A Novel CDK9 Inhibitor Shows Potent Antitumor Efficacy in Preclinical Hematologic Tumor Models


Abstract

DNA-dependent RNA polymerase II (RNAP II) largest subunit RPB1 C-terminal domain (CTD) kinases, including CDK9, are serine/threonine kinases known to regulate transcriptional initiation and elongation by phosphorylating Ser 2, 5, and 7 residues on CTD. Given the reported dysregulation of these kinases in some cancers, we asked whether inhibiting CDK9 may induce stress response and preferentially kill tumor cells. Herein, we describe a potent CDK9 inhibitor, LY2857785, that significantly reduces RNAP II CTD phosphorylation and dramatically decreases MCL1 protein levels to result in apoptosis in a variety of leukemia and solid tumor cell lines. This molecule inhibits the growth of a broad panel of cancer cell lines, and is particularly efficacious in leukemia cells, including orthotopic leukemia preclinical models as well as in ex vivo acute myeloid leukemia and chronic lymphocytic leukemia patient tumor samples. Thus, inhibition of CDK9 may represent an interesting approach as a cancer therapeutic target, especially in hematologic malignancies.

Introduction

Cyclin-dependent kinases (CDK) are involved in cell-cycle control (CDK1, 2, 4, and 6; ref. 1), and transcription regulation (CDK 7, 8, and 9; ref. 2). In cancer cells, loss of cell-cycle control through deregulation of CDK activities is considered a fundamental aspect of tumorigenesis. Multiple CDK inhibitors have been tested in clinical trials including flavopiridol (alvocidib), R-roscovitine (CYC202, Seliciclib), SNS-032, PD-0332991, AZD5438, AG-024322, and AT7519. Some have demonstrated antitumor efficacy, for example, flavopiridol demonstrated significant activity in patients with chronic lymphocytic leukemia (3–5). Although originally developed as pan-CDK cell-cycle inhibitors, the antitumor activity of several of these compounds, including flavopiridol (alvocidib), has been hypothesized to incorporate a component of inhibition of transcription as a result of potent inhibitory activity against CDK7 and CDK9 (4, 5). This type of pan-CDK inhibitors has shown complex and challenging toxicology profiles that have limited their clinical utility (3–5). We hypothesized that the identification and development of a CDK9 inhibitor through a target-based pharmacologic approach based on exclusive in vivo inhibition of transcription and not cell cycle could yield significant antitumor activity while potentially clarifying the toxicologic profile.

DNA-dependent RNA polymerase II (RNAP II) largest subunit RPB1 contains a carboxyl-terminal domain (CTD) consisting of multiple heptapeptide repeats with consensu amino acid sequence YSPTSPS. Transcription activation requires phosphorylation of CTD, by a variety of kinases including CDK7, CDK8, and CDK9 (6–8). CDK7, together with its cofactors cyclin H and Mat1, forms a tightly associated protein complex that is part of RNAP II general transcription factor TFIIH. When associated with the pre-initiation complex, CDK7 can phosphorylate the CTD at serine residues 7, 5, and 2 in vitro and in vivo, depending on the interactions with its partners and release of the transcription pre-initiation complex into the initiation complex (9–12). CDK7 in association with cyclin H and Mat1 in the core complex is also involved in cell-cycle progression by phosphorylating other CDKs in their activation loop (T-loop), termed CDK-activating kinase (CAK) activity (13). This complex is further involved in DNA nucleotide excision repair, RNA melting, and other RNA processes (14–16).

CDK8 and cyclin C, together with other factors, are associated in mediator complex that, depending on the
context, functions in either transcription repression or activation. CDK8/cyclin C can phosphorylate CTD at Ser2 and Ser5 (7, 11, 17). CDK9, binding with its partners cyclin T or cyclin K, forms the protein complex positive transcription elongation factor b (P-TEFb), which is required for transcription elongation (18, 19). Once the transcription pre-initiation complex has been phosphorylated at CTD Ser5 by CDK7 in the TFIIH complex, the rate-limiting promoter-proximal pausing of RNAP II is further phosphorylated at CTD Ser2 by CDK9 complex to facilitate promoter clearance and elongation. Apart from regulating transcriptional initiation and elongation, P-TEFb has additional functions in pre-mRNA splicing, and transcription termination (20, 21).

Multiple lines of evidence point to the critical functions of CDK7 and CDK9 transcription regulation in various human cancer types (22). CDK7 and 9 protein levels and kinase activities are elevated in various tissues and dysregulation of their function correlate with aberrant transcription profiles observed in lung and breast adenocarcinoma, endometrial carcinoma, leukemia, lymphoma, multiple myeloma, and neuroblastoma (23–30). The CDK7 inhibitor flavopiridol has shown both preclinical and clinical activity, especially in hematologic malignancies (31–36). Herein we describe the discovery and characterization of a novel CDK9 inhibitor, LY2857785, with reduced CDK7 inhibitory activity, which potently inhibits CTD phosphorylation and exhibits antitumor efficacy in several preclinical models, including orthotopic models of leukemia.

Materials and Methods

Cell lines and antibodies

Human A549 (lung carcinoma), U2OS (osteosarcoma), HCT116 (colorectal carcinoma), U87MG (glioblastoma), A375 (malignant melanoma), MV-4-11 (biphenotypic B-myelomonocytic leukemia), RPMI 8226 (myeloma), L363 (multiple myeloma) cancer cell lines were purchased from ATCC first in 2005 and later as needed, and cultured following their recommendations at 37°C and 5% CO2 in a humidified atmosphere.

The following antibodies against corresponding proteins were purchased and used according to the manufacture’s instruction: RNAP II CTD (MMS126R Covance and ab26721 Abcam); CTD P-Ser2 (ab5095 Abcam and H5 Covance); CTD P-Ser5 (ab5131, Abcam); MCL1 (SC-819), C-MYC (SC-764), and p21 (SC-397) are from Santa Cruz; p53 (9282), BCL2 (2872), and XIAP (2042) from Cell Signaling; cleaved PARP (ab4830) and glyceraldehyde-3-phosphate dehydrogenase antibodies (ab9485) from Abcam; horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin G (IgG; Amersham); goat anti-mouse IgM Alexa Fluor 488, anti-mouse-Alexa Fluor-647, anti-rabbit-Alexa Fluor-488, and Hoechst 33342 are from Molecular Probes.

Test compounds

LY2857785 was synthesized at Lilly Research Laboratories chemistry labs. Flavopiridol was purchased from AmFineCom, Inc. and Rapamycin from LC Laboratories. LY2857785 and flavopiridol were formulated as a solution in sterile normal saline. Rapamycin was formulated daily in hydroxyethyl cellulose (HEC) 1%/Tween 80 0.25%/Antifoam 0.05%. pH was adjusted to a pH > 4.5 before injection for all compounds.

CDK7, CDK8, CDK9, and profiling biochemical kinase assays

CDK7 and CDK9 reaction mixtures contained 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L HEPES, 5 mmol/L DTT, 10 μmol/L ATP, 0.5 μCi 33p-ATP, 10 mmol/L MnCl2, 150 mmol/L NaCl, 0.01% Triton X-100, 2% dimethylsulfoxide (DMSO), 0.05 mmol/L CDK7/9ptide, and 2 mmol/L CDK7/Mat1/cyclin H (14-476M, Upstate), or 2 nmol/L CDK9/cyclin T1 (14-685M, Upstate), respectively. CDK8/cyclin C reaction is performed in HEPES 30 mmol/L, DTT 2 mmol/L, MgCl2 5 mmol/L, 0.015% Triton X-100, 5 μmol/L ATP, and 400 nmol/L of RB-HER-CHKStide containing 20 nmol/L of enzyme.

Compound in DMSO was diluted serially 1:3 for dose response. Reactions were carried out in 96-well polystyrene plates. The reactions were incubated at room temperature for 60 minutes and followed by termination with 10% H3PO4 or 10% trichloroacetic acid (TCA). For the filter binding assay, reactions were transferred to 96-well filter plates (MAPHNLOB; Millipore) and measured by Microbeta scintillation counter (Perkin Elmer Life and Analytical Sciences). For ADP Transcreener Fluorescent Polarization Assays, reactions were quenched with ADP detection mix (BellBrook Labs), incubated 2 hours at room temperature and then FP was measured at λex = 610 nm, λem = 670 nm on a Tecan Ultra 384 (Tecan) plate reader. The concentration of ADP product was calculated from millipolarization (μP) using a prepared ADP/ATP dilution series as a standard curve.

Kinase profiling (CEREP and Millipore kinase panels) were carried out in 96-well polystyrene plates, according to the manufacturer specifications. Briefly, in a final volume of 25 μL the enzyme was incubated with the appropriate buffer, peptide substrate, and the diluted compound. Reactions were initiated by the addition of ATP/[35P] and the ATP mix was incubated at room temperature for 40 minutes. Reactions were quenched with the addition 5 μL of 3% phosphoric acid, 10 μL of the reaction were spotted onto a filtermat, washed 3 times for 5 minutes in 75 mmol/L phosphoric acid and once in methanol. Once the filters were dry, they were submitted to scintillation counting.

RNAP II CTD phospho-Ser2 and -Ser5 multiplexing cell-based Acumen assay

U2OS cells were plated in 96-well plates and incubated overnight. Compounds in DMSO stock were diluted serially and incubated with cells for 16 hours for multiplexing.
LY2857785 is a highly potent and selective inhibitor of CDK9. A, chemical structure of LY2857785. B, LY2857785 kinase activity profile with enzymatic assays. Biochemical IC₅₀s obtained from internal assays developed at Eli Lilly and Company or CEREP kinase panel. All kinases with IC₅₀ less than 1 μmol/L are listed and compared with flavopiridol. C, dose responsive curves of LY2857785 against (left) CTD P-ser2 (green) and cell proliferation (red), (middle) P-Ser5, and (right) 4N (green)/2N (red) DNA content in U2OS osteosarcoma cells. D, pairwise correlations between CTD P-Ser2 or cell proliferation inhibition potency versus CDK7 or CDK9 enzymatic inhibition potency in U2OS cells. E, multiple regression results for either cell potency versus both CDK7 and CDK9 enzymatic assay potencies.

Figure 1. LY2857785 is a CDK9 Inhibitor with Preclinical Antitumor Activity
CTD P-Ser2/DNA content, and 2.5 hours for CTD P-Ser5 inhibitions. Cells were fixed with Prefer (Anatech Ltd.) for 30 minutes or with 4.8% paraformaldehyde for 45 minutes at room temperature and permeated with 0.1% Triton-X 100 in PBS or cold methanol (−20°C) for 15 minutes, respectively. Primary antibody [H5 (CTD P-Ser2) or ab5131 (CTD P-Ser5)] in 1% BSA/ PBS containing 50 μg/mL RNase (R6513; Sigma) was incubated at 4°C overnight, then with the secondary antibody (Goat antimouse IgM Alexa Fluor 488) for 1 hour at room temperature and cocomposed with propidium iodide (Sigma) before being analyzed on Acumen Explorer Laser-scanning fluorescence microplate cytometer (TPP LabTech Ltd.) for P-CTD and DNA histogram profiles.

**Cell proliferation and apoptosis assays**

Solid tumor cells were plated in poly-l-lysine coated and hematologic cell lines were seeded in noncoated 96-well plates overnight before being treated with compounds. Solid tumor cells were fixed with Prefer for 20 minutes at room temperature and permeated with 0.1% Triton X-100 in PBS for 15 minutes. Caspase-3 expression was measured by immunofluorescence with antiactivated caspase-3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) activity was measured with In Situ Cell Death Detection Kit (Roche). Both assays were analyzed on Acumen Explorer laser-scanning fluorescence microplate cytometer (TPP LabTech Inc.). Hematologic tumor cells were assayed for cell viability with CellTiter-Glo Luminescent Cell Viability Assay (Promega). TCA and monolayer assay (FA) in Oncotest GmbH were previous described (37).

For the human bone marrow granulocyte-macrophage colony formation (CFU-GM) assay, normal human bone marrow myeloid progenitor cells were isolated and cells with >90% viability were seeded in methylcellulose-based media with supplemental cytokines (Stem Cell Technologies). Cells were treated with varying concentrations of compound for varying durations ranging from 4 to 24 hours. Following incubation for 10 to 14 days, colonies were manually enumerated and inhibition of colony formation was calculated relative to vehicle-treated cells.

The human cancer cell lines used in Fig. 2A from Oncotest GmbH are: AHS NSB017, AHS NSB019, AHS NSB020 (hematopoietic stem cells); BXF 1128, BXF 1228 (bladder cancer); CXF 1103, CXF 1729, CXF 1783, CXF 243, CXF 280, CXF 676, CXF 975 (colon cancer); CXF 1712, CXF 209, GXF 97 (gastric cancer); HNXF 536, HNXF 908 (head and neck cancer); LFAX 1041, LFAX 1584, LFAX 526, LFAX 629, LFAX 983 (lung adenocarcinoma); LFAX 1422, LFAX 211 (lung squama cell carcinoma); LFXL 1072, LFXL 430, LFXL 529 (large cell lung cancer); MAXF 1322, MAXF 1384, MAXF 401, MAXF 583 (mammary cancer); MEXF 1539, MEXF 276, MEXF 462, MEXF 989 (melanoma); OVXF 1353, OVXF 550, OVXF 899 (ovary cancer); PAXF 546, PAXF 736 (pancreatic); PRXF, DU143, PRXF PC3M (prostate cancer); PXF 1752, PXF 541 (pleuramesothlioma); RXF 1220, RXF 393, RXF 486, RXF 631 (renal cancer); SXF 1186, SXF 1301, SXF 627 (sarcoma). In Fig. 2B, the cell lines are A2780 (ovarian carcinoma), MV-4-11 (acute myeloid leukemia, AML), A549 (lung carcinoma), HEPG2 (hepatocellular liver carcinoma), HLE (hepatoma), HCT116 (colorectal cancer), MDA-MB-436 (breast carcinoma), ASPC1 (pancreas adenocarcinoma), BT474 (breast carcinoma), HT29 (colorectal adenocarcinoma), and KATOIII (gastric carcinoma). They were obtained from ATCC Inc.

In Fig. 2D, the hematologic cancer cell lines from Oncotest GmbH are: (AML) KG1, HL60, MV411, NOMO1, OCIAML2, PL21; (ALL) CCRFCEM/VCR, CCRFCEM, JURKAT, MOLT4; (CML) EM2, K562, KCL22, JURLMK1, MEG01; (multiple myeloma) IM9, L363, LP1, NCISH929, RPMI8226; and (NHL) HUT78, MYLA, RAJI, U937.

**RNAP II CTD Ser2 phosphorylation inhibition for in vivo target inhibition assay**

Four animals were used for each treatment group and 6 animals in the vehicle group for in vivo target inhibition (IVT). Formulated LY2857785 was administered intravenously (i.v.) in animals carrying xenograft tumors in a final volume of 0.2 mL/mouse or 0.6 mL/rat when the mean tumor volume was approximately 150 to 200 mm³. Subsequently, animals were asphyxiated with CO₂ and the xenograft tumors harvested by surgical removal, flash frozen in liquid nitrogen, and stored at −80°C until analyzed. Tumors were homogenized in a room temperature in high-salt lysis buffer containing 50 mmol/L Tris, pH 8.0, 400 mmol/L NaCl, 0.5% NP-40, Phosphatase Inhibitor cocktails I and II (Sigma Aldrich), and protease inhibitors (Roche). The homogenate was subsequently centrifuged at 13,200 rpm for 10 minutes at 4°C. Protein concentration was determined by Coomassie Protein Assay Reagent (#1856210; Thermo Scientific). The ELISA plates were prepared by coating antibody (donkey anti-mouse IgM, IgG, 715-005-150; Jackson ImmunoResearch Laboratories) and secondary antibody (Goat anti-mouse IgG, 715-005-150; Jackson ImmunoResearch Laboratories) to capture antibody (mouse anti-RNAP II, MMS126R; Covance) overnight at 4°C. The test samples were added to the plates and incubated with detection antibodies (CTD P-Ser2 or total CTD) at room temperature. HRP-conjugated antibody was used to develop the signal with chemiluminescence, and the plate was read with the Envision Multilabel Reader (PerkinElmer).

**Xenograft tumor and orthotopic myr-AKT/Ep-Myc and Bcl2/Ep-Myc leukemia models**

All in vivo studies were performed according to the Institutional Animal Care and Use Protocols. For xenograft models, human cancer cells U87MG, MV-4-11, A375, and HCT116 were implanted into female nude rats or athymic nude female mice. For orthotopic leukemia models, myr-AKT/Ep-Myc or Bcl2/Ep-Myc cells from the blood leukemic animals were injected into wild-type female C57BL/6 host mice (38). All in vivo studies were performed according to the Institutional Animal Care and Use Protocols. Human cancer cells U87MG, MV-4-11,
A375, and HCT116 were maintained as recommended by vendor, harvested, washed, and resuspended in a 1:1 mixture of serum free media and Matrigel (354234; Becton Dickinson). For xenograft tumor models, female nude rats (NIHRNU-M, 160–200 g; Taconic) were injected subcutaneously in rear flank at 2 \times 10^6 cells/rat (U87MG) or 1 \times 10^6 cells/rat (MV4-11), respectively. Athymic nude female mice (Harlan, 7–8 weeks) were injected subcutaneously in the rear flank at 5 \times 10^6 cells/mouse (U87MG), 5 \times 10^6/mouse (MV4-11), or 1 \times 10^7/mouse (A375), respectively. Tumor volume was estimated by using the formula: \( v = l \times w^2 \times 0.536 \), where \( l \) is the larger measured diameter and \( w \) is the smaller of perpendicular diameter and analyzed with SAS software (SAS Institute Inc.).

B6.Cg-Tg[IghMyc]22Bri/J (Jackson Laboratory) transgenic mice embryonic liver cells were transduced (for ABCD).
myr-AKT/Eμ-Myc leukemia model) with a retrovirus expressing a myristoylated form of human AKT1 (myr-AKT) under control of the viral 5' long terminal repeat (LTR) and expressing GFP under control of the PGK promoter (MSCV6 myr-AKT PGK/GFP), or (for Bcl2/ Eμ-Myc Leukemia Model) with a retrovirus expressing the human Bcl2 antiapoptotic gene under control of the viral 5' LTR and expressing GFP under control of the PGK promoter (Bcl2 PGK/GFP). Transduced cells were transferred into lethally irradiated female C57BL/6 mice (Taconic), 8 to 10 weeks old and 20 to 22 g in weight. Hematopoietic stem cells among the transduced cells repopulated the bone marrow of the recipient. The "rescued" primary animals were observed for development of leukemia. When leukemia was confirmed in the subject animal, blood from primary animals was serially passed to secondary (nonirradiated) host animals. Mice were inoculated retro-orbitally with a predetermined number of leukemic cells obtained from donor animals (day 0) and were monitored by observing a small amount of blood obtained retro-orbitally as noted. Forty-five microliters of aliquots of heparinized whole blood obtained from myr-AKT/Eμ-Myc leukemia animals at each time point were stained by PerCP Rat anti-Mouse CD45R/B220 antibody (BD Biosciences Pharmingen). Ten microliters of aliquots of heparinized whole blood obtained from Bcl2/Eμ-Myc leukemia animals at each time point were fixed with 1% paraformaldehyde in PBS. Red blood cells were lysed and remaining nucleated leukocytes were fixed on a Coulter TQ-Prep (Coulter Corporation) for fluorescent antibody cell sorting analysis using a Cytomics FC 500 (Beckman Coulter). Leukemic cells within a specific region of the forward scatter/side-scatter (FS/SS) plot were counted per sample.

The animals were dosed with saline, rapamycin, or LY2857785, respectively, according to the schedules described in the text. An untreated vehicle control group was administered saline i.v. every 3 days. Flow cytometry analysis was conducted using Beckman Coulter’s CXP software. Statistical significance of the effect of LY2857785 and/or control compounds was assessed by Dunnett method, one-way ANOVA (JMP Statistical Discovery Software; SAS Institute).

Antitumor growth efficacy with xenograft models

When the mean tumor volume reached 150 mm³ for xenograft tumors in nude rats and mice, animals were randomized by tumor volume and compound was administered. Seven animals were used per vehicle and treatment group for U87MG bearing nude rat model. Ten animals were used per vehicle and treatment group for each treatment group.

Western blot analysis

Cells were lysed with high salt buffer and supernatants were collected after centrifuging at 14,000 rpm for 10 minutes at 4°C. Proteins were separated on 4% to 20% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and blotted for Western blot analysis with antibodies indicated in the results.

Bioinformatics analysis

Gene expression data generated from DNA microarray analysis for all cancer cell lines from Oncotest TCA assay and internal cancer cell panel (GEO accession number GSE36139) were used to examine sensitive versus resistant cell lines using Ingenuity Pathway Analysis (Eli Lilly and Company) and Molecular Concepts Analysis (37, 39). The microarray data were downloaded from Cancer Cell Line Encyclopedia (CCLE) website (http://www.broadinstitute.org/ccle/data; GEO accession number GSE36139). This is robust microarray average (RMA)-normalized mRNA expression data normalized from Raw Affymetrix CEL files using standard Affymetrix annotation. The Affymetrix CEL files were generated using Affymetrix U133+2 platform. Gene expression data are filtered to exclude probesets: (i) that do not sufficiently map to exonic regions of transcripts as per Ensembl release 71 and described at http://www.ensembl.org/info/docs/microarray_probe_set_mapping.html; (ii) that do not vary significantly across samples (30% of probesets with lowest SD); and (iii) for which 75th percentile signal was lower than 8 (log base 2). Furthermore, the probesets that change by greater than 2-fold between the 2 classes with false discovery rate <0.1.

The statistical method to generate the probability of chemotherapeutic sensitivity uses Bayesian probit...
regression model combining with singular value decompositions (47). First, the probeset selection from the training data is done as described above. Training data are constructed with samples in columns and probesets (or genes) in rows. Principal components of the training data are used to compute the metagene and metasample values. Bayesian binary probit regression models are fitted to the metagene signatures for assessing the relevance of each metagene in within-sample classification. Given the independent unknown test dataset, the gene expression data with probesets matched with signature is projected onto the metagenes from the training data, and relative probability of sensitivity (POS) is predicted using fitted Bayesian binary probit regression model from the metagene signature. The classes are always defined as “0” for resistant and “1” for sensitive for training such that low POS scores would be suggestive of a sample being resistant and vice versa. The implementation of the software was done in R environment (compatible with version 2.15 and above).

Results

**Discovery of LY2857785**

LY2857785 [N1-[4-(3-isopropyl-2-methyl-indazol-5-yl)pyrimidin-2-yl]-N4-tetrahydropryan-4-y1-cyclohexane-trans-1, 4-diamine methanesulfonic acid salt; Fig. 1A] was identified through structure based design and Medicinal Chemistry structure-activity-relationship (SAR) studies as a type I reversible and competitive ATP kinase inhibitor against CDK9 (IC_{50} 0.011 μmol/L) and other transcription kinases CDK8 (IC_{50} 0.016 μmol/L), and CDK7 (IC_{50} 0.246 μmol/L), at levels comparable to or more potent than flavopiridol (Fig. 1B). LY2857785 showed good selectivity against a panel of 114 protein kinases, with only 5 other flavopiridol (Fig. 1B). LY2857785 was identified through structure based design and Medicinal Chemistry structure-activity-relationship (SAR) studies as a type I reversible and competitive ATP kinase inhibitor against CDK9 (IC_{50} 0.011 μmol/L) and other transcription kinases CDK7 (IC_{50} 0.016 μmol/L), and CDK8 (IC_{50} 0.246 μmol/L), at levels comparable to or more potent than flavopiridol (Fig. 1B). LY2857785 exhibited high aqueous solubility (>2.0 mg/mL at pH 7.4) and good solution stability, enabling in vivo formulation using 0.9% NaCl normal saline at pH 5.5 to 6. This inhibitor showed excellent physicochemical properties with log D (pH 7.4) = 2.11, with 22% unbound fraction in both human and rat plasma.

At the cellular level, LY2857785 inhibits CTD P-Ser2 and CTD P-Ser5 in U2OS cells at IC_{50}s 0.089 (n = 13) and 0.042 (n = 1) μmol/L, respectively, with comparable potency to flavopiridol (0.147 (n = 7) and 0.196 (n = 1) μmol/L for Ser2 and Ser5, respectively). This is consistent with the CDK7 and CDK9 knockdown profiles of CTD phosphorylation in the same cell line (Supplementary Fig. S1). The LY2857785 cell proliferation inhibition (IC_{50} 0.076 μmol/L) closely correlates with the P-Ser2 inhibition (IC_{50} 0.089 μmol/L; Fig. 1C, left), suggesting that the CTD phosphorylation inhibition directly leads to cell proliferation inhibition. At the time of this study, the specific role or component of CDK8 in CTD phosphorylation was not clearly defined. Although LY2857785 inhibited CDK4, CDK6, and CDK2 enzymatic activities, it does not induce G1-S cell-cycle arrest (Fig. 1C DNA content, right). Activity against CDK1 (Histone H1 P-T153 inhibition IC_{50} 0.241 μmol/L) was also observed, however LY2857785 only induces a moderate G_{2}-M DNA content increase, from 35% to 55%, with EC_{50} 0.135 μmol/L (Fig. 1C, right). Once that we determined that LY2857785 does not show any significant cell-cycle phenotype that could explain its cell antiproliferative activity, we decided to focus on the role of CDK9, and to a less extent CDK7, on CTD Ser2 phosphorylation because, in our hands, the knockdown of these 2 CDK isoforms was sufficient to strongly inhibit RNAP II P-CTD (Supplementary Fig. S1; ref. 40).

In this context and to elucidate the individual contributions of CDK7 and CDK9 biochemical activities present in our chemical scaffold to CTD P-Ser2 phosphorylation and cell proliferation inhibition, we analyzed more than 900 SAR compounds. The statistical analysis, through pairwise correlation (Fig. 1D) and multiple regression methods (Fig. 1E), helped to dissect the individual contributions of these targets to the CTD P-Ser2 or cell proliferation inhibition. In the pairwise correlation analysis (Fig. 1D), CDK9 enzymatic inhibition had higher pairwise correlations than CDK7 with both CTD P-Ser2 (r = 0.76 vs. r = 0.62) and cell proliferation (r = 0.77 vs. r = 0.63) inhibition potencies. Using multiple linear regression analysis with both biochemical potencies as possible predictors (Fig. 1E), CDK9 biochemical potency was more significant and had a larger coefficient than CDK7 for both cell proliferation (0.564 vs. 0.202) and CTD P-S2 inhibition potencies (0.561 vs. 0.205). This regression estimates indicate that improving CDK9 potency by 10-fold (1 log unit) would have an expected improvement in cell potency of 1.6-fold (10^{0.564}), whereas improving CDK9 potency by 10-fold would have an expected improvement of 3.7-fold (10^{0.564}) in cell potency (Fig. 1E). Both analyses suggest that CDK9 is the main driver for CTD P-S2 and cell proliferation inhibition. The interaction effect between CDK7 and CDK9 enzymatic assays was not statistically significant and thus was not included in either model. The RNAP II P-Ser2 and cell growth inhibition cell-based assay potencies correlated with each other at r = 0.97, again suggesting that CTD inhibition is directly responsible for the observed inhibition of cell proliferation.

LY2857785 inhibits hematologic and solid tumor cell proliferation and induces apoptosis in vitro

LY2857785 showed potent compound exposure- and time-dependent cell proliferation inhibition in MV-4-11, RPMI8226, and L363 cells. When incubated between 4 to 24 hours, the cell growth inhibition potency reached a maximal effect at 8 hours with IC_{50}s 0.04, 0.2, and 0.5 μmol/L for MV-4-11, RPMI8226, and L363 cells, respectively (Table 1). LY2857785-induced cancer cell apoptosis is also time dependent, reaching maximal potency at 8 hours with IC_{50} 0.5 μmol/L in L363 cells.

LY2857785 also inhibited proliferation in several solid tumor cell lines, including U2OS, HCT116, and A549 with IC_{50} of 0.05, 0.03, and 0.01 μmol/L after 24 hours of
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Table 1. LY2857785 hematologic cancer cell proliferation inhibition potency is time dependent

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<th>Time (h)</th>
<th>MV4-11 (IC_{50} μmol/L)</th>
<th>RPMI8226 (IC_{50} μmol/L)</th>
<th>L363 (IC_{50} μmol/L)</th>
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NOTE: Cell proliferation inhibition IC_{50} was measured using CellTiter Glo assay.

continuing exposure, respectively (Supplementary Table S1). Treatment of LY2857785 for various exposure times from 2 to 72 hours showed that LY2857785 exhibited increased inhibition of cell proliferation over time, reaching maximal potency at around 8 to 12 hours (Supplementary Table S1).

Similarly, LY2857785 potently induced apoptosis in these cancer cell lines. The ability to induce caspase-3 (CASP-3) expression and TUNEL activity also increased over time, reaching maximal potencies of 0.02 to 0.2 μmol/L IC_{50}s at around 16 to 24 hours for the same solid tumor cells (Supplementary Table S1). LY2857785 reached its highest potency for cell growth inhibition and apoptosis more rapidly in hematologic cancer cells (8 hours of exposure; Table 1) than in solid tumor cells (12–24 hours of exposure; Supplementary Table S1).

Western blot analysis showed that the level of the antiapoptosis proteins MCL1 and XIAP were dramatically reduced, and the apoptosis markers CASP-3 and cleaved PARP were induced by LY2857785 in these cancer cell lines as shown for MV-4-11 in Supplementary Fig. S2A. Although both flavopiridol and LY2857785 inhibited the MCL-1 protein level to a similar extent, at 48 hours after the compound washout, LY2857785 still showed persistent MCL-1 inhibition at 0.6 μmol/L or higher, whereas flavopiridol did not (Supplementary Fig. S2A). Correspondingly, LY2857785 decreased cell viability in a dose-dependent manner (Supplementary Fig. S2B).

**LY2857785 antiproliferation profile predicts hematologic cancer cell sensitivity**

The above data prompted us to identify cell types sensitive to LY2857785 by screening the compound in 48 patient-derived solid tumor cell lines using soft agar colony-formation assay and 24 hematologic tumor cell lines using a modified propidium iodide assay at Oncoset GmbH (41). The solid tumor test panel covered 13 different human tumor histologies including, colon, non–small cell lung (adeno, squamous cell, and large cell), mamma- ry, and prostate cancers. The hematologic tumor panel included acute lymphoblastic leukemia (ALL), AML, chronic myelogenous leukemia (CML), non-Hodgkin lymphoma (NHL), and multiple myeloma (37, 41).

In the solid tumor soft-agar colony formation assay, LY2857785 displayed a mean IC_{50} value of 0.22 μmol/L for the tested tumor cell lines. The IC_{50} values ranged from 0.02 to >3 μmol/L. In 17 of 48 cell lines (35%), LY2857785 showed better than average antitumor activity against several tumor models of sarcoma, large cell lung cancer, and melanoma. The IC_{50} values in these sensitive tumors were on average about 4.5-fold lower than the mean IC_{50} of all models tested, and about 1.9-fold lower than those in normal hematopoietic stem cells (AHS NSB017, AHS NSB019, AHS NSB020; Fig. 2A).

The LY2857785 sensitive and resistant cell lines identified from Oncoset tumor colony formation assay (Fig. 2A) as well as from an internal cancer cell panel (including MV-4-11, A549, A2780, HEPG2, MDA-MB-436, HLE, HCT116, ASPC1, BT474, HT29 and KATOIII; Fig. 2B) were selected for gene expression pathway analysis to identify a 261 gene signature panel that best differentiates sensitivity (Fig. 2B and Supplementary Table S2). The gene panel predictor was applied to all other available solid and hematologic tumor type gene expression profile databases to predict the potential sensitive tumor types. This gene profiling analysis indicated that hematologic tumor types, as well as skin, bone, and central nervous system (CNS) tumors are most sensitive to LY2857785, whereas pancreas, esophageal, and large intestine cancers are resistant (Fig. 2C).

Indeed, LY2857785 exhibited significant proliferation inhibition for all 24 hematologic cancer cell lines with a geometric IC_{50} value of 0.197 μmol/L. The IC_{50} values ranged from 0.049 to 1.1 μmol/L. Among hematologic tumors, 21 of 24 (87%) of the tested cell lines showed intermediate to high sensitivity toward LY2857785 (Fig. 2D). Three AML cell lines, namely MV-4-11 (IC_{50} = 0.049 μmol/L), OCIAML2 (IC_{50} = 0.063 μmol/L), and PL21 (IC_{50} = 0.072 μmol/L) were the most sensitive to LY2857785. Five AML cell lines were among the 8 most sensitive lines to LY2857785 (Fig. 2D). The P-glycoprotein (P-gp) overexpressing cell line CCRFCEM/VCR was ~4.5-fold less sensitive compared with the parent cell line CCRFCEM, indicating an interaction with the P-gp efflux pump (Fig. 2D). Accordingly, using Madin–Darby canine kidney cell lines expressing P-gp, LY2857785 was demonstrated as a P-gp substrate (data not shown).

**LY2857785 treatment leads to strong inhibition of CTD phosphorylation and induction of cell death in AML and CLL patient peripheral blood mononuclear cells**

LY2857785 was shown to be a highly potent CTD P-Ser2 inhibitor in AML and CLL patient PBMC samples, as measured by respective IC_{50}s of 0.0435 ± 0.0177 μmol/L and 0.1449 ± 0.1066 μmol/L (Fig. 3A), which were similar to those of flavopiridol (0.0681 and 0.15 μmol/L, respectively). In addition, at 0.5 μmol/L, LY2857785 inhibition...
LY2857785 is a CDK9 inhibitor with preclinical antitumor activity. Figure 3. LY2857785 actively inhibits AML and CLL patient PBMC P-Ser2 and induces cell death ex vivo. A, representative PBMCs from AML, CLL, and normal human blood donors treated with LY2857785 for 4 hours and analyzed for RNAP II CTD P-Ser2 inhibition. B, AML patient PBMC cells were treated with LY2857785 at the indicated concentrations and exposure durations. The cell lysates were generated for Western blot analysis with proteins indicated on the right. C, patients with CLL and normal blood donors PBMCs were treated with LY2857785 at indicated concentrations for 24 (left) or 48 (right), respectively. The cells stained by trypan blue and the dead cells were counted. At least 500 cells from each sample were counted. Five normal blood and 6 patient with CLL PBMC samples were collected for statistical analysis.
potency increased with longer compound incubation from 1 to 48 hours (Fig. 3B). In comparison with CTD P-Ser5 inhibition, CTD P-Ser2 inhibition is more sensitive to LY2857785 (Fig. 3B). Notably at 0.5 μmol/L incubation within 1 and 3 hours, LY2857785 significantly inhibited CTD P-Ser2 but not P-Ser5. At 4 hours of treatment, P-Ser2 reached complete inhibition at 0.032 μmol/L LY2857785, whereas total inhibition of P-Ser5 was achieved only at the highest concentration of 5 μmol/L (Fig. 3B). The significant inhibition of RNAP II CTD phosphorylation correlates with the induction of apoptosis as measured by PARP cleavage in patient with AML samples (Fig. 3B), as well as the antiapoptosis MCL1 and XIAP proteins expression inhibition at 0.5 μmol/L with longer than 3 hours of incubation (Fig. 3B).

Potent CTD P-Ser2 inhibition by LY2857785 also correlated with CLL cell killing ex vivo in patient with cancer samples. The leukemia cell death originating from LY2857785 treatment was exposure and time dependent (Fig. 3C). The cell death induction for CLL at 0.32 and 0.12 μmol/L of LY2857785 is significantly higher than for the normal blood cells with P < 0.001 and <0.004, respectively (Fig. 3C). With 48 hours treatment of LY2857785, dose-dependent death induction of CLL cells was more pronounced (Fig. 3C).

LY2857785 inhibits proliferation of normal human hematopoietic cells in vitro

To investigate whether the antiproliferative effect of LY2857785 (Fig. 2A, Fig. 3C) was selective against malignant versus normal proliferating cells, LY2857785 was evaluated in the human bone marrow myeloid progenitor colony formation assay (CFU-GM). The hematopoietic colony formation assay is a relevant model to assess potential toxic effects of new compounds in proliferating normal human cells (42–44), and the assay has been validated as an in vitro model to estimate maximum tolerated dose levels in humans for cytotoxic agents (45, 46).

In washout experiments, LY2857785 exhibited concentration- and time-dependent antiproliferation of human granulocyte-macrophage colonies (Supplementary Table S3). No effect on proliferation was observed on the human bone marrow progenitor cells within a 4-hour exposure period (IC50 > 10 μmol/L). However, marked inhibition of colony formation occurred following 8 to 24 hours of LY2857785 exposure (Supplementary Table S3). Importantly, the potency for antiproliferation of normal human bone marrow progenitor cells associated with >8 hours of compound treatment was comparable to the potencies of LY2857785 for inhibition of CTD phosphorylation and antiproliferation in tumor cells. This result suggests that significant myelosuppression would likely occur at exposure to pharmacologic levels of LY2857785 for more than 8 hours. In terms of relative potency and the time course of response, LY2857785 and flavopiridol exhibited similar in vitro antiproliferation profiles of normal human bone marrow myeloid colonies (Supplementary Table S3), suggesting the heme toxicity is likely related to transcriptional CDK inhibition.

LY2857785 inhibits RNAP II CTD Ser2 phosphorylation in vivo

In HCT116 xenograft tumor-bearing mice, LY2857785 demonstrated dose-dependent RNAP II CTD P-Ser2 inhibition potently in vivo with TED50 of 4.4 mg/kg [Threshold Effective Dose (dose that produces an effect equal to 50% CTD phosphorylation inhibition)] and TEC50 of 0.36 μmol/L [Threshold Effective Concentration (plasma concentration that produces an effect equal to 50% CTD phosphorylation inhibition); Fig. 4A, left]. LY2857785 also showed significant duration of CTD P-Ser2 inhibition for 3 to 6 hours at TED70 (8 mg/kg) in HCT116 and MV-4-11 nude mice xenograft models (Fig. 4A and B, respectively). At increased dose, TED90 (18 mg/kg), significant CTD P-Ser2 inhibition was sustained for 8 hours in MV-4-11 model (Fig. 4B, right). In the nude rat MV-4-11 xenograft model, LY2857785 similarly showed dose-dependent CTD P-Ser2 inhibition for 8 hours at TED70 (7 mg/kg) and TED90 (10 mg/kg; data not shown). Collectively, these data consistently demonstrated that LY2857785 is a potent CDK9 inhibitor in vivo.

LY2857785 demonstrates potent antitumor growth efficacy in preclinical tumor models

As described above, LY2857785 produced maximum inhibition of cell proliferation and induction of apoptosis within 8 hours for hematologic tumor cells, and within 12 to 24 hours for solid tumor cell lines. Taking this into account and the fact that the compound also exhibited reduced in vitro toxicity in normal bone marrow cells with shorter duration of compound treatment, we next tested LY2857785 by either 4-hour infusion or i.v. bolus in an AML (MV-4-11) model, and either 16-hour infusion or i.v. bolus for solid tumor models (U87MG, A375) in nude rats or mice.

LY2857785 demonstrated the most dramatic tumor regression in the AML MV-4-11 xenograft tumor model either by i.v. bolus in mice or i.v. infusion in rats (Fig. 5A and B). The tumor growth inhibition was dose-dependent across a dose range corresponding to TED50 to TED90. In nude mice with i.v. bolus dosing (Fig. 5A), the TED90 dose (18 mg/kg) produced tumor growth regression, whereas the TED70 (8 mg/kg) resulted in a flat tumor growth curve and the TED50 (4 mg/kg) dose did not completely suppress tumor growth. In the nude rat model with i.v. infusion dosing (Fig. 5B), all dose levels were associated with tumor regression during treatment. However, following the completion of treatment, the tumor growth rebounded in all dose groups, although the highest dose of LY2857785 still exhibited the greatest tumor growth inhibition (Fig. 5B). Animal weight loss was not observed in the nude rat MV-4-11 efficacy study (maximal weight loss 0% in all groups; Fig. 5B), suggesting LY2857785 was tolerated given at these doses and schedule. Similarly, no
significant weight loss was observed in the nude mouse study (maximal 9% only in the 18 mg/kg group; Fig. 5A), however, animal mortality was observed in these studies at LY2957785 high-dose groups (indicated in the tumor growth curves with dotted lines).

LY2857785 also showed significant tumor growth inhibition in U87MG glioblastoma xenograft tumors with both i.v. infusion and i.v. bolus dosing in both nude rat and mouse models (Fig. 5C). In addition, LY2857785 inhibited tumor growth in the melanoma A375 xenograft model in a mouse i.v. bolus study (Fig. 5C). Similarly, animal mortality was observed in LY2857785 high-dose groups in these models. Taken together, LY2857785 was efficacious in several xenograft tumor models, demonstrating significant tumor growth regression and/or inhibition of tumor growth (Fig. 5C).

The anticancer activity of LY2857785 was also studied in target-driven murine leukemia models, which were created by transferring transduced mouse embryonic liver cells with a retrovirus expressing human Bcl2 or a myristoylated form of human AKT1 (myr-AKT) into lethally irradiated recipients and then transplanting the resulting leukemia lines into the recipient wild-type female C57BL/6 host mice. Dose-dependent inhibition of tumor proliferation was demonstrated in the myr-AKT/Eμ-Myc orthotopic leukemia model at the TED70 (18 mg/kg) dose with every 3 days, every 2 days, and every 7 days i.v. dosing regimen (Fig. 5D, columns 2, 3, and 4, respectively), or at the 10 mg/kg dose with every day, every 2 days, and every 3 days intraperitoneal dosing (Fig. 5D, columns 8–10, respectively). The more frequent dosing regimen yielded more efficient tumor growth inhibition. LY2857785 also demonstrated dose–response efficacy at TED50 (4 mg/kg), 70 (8 mg/kg), 80 (15 mg/kg), and 90 (18 mg/kg) with i.v. bolus on a every 3 days dosing schedule (Fig. 5D, columns 2, 5, 6, and 7, respectively). All LY2857785 dosing regimens tested in this orthotopic models demonstrated better efficacy than rapamycin at 25 mg/kg every day oral dose (maximal tolerated dosing, MTD; Fig. 5D, column 1).

In addition to the myr-AKT/Eμ-Myc model, LY2857785 demonstrated significant inhibition of tumor proliferation in the Bcl2/Eμ-Myc orthotopic leukemia model, which translated to enhanced animal survival in animals carrying the leukemic line (Fig. 5E). This model is driven by human Bcl2 antiapoptotic gene overexpression, and mimics human B-cell lymphoma and CLL tumorigenesis. At all time points measured (days 12, 20, 26, 33, 40, and 46), LY2857785 at 20 mg/kg (>TED90) given on a 3 doses at the interval of every 4 days dosing schedule showed significant efficacy using a tumor growth inhibition endpoint (tumor growth inhibition of 99%, 99%, 91%, 89%, 100%, and 99%, respectively, in surviving animals). As shown in Fig. 5E, LY2857785 dosed at 20 mg/kg every 4 days also significantly increased animal survival compared with the vehicle control group ($P < 0.049$). In contrast,
Figure 5. LY2857785 treatment significantly inhibits tumor growth in vivo. A, MV-4-11 xenografts in nude mice were treated by LY2857785 i.v. bolus. B, MV-4-11 xenografts in nude rats were treated with LY2857785 4-hour i.v. infusion. Each red arrow indicates a compound dosing and the tumor volumes relative to the beginning of treatment are shown in A and B. The dotted tumor growth lines in A and B indicate animal loss (total 4 in 18 mg/kg group in A, and 3, 4 in 6 and 9 mg/kg groups in B, respectively). C, the summary of tumor growth inhibition efficacy by LY2857785 in several xenograft tumor models tested. D, AKT/Em-Myc orthotopic leukemia cells were inoculated in C57BL/6 mice and the animals were treated with vehicle, rapamycin, or LY2857785 at the indicated doses and schedules, respectively. At day 12, the tumor cell proliferation inhibition normalized to the vehicle control was measured. The statistical significance is indicated by the asterisk (*) for C and D. E, Bcl2/Em-Myc orthotopic leukemia cells were inoculated in C57BL/6 mice and the animals were treated with vehicle, LY2857785 or flavopiridol. Kaplan–Meier animal survival plot is depicted. QD, every day; Q2D, every 2 days; Q3D, every 3 days; Q4D, every 4 days; Q7D, every 7 days; Q3D x5, 5 doses at the interval of every 3 days.
flavopiridol at 15 mg/kg given every 3 days (MTD dose) did not significantly improved animal survival (Fig. 5E).

Discussion

LY2857785 is a reversible ATP competitive inhibitor for CDK9 (to a less extent also CDK7) that potently inhibits RNAP II RPB1 CTD domain P-Ser2 and P-Ser5 \textit{in vitro} and \textit{in vivo}. It also inhibits the antiapoptosis expression of the protein MCL-1 (Figs. 1 and 3). The fact that there is no significant G1 or G2–M cell-cycle arrest (Fig. 1), together with rapid inhibition of cell proliferation and induction of apoptosis (Table 1 and Supplementary Table S1; Fig. 3 and Supplementary Fig. S2), suggest that the effects of this compound are unlikely to be mediated by a cell-cycle mechanism but rather because of its function as a transcription inhibitor.

We have performed the gene set enrichment analysis (GSEA; ref. 47) using ingenuity pathway analysis (IPA) on the 261 signature genes (Fig. 2B). The analysis showed that these genes are highly enriched in cancer-related diseases, pathways, and molecular networks. The top 10 significantly (P-value < 1.0e-4) enriched canonical pathways include AML signaling, as well as IL-15, FGF, p53, IGF-1, and FGF signalings. In sensitive cells, the cancer and cellular growth and proliferation genes are the most significantly enriched, including cyclin A, cyclin E, estrogen receptor, NF-xB, and AKT1. The hyperactive of receptor signal and cell-cycle activity may indicate that these cells have active cell-cycle progression and transcription regulation, rendering the transcription inhibition sensitivity. Among apoptosis pathways, the MCL-1/BCL2L1 and caspase-3/-7 molecular pathways are downregulated with rapid inhibition of cell proliferation and induction of apoptosis (Table 1 and Supplementary Table S3). The mechanism but rather because of its function as a transcription inhibitor.

These may indicate apoptosis pathway activity contributed to LY2857785-induced CDK9-dependent cell-proliferation inhibition. The molecular gene signature prediction (Fig. 2), \textit{in vitro} and \textit{in vivo} activities of the compound in hematologic tumor proliferation inhibition and apoptosis induction (Table 1 and Fig. 5), suggest that LY2857785 represents a novel compound with unique transcription inhibitor activity that demonstrates promise as a targeted chemotherapeutic agent for AML. The molecule’s relatively short exposure time (8 hours) for hematologic tumor cell death induction (Table 1), together with the significantly greater CLL cancer cell killing potency than for normal PBMC \textit{ex vivo} (Fig. 3), suggests the possible therapeutic margin for LY2857785 may be in treating patients with hematologic tumors, particularly AML and CLL.

This enthusiasm must be tempered, however, in that the \textit{in vitro} human bone marrow colony-formation assay and animal toxicity studies indicate LY2857785 also inhibits proliferation of normal hematopoietic cells in an exposure- and time-dependent manner (Supplementary Table S3). The \textit{in vitro} bone marrow colony-formation assay is also useful to investigate potential species differences in heme toxicity (43, 44). LY2857785 was tested in normal hematopoietic progenitor cells from rats and dogs and potent antiproliferation was observed in both species cells following \textit{in vitro} treatment (data not shown). Human bone marrow cells, however, were found to be less sensitive (2- to 5-fold) than those of rats and dogs. When LY2857785 was evaluated in rat and dog toxicology studies, the potent \textit{in vitro} myelotoxicity also occurred \textit{in vivo} in both species with a steep dose-response and narrow safety margin relative to pharmacologic exposure levels that induced antitumor activity in xenograft models. Based on the \textit{in vitro} and \textit{in vivo} profiles, the dog was the most sensitive species to LY2857785-related hemotoxicity and was utilized in more extensive toxicology studies. Data obtained from these studies revealed a steep dose response to LY2857785-mediated toxicity that included bone marrow, gastrointestinal tract, and other target organs. Thus, in spite of the novel mechanism of action and impressive antitumor activity displayed by LY2857785, the decision was made to discontinue its clinical development. Our findings suggest CDK9 target-related toxicities and future efforts may benefit by taking this into consideration. In summary, following a rational, target-based, \textit{in vitro} and \textit{in vivo} pharmacology testing paradigm quite different from that of other pan-CDK inhibitors described in the literature, we have identified and characterized LY2857785, an inhibitor of CDK9 showing potent transcription-related antitumor efficacy in preclinical hematologic tumor models.

Disclosure of Potential Conflicts of Interest

T.I. Meier has ownership interest (including patents) in the Eli Lilly and Company. G.P. Donoho has ownership interest (including patents) in the Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

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