in the efficiencies of silencing are best explained by intrinsic differences in CCND1 expression between the cell lines as well as by the method of transfection. The methods of transfection used were lipofection for Granta-519 and electroporation for Jeko-1, but neither of these methods is suitable for *in vivo* or clinical delivery. It is also not clear whether a gene dosage effect exists for CCND1 expression since the degree of downregulation did not necessarily correlate with the degree of enhancement in cytotoxicity when the siRNAs were combined with chemotherapy agents. CCND1 need not be silenced completely in MCL to achieve a beneficial effect such as lowering the IC₅₀ values for chemotherapeutic agents. Nevertheless, it would be of interest to see how much more effective chemotherapy could become if CCND1 were silenced more efficiently. We believe the lack of complete silencing is caused by difficulties in delivery of siRNAs to MCL cells rather than in the siRNA designs. There are several methods to improve the delivery of siRNAs into targeted cell, which can be utilized *in vivo*: siRNAs can be covalently conjugated to targeting ligands [23] such as aptamers [24–27] and antibodies [28], or incorporated into nanoparticles [29–31]. To this end we are developing an aptamer approach for delivery of therapeutic siRNAs to mantle cell and B-cell lymphomas. Efficient targeted delivery will allow a more effective downregulation of the mRNA targets as well as providing a vehicle for *in vivo* testing of the siRNAs. Phase I clinical trials testing both nanoparticle and aptamer delivery methods for siRNA targeted to lymphoma cells are currently under development at this institution.

The enhancement of the cytotoxicity of chemotherapy via anti-CCND1/2 siRNAs is also specific to the chemotherapy agent used. This can be partly explained by the intrinsic differences in anti-lymphoma chemotherapy. Doxorubicin, an anthracycline, intercalates into DNA and inhibits DNA synthesis. Etoposide, a topoisomerase II inhibitor, is cell cycle-specific and acts in the late S and G2 phase. Downregulation of CCND1 by siRNAs can alter cell cycle progression and perhaps inhibit the effectiveness of a cell cycle specific agent. There are also many other chemotherapeutic agents used in lymphoma therapy including methotrexate, cytarabine, fludarabine, and rituximab. Each of these agents targets different tumor survival pathways. It is entirely possible that siRNAs against CCND1 will improve cytotoxicity of some agents but reduce the effectiveness of others. Future studies are required to elucidate the mechanism by which downregulation of CCND1 alters the chemosensitivity of certain agents.

Finally, we have confirmed the existence of a feedback mechanism in CCND1/CCND2. The D-type cyclins, cyclin D1, D2, and D3, are highly homologous to one another and have similar expression patterns during G1 phase [32]. This group of cyclins is functionally redundant and most tissue types will express one type of cyclin D [33]. In fact, MCL cell lines with the highest overexpression of CCND1 also have the lowest CCND2 expression. JVM-2, the MCL cell line with the lowest expression of CCND1, has the highest expression of CCND2. This phenomenon has also been observed in MCL patient samples [34]. 95% of the MCL patients overexpress CCND1 through the t(11;14) translocation, but the other 5%

actually have CCND2 or CCND3 overexpression with no alterations in CCND1 [34]. The D-type cyclins may not be the only cyclins involved in this feedback loop. Several other cyclins have been implicated in lymphoma pathogenesis. It is also known that CCND1 regulates the entry from G1 to S phase, and CCND2 regulates entry from S phase to G2 phase [5]. It is plausible that downregulation of additional cyclins may enhance anti-lymphoma therapy. Also, several novel biologic agents in MCL therapy belong to the class of cyclin-dependent kinase inhibitors. We were unable to completely arrest cell cycle in MCL cell lines with siRNA against CCND1 (data not shown). Perhaps combination treatment of siRNA against cyclins and cyclin-dependent kinase inhibitors [35] will be synergistic in arresting the cell cycle and could further enhance cytotoxicity in MCL. Simultaneous efforts to develop effective methods for siRNA delivery in the clinical setting are underway and will allow the investigation of the effects of CCND1 inhibition on mantle cell lymphoma patients in the future.

In conclusion, we were able to show that siRNA mediated downregulation of CCND1 expression in MCL. Reduction of cyclin D1 in MCL with using siRNA lowers the IC₅₀ values of chemotherapeutic agents. Dual inhibition of cyclin D1 and D2 further enhances the cytotoxic effect of doxorubicin and etoposide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Campo E, Raffeld M, Jaffe ES. Mantle-cell lymphoma. *Semin Hematol.* 1999; 36:115–27. [PubMed: 10319380]
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2007. *CA Cancer J Clin.* 2007; 57:43–66. [PubMed: 17237035]
3. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin.* 2009; 59:225–49. [PubMed: 19474385]
4. Bertoni F, Rinaldi A, Zucca E, Cavalli F. Update on the molecular biology of mantle cell lymphoma. *Hematol Oncol.* 2006; 24:22–7. [PubMed: 16402392]
5. Knudsen KE, Diehl JA, Haiman CA, Knudsen ES. Cyclin D1: polymorphism, aberrant splicing and cancer risk. *Oncogene.* 2006; 25:1620–8. [PubMed: 16550162]
6. Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell.* 2003; 3:185–97. [PubMed: 12620412]
7. Chen RW, Bemis LT, Amato CM, et al. Truncation in CCND1 mRNA alters miR-16–1 regulation in mantle cell lymphoma. *Blood.* 2008; 112:822–9. [PubMed: 18483394]
8. Lin TS, Blum KA, Fischer DB, et al. Flavopiridol, fludarabine, and rituximab in mantle cell lymphoma and indolent B-cell lymphoproliferative disorders. *J Clin Oncol.* 28:418–23. [PubMed: 20008633]
9. Kawamata N, Chen J, Koeffler HP. Suberoylanilide hydroxamic acid (SAHA; vorinostat) suppresses translation of cyclin D1 in mantle cell lymphoma cells. *Blood.* 2007; 110:2667–73. [PubMed: 17606765]

10. Fisher RI, Bernstein SH, Kahl BS, et al. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. *J Clin Oncol*. 2006; 24:4867–74. [PubMed: 17001068]
11. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. *Nature*. 2004; 431:343–9. [PubMed: 15372041]
12. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*. 2000; 101:25–33. [PubMed: 10778853]
13. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*. 2005; 123:607–20. [PubMed: 16271386]
14. Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*. 2001; 107:309–21. [PubMed: 11701122]
15. Klier M, Anastasov N, Hermann A, et al. Specific lentiviral shRNA-mediated knockdown of cyclin D1 in mantle cell lymphoma has minimal effects on cell survival and reveals a regulatory circuit with cyclin D2. *Leukemia*. 2008; 22:2097–105. [PubMed: 18685613]
16. Tiemann K, Hohn B, Ehsani A, et al. Dual-targeting siRNAs. *RNA*. 16:1275–84. [PubMed: 20410240]
17. Hsi ED, Jung SH, Lai R, et al. Ki67 and PIM1 expression predict outcome in mantle cell lymphoma treated with high dose therapy, stem cell transplantation and rituximab: a Cancer and Leukemia Group B 59909 correlative science study. *Leuk Lymphoma*. 2008; 49:2081–90. [PubMed: 19021050]
18. Determann O, Hoster E, Ott G, et al. Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood*. 2008; 111:2385–7. [PubMed: 18077791]
19. Salaverria I, Zettl A, Bea S, et al. Specific secondary genetic alterations in mantle cell lymphoma provide prognostic information independent of the gene expression-based proliferation signature. *J Clin Oncol*. 2007; 25:1216–22. [PubMed: 17296973]
20. Kienle D, Katzenberger T, Ott G, et al. Quantitative gene expression deregulation in mantle-cell lymphoma: correlation with clinical and biologic factors. *J Clin Oncol*. 2007; 25:2770–7. [PubMed: 17563396]
21. Hartmann E, Fernandez V, Moreno V, et al. Five-gene model to predict survival in mantle-cell lymphoma using frozen or formalin-fixed, paraffin-embedded tissue. *J Clin Oncol*. 2008; 26:4966–72. [PubMed: 18606985]
22. Zuckerman JE, Hsueh T, Koya RC, Davis ME, Ribas A. siRNA Knockdown of Ribonucleotide Reductase Inhibits Melanoma Cell Line Proliferation Alone or Synergistically with Temozolomide. *J Invest Dermatol*. 2010
23. Sioud M. What are the key targeted delivery technologies of siRNA now? *Methods Mol Biol*. 629:93–107. [PubMed: 20387145]
24. Zhou J, Li H, Li S, Zaia J, Rossi JJ. Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Mol Ther*. 2008; 16:1481–9. [PubMed: 18461053]
25. Shaw BR, Moussa L, Sharaf M, Cheek M, Dobrikov M. Boranophosphate siRNA-aptamer chimeras for tumor-specific downregulation of cancer receptors and modulators. *Nucleic Acids Symp Ser (Oxf)*. 2008:655–6.
26. McNamara JO 2nd, Andrechek ER, Wang Y, et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol*. 2006; 24:1005–15. [PubMed: 16823371]
27. Zhou J, Rossi JJ. Aptamer-targeted cell-specific RNA interference. *Silence*. 1:4. [PubMed: 20226078]
28. Kumar P, Ban HS, Kim SS, et al. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell*. 2008; 134:577–86. [PubMed: 18691745]
29. Kenny GD, Kamaly N, Kalber TL, et al. Novel multifunctional nanoparticle mediates siRNA tumour delivery, visualisation and therapeutic tumour reduction in vivo. *J Control Release*.

30. Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 464:1067–70. [PubMed: 20305636]
31. Tamura A, Nagasaki Y. Smart siRNA delivery systems based on polymeric nanoassemblies and nanoparticles. *Nanomedicine (Lond)*. 5:1089–102. [PubMed: 20874023]
32. Lahti JM, Li H, Kidd VJ. Elimination of cyclin D1 in vertebrate cells leads to an altered cell cycle phenotype, which is rescued by overexpression of murine cyclins D1, D2, or D3 but not by a mutant cyclin D1. *J Biol Chem*. 1997; 272:10859–69. [PubMed: 9099742]
33. Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. *Cell*. 2004; 118:477–91. [PubMed: 15315760]
34. Wlodarska I, Dierickx D, Vanhentenrijk V, et al. Translocations targeting CCND2, CCND3, and MYCN do occur in t(11;14)-negative mantle cell lymphomas. *Blood*. 2008; 111:5683–90. [PubMed: 18391076]
35. Lacrima K, Valentini A, Lambertini C, et al. In vitro activity of cyclin-dependent kinase inhibitor CYC202 (Seliciclib, R-roscovitine) in mantle cell lymphomas. *Ann Oncol*. 2005; 16:1169–76. [PubMed: 15851403]

Figure 1. Cyclin D1 protein expression of Non-Hodgkin's Lymphoma cell lines
Western blot analysis was performed on 8 cell lines derived from Mantle Cell Lymphoma (Jeko-1, Granta-519, JVM-2, Rec-1 and Z138), Burkitt's Lymphoma (Raji, Daudi) and Diffuse Large B-cell Lymphoma (SUDHL-4, SUDHL-6).

Figure 2. Downregulation of Cyclin D1 in Jeko-1 (C,D) and Granta-519 (A,B) cells with four siRNAs (CCND1 224, CCND1 391, CCND1 778 and CCND1 trunc)
 (A) Cyclin D1 mRNA levels of Granta-519 cells transfected with siRNAs targeting Cyclin D1. The mRNA levels were examined by real-time PCR and calculated relative to internal control RPLP0. (B) Cyclin D1 protein levels of Granta-519 cells transfected with four siRNAs. Protein levels were determined by Western blot analysis using α -tubulin as internal control. (C) Relative Cyclin D1 mRNA levels of Jeko-1 cells transfected with four different siRNAs. (D) Relative Cyclin D1 protein levels of Jeko-1 transfected cells.

Figure 3. Cyclin D2 upregulation after treatment with Cyclin D1 targeting siRNAs (CCND1 224, CCND1 391, CCND1 778, CCND1 trunc) in Granta-519 (A) and Jeko-1 (B)
Relative Cyclin D2 mRNA determined by real time PCR after treatment with 4 siRNAs targeting Cyclin D1. RPLP0 was employed as internal control.

Figure 4. Effect of siRNAs targeting CCND1 and D2 in combination (CCND1 778, CCND1 778+D2, CCND1 224+D2, siD1/D2) on Cyclin D1 and Cyclin D2 expression in Granta-519 cells (A) Relative mRNA levels of Cyclin D1 and Cyclin D2 48h post transfection with CCND1 778 alone, CCND1 778 in combination with D2, CCND1 224 in combination with D2 siRNA and dual-targeting siD1/D2. RPLP0 was employed as internal control. (B) Cyclin D1 protein levels were detected with Western blotting 48h post transfection. Relative Cyclin D1 levels were calculated with α -tubulin as loading control.

Figure 5. Effect of siRNAs targeting CCND1 and D2 in combination (CCND1 778, CCND1 778+D2, CCND1 224+D2, siD1/D2) on Cyclin D1 and Cyclin D2 expression in Jeko-1 cells
 (A) Relative mRNA levels of Cyclin D1 and Cyclin D2 48h post transfection with CCND1 778 alone, CCND1 778 in combination with D2, CCND1 224 in combination with D2 siRNA and dual-targeting siD1/D2. RPLP0 was employed as internal control. (B) Cyclin D1 protein levels were detected with Western blotting 48h post transfection. Relative Cyclin D1 levels were calculated with α -tubulin as loading control.

Table 1 Effects of cyclin siRNAs on IC₅₀ of chemotherapeutic agents

SiRNAs	Granta-519		Jeko-1	
	IC ₅₀ Doxorubicin	IC ₅₀ Etoposide	IC ₅₀ Doxorubicin	IC ₅₀ Etoposide
Control	212.7nM	564 nM	487.3 nM	307 nM
CCND1 224	146.2 nM (−32%)	401.4 nM (−28%)	249.6 nM (−49%)	391.2 nM (+27%)
CCND1 391	123.2 nM (−42%)	266.3 nM (−53%)	306.8 nM (−37%)	269.8 nM (−12%)
CCND1 778	238.6 nM (+12%)	378.5. nM (−33%)	238.8 nM (−51%)	266.8 nM (−13%)
CCND1 224+D2	93.5 nM (−57%)	348.3 nM (−39%)	195.8 nM (−60%)	377.3 nM (+22.8%)
CCND1 778+D2	91.1 nM (−58%)	479.2 nM (−16%)	283.2 nM (−42%)	303.5 nM (−1%)
siD1/D2	118.6nM (−45%)	296.1 nM (−48%)	332.4 nM (−32%)	325.6 nM (+6%)