Dinaciclib (SCH727965) Inhibits the Unfolded Protein Response through a CDK1- and 5-Dependent Mechanism

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Abstract
Evidence implicating dysregulation of the IRE1/XBP-1s arm of the unfolded protein response (UPR) in cancer pathogenesis (e.g., multiple myeloma) has prompted the development of IRE1 RNase inhibitors. Here, effects of cyclin-dependent kinase (CDK) inhibitor SCH727965 (dinaciclib) on the IRE1 arm of the UPR were examined in human leukemia and myeloma cells. Exposure of cells to extremely low (e.g., nmol/L) concentrations of SCH727965, a potent inhibitor of CDKs 1/2/5/9, diminished XBP-1s and Grp78 induction by the endoplasmic reticulum (ER) stress-inducers thapsigargin and tunicamycin, while sharply inducing cell death. SCH727965, in contrast to IRE1 RNase inhibitors, inhibited the UPR in association with attenuation of XBP-1s nuclear localization and accumulation rather than transcription, translation, or XBP-1 splicing. Notably, in human leukemia cells, CDK1 and 5 short hairpin RNA (shRNA) knockdown diminished Grp78 and XBP-1s upregulation while increasing thapsigargin lethality, arguing for a functional role for CDK1/5 in activation of the cytoprotective IRE1/XBP-1s arm of the UPR. In contrast, CDK9 or 2 inhibitors or shRNA knockdown failed to downregulate XBP-1s or Grp78. Furthermore, IRE1, XBP-1, or Grp78 knockdown significantly increased thapsigargin lethality, as observed with CDK1/5 inhibition/knockdown. Finally, SCH727965 diminished myeloma cell growth in vitro in association with XBP-1s downregulation. Together, these findings demonstrate that SCH727965 acts at extremely low concentrations to attenuate XBP-1s nuclear accumulation and Grp78 upregulation in response to ER stress inducers. They also highlight a link between specific components of the cell-cycle regulatory apparatus (e.g., CDK1/5) and the cytoprotective IRE1/XBP-1s/Grp78 arm of the UPR that may be exploited therapeutically in UPR-driven malignancies. Mol Cancer Ther; 13(3); 662–74. ©2013 AACR.
promising UPR targets for therapeutic intervention in cancer therapy. These considerations have prompted extensive efforts to develop XBP-1 inhibitors, including agents that act as IRE1 RNase inhibitors and block XBP-1 splicing, for example, STF-083010, MK0186893, and MKC-3946 (12–14). Indeed, such agents have been shown to induce multiple myeloma cell death and to potentiate the activity of anti-myeloma agents that trigger ER stress (12–14). However, the high concentrations (e.g., 10–100 μmol/L) of these agents generally required to inhibit XBP-1s expression could limit their potential.

The observation that deregulation of cell-cycle control due to aberrant cyclin-dependent kinase (CDK) activity is a common feature of most cancers prompted the development of CDK inhibitors such as the pan-CDK inhibitor flavopiridol, the first of its class to enter the clinic (15). More recently, newer-generation CDK inhibitors have been developed, including dinaciclib (SCH727965; ref. 16), an inhibitor of CDKs 1, 2, 5, and 9, which display greater potency than flavopiridol. Although SCH727965 has shown promising preclinical activity against a variety of tumor cell types (16), and is currently undergoing phase I/II clinical trials in several malignancies, the mechanisms responsible for its antitumor activity remain to be fully elucidated. Furthermore, connections between the ER stress response and the cell-cycle regulatory apparatus are not well understood. Here, we report that extremely low concentrations (e.g., 2–10 nmol/L) of the CDK inhibitor SCH727965, or either CDK1 or 5 knockdown, disrupt the IRE1 arm of the UPR in human myeloma and leukemia cells through a novel mechanism, that is, inhibition of XBP-1s nuclear localization and accumulation, rather than XBP1 splicing as observed with IRE1 endonuclease inhibitors (12–14), accompanied by downregulation of Grp78. Significantly, these events increase the lethality of established ER stress inducers (e.g., thapsigargin and tunicamycin). Such observations argue that specific CDKs (e.g., CDK1 and 5) play key roles in the UPR, thus identifying a common feature of most cancers prompted the development of CDK inhibitors such as SCH727965 may disable the IRE1 arm of the UPR and in doing so, potentiate the activity of agents that elicit this cytoprotective process.

Materials and Methods

Cell lines

BaF3/Bcr-Abl was obtained as previously described (17). Jurkat, U937, K562 (myeloid leukemia cells), RPMI-8226, U266, H929, and J558 (multiple myeloma cells) were purchased from American Type Culture Collection (ATCC) or DSMZ. J558 cells were cultured in ATCC-formulated Dulbecco’s modified Eagle medium (ATCC; #30-2002) supplemented with 10% horse serum. All other cell lines were cultured in RPMI-1640 medium containing 10% FBS. Older frozen stocks were authenticated by DNA profiling [short tandem repeat (STR) analysis] using Promega PowerPlexx16HS assay with 15 autosomal loci plus X/Y. All lines were frozen within 2 months of receipt, and fresh aliquot were thawed before lines reached 6 months in culture.

Patient samples

Peripheral blood was obtained with informed consent from all patients; these studies have been approved by the Virginia Commonwealth University (VCU; Richmond, VA) Institutional Review Board. Mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich) density gradient separation as described previously (18).

Reagents

SCH727965 ((S)-((2S,4S,6S)-4-cyclohexyl-6-ethyl-2-oxo-1,2,3,4-tetrahydro-5H-pyridin-3-yl)methyl)(2-(3-ethyl-7-

(1-oxy-pyridin-3-ylmethyl)aminol) pyrazolo[1,5-a]pyrimidin-5-yl)piperidin-2-yl)(ethanol), structure shown in Supplementary Fig. S1A), was provided by Merck in association with the Cancer Treatment and Evaluation Program (CTEP), National Cancer Institute (NCI; Bethesda, MD). STF-083010, thapsigargin, and tunicamycin were purchased from Sigma-Aldrich. MG-132, RO-3306, and Polybrene were purchased from Santa Cruz Biotechnology and EMD Millipore, respectively. PHA-848125, PD-0332991, AZD5438, JNJ7706621, and PHA793887 were purchased from Selleck Chemicals. All compounds were dissolved in dimethyl sulfoxide (DMSO) for in vitro study.

Plasmids

IRE1s-pcDNA3.EGFP (Addgene; #13009; Lipson and colleagues; ref. 19) was a gift from Addgene. P3xFLAG-CML-10 was purchased from Sigma-Aldrich. Knockdown CDK1-pLKO.1, CDK2-pLKO.1, CDK5-pLKO.1, IRE1-pLKO.1, and CDK9-pLKO.1 were purchased from Thermo Scientific. Luciferase/pLKO.1 or scramble shRNA/pLKO.1 was used as control. shXBP1-s-pSR was constructed by inserting the target sequence for human XBP1 (5′-GGAGGACAGGATGTAGATTT-3′) into pSUPER.retro.puro (Oligogene, Seattle) according to the manufacturer’s protocol. Similarly, shGRP78-pSR was constructed by inserting the target sequence (5′-GCTCGACTCTAGATCCTAAAAG-3′) and (5′-GGTCACTTGATTGAGATTTG-3′) into pSUPER.retro.puro.

Transfection

Plasmids IRE1α/p-cDNA3, shGRF78-pSR, and shXBP1-pSR were transfected by Amaxa nucleofector according to the manufacturer’s protocol (Lonza). Knockdown of CDK1, 2, 5, 9, and IRE1/pLKO.1 were followed the Addgene protocol. Briefly, cocktails of pLKO.1 short hairpin RNA (shRNA; 3 μg), psPAX2 (1.5 μg), pMD2.G (0.5 μg), Opti-MEM 40 μL [Invitrogen (Life Technologies) #31985], and FuGENE6 12 μL (Roche Applied Science; #1181443001) were mixed at appropriate concentrations and dropped evenly via pipette onto 6 mL of HEK293T cells in Petri dishes. The harvested media (containing viral production) was collected at 24 and 48 hours, and then mixed with Lentivector (Clontech; #
631231), centrifuged, dissolved in a small amount of RPMI, and stored at −80°C. Target cells were added to the lentiviral particle solution with Polybrene (1–10 μg/mL). After 48 hours, the cells were collected for experiments.

**Nuclear and cytoplasmic extraction**

Nuclear fractions were prepared by using the nuclear extraction kit (Active Motif). Briefly, after drug treatment, cells were pelleted and lysed by vigorous vortex in hypotonic buffer for 15 minutes. The samples were then centrifuged at 14,000 × g for 1 minute; the supernatant was considered cytoplasmic. Insoluble pellets were further lysed in complete lysis buffer for 30 minutes, and nuclear extracts (supernatant) were collected after a 10-minute centrifugation at 14,000 × g. Both cytoplasmic and nuclear fractions were quantified and subjected to Western blot analysis.

**Polymerase chain reaction**

Total RNA was extracted using TRizol (Life Technologies; # 15996–018) and followed a standard protocol. Polymerase chain reaction (PCR) was amplified using SuperScript III one-step real-time PCR (RT-PCR) system with Platinum Taq polymerase (Life Technologies; # 12574–018). Primer sets for human XBP-1, Grp78 were XBP-1/PCR/F 5′-AGGCAACAGTGTCAGAGTCC-3′; XBP-1/PCR/R 5′-CCTGGTTGCTGAAGAGGAGC-3′; GRP78/PCR/F 5′-TAGCGTATGGTGCGTCTGTC-3′; GRP78/PCR/R 5′-GAACCAGGAGTTAAGAACCG-3′; XBP-1s primer set for mouse XBP-1 was XBP1-S 5′-GAACCAGGAGTTAAGAACCG-3′; XBP1-1-R 5′-AGGCAACAGTGTCAGAGTCC-3′. The program for the thermal cycler was 45°C 30 minutes, 94°C 2 minutes, 40 cycles of 94°C 15 seconds, 58°C 30 seconds, 68°C 1 minute, and then 68°C for 5 minutes.

**Assessment of apoptosis**

The extent of apoptosis was evaluated by either annexin V–fluorescein isothiocyanate staining [BD Biosciences (Pharmingen)] or 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) by flow cytometry as described previously (18).

**Confocal microscopy**

Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. XBP-1s was detected by anti-XBP1 (Santa Cruz Biotechnology) and amplified by Alexa Fluor 488–conjugated secondary antibodies (Molecular Probes, Life Technologies). Slides were mounted in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Southern Biotechnology Associates). Slides were analyzed using a Zeiss LSM 700 confocal microscope and software (Carl Zeiss [Zen 2011]).

**Immunoblot (Western blot analysis)**

Western blot analysis was carried out as previously described (18). The following were used as primary antibodies: IRE1, p-eIF2α (Ser51), p-JNK, p70-S6K (Ser421/424), p70-S6K (T389), eIF4E (Ser209), 4E-BP1 (Thr70), 4E-BP1 (Ser65), caspase-3, cleaved PARP (Cell Signaling Technology); XBP-1s, Grp78, CDK1, CDK9 (Santa Cruz Biotechnology); Mcl-1 [BD Biosciences (Pharmingen)]; actin, FLAG (M2), α-tubulin (Sigma-Aldrich). Blots were stripped and reprobed with actin or tubulin antibodies to ensure equal loading and transfer of proteins.

**Animal studies**

Animal studies were conducted under an approved protocol by the VCU Institutional Animal Care and Use Committee. Athymic NCr-nu/nu mice (NCI) were subcutaneously inoculated in the right rear flank with 3 × 10⁶ J558 cells. SCH727965 was dissolved in 20% hydroxypropyl β-cyclodextrin. Once tumors became apparent, mice were treated 4 days per week with SCH727965 45 mg/kg administered via intraperitoneal (i.p.) injection. Control animals were injected with equal volumes of vehicle. Mice were monitored for tumor growth every other day by caliper measurement. Tumor volumes were calculated using the formula (length × width²)/2. When tumor length or width reached 20 mm, mice were euthanized in accordance with institutional guidelines.

**Statistical analysis**

The significance of differences between experimental conditions was determined by using the Student t test.

**Results**

SCH727965 blocks induction of XBP-1s, Grp78, and downstream targets

U937 cells exposed (3 hours) to the ER stress-inducer thapsigargin displayed a clear induction of XBP-1s accompanied by increased expression of two of its downstream targets, for example, ERjd4 mRNA and Grp78 protein (ref. 8; Fig. 1A). Notably, each of these events (i.e., XBP-1s, Grp78, and ERd4 upregulation) was blocked by coadministration of SCH727965 (2–10 nmol/L). Similarly, SCH727965 markedly attenuated XBP-1s and reduced Grp78 expression in tunicamycin- or thapsigargin-treated 8226 and H929 multiple myeloma cells (Fig. 1B and Supplementary Fig. S1B) as well as in K562 and BaF3/Bcr-Ab1 leukemia cells (Fig. 1C). Time-course studies indicated that in U937 cells, SCH727965 (2 nmol/L) prevented upregulation of XBP-1s and Grp78 at 3 hours, and further reductions were observed over the ensuing 16 hours (Fig. 1D, left). Similarly, coadministration of SCH727965 (2 nmol/L) and thapsigargin for 16 hours blocked thapsigargin-induced XBP-1s and Grp78 induction in K562 cells (Fig. 1D, right). SCH727965-mediated reductions in XBP-1s and Grp78 expression were also observed in U937 cells exposed to another ER stress inducer, tunicamycin (Fig. 1E). In addition, exposure of J558 multiple myeloma cells, which display high basal XBP-1s expression (20), to SCH727965 induced dose- and time-dependent declines in XBP-1s protein expression.
Finally, exposure to SCH727965 (1.6 nmol/L) blocked thapsigargin-mediated Grp78 induction in three primary acute myelogenous leukemia (AML) patient samples (Fig. 1G). Together, these findings indicate that very low (e.g., nmol/L) concentrations of SCH727965 effectively block induction of multiple IRE1 targets in...
human myeloma and leukemia cells exposed to ER stress inducers.

**SCH727965 does not inhibit IRE1 activation or XBP-1s splicing**

Previous studies have identified several compounds that inhibit IRE1 activity and as a consequence, XBP-1s splicing (12–14). Consequently, several approaches were used to compare the effects of SCH727965 with those of known IRE1 inhibitors with respect to inhibition of XBP-1s generation. First, U937 cells were exposed to thapsigargin in the presence or absence of SCH727965 (1.5–8 nmol/L), after which XBP-1s transcription was monitored by RT-PCR. As shown in Fig. 2A, exposure to SCH727965 failed to block XBP-1s mRNA induction in multiple thapsigargin-treated leukemia and myeloma cell lines, for example, U937, K562, 8226, and BaF3/T315I. Furthermore, the absence of XBP-1s mRNA inhibition persisted over the entire 16 hours of SCH727965 exposure interval (Fig. 2B). Consistent with these findings, SCH727965 also failed to diminish XBP-1s mRNA expression in J558 cells, which constitutively express high levels of this protein (Fig. 2C). Notably, the failure of SCH727965 to block XBP-1s mRNA induction by thapsigargin contrasted sharply with the actions of bona fide endonuclease inhibitors such as STF-083010 (14), which markedly inhibited XBP-1s mRNA formation in several cell lines (Fig. 2D).

Second, as shown in Fig. 1D, lines 8, thapsigargin induced IRE1 activity, reflected by increased splicing of XBP-1s and IRE1 phosphorylation/dimerization in K562 cells. Interestingly, coadministration of SCH727965 with thapsigargin resulted in further increases in IRE1 activation, manifested by IRE1 phosphorylation/dimerization and upregulation of its downstream target p-JNK (21), although XBP-1s expression was completely abrogated (Fig. 1D, lines 9; and data not shown). These results argue that SCH727965 does not inhibit XBP-1s by blocking IRE1 activation. Finally, SCH727965 sharply downregulated XBP-1s expression in cells ectopically expressing IRE1 to an equivalent extent as observed in empty-vector control cells (Supplementary Fig. S1B), implying that SCH727965 inhibits XBP-1s formation through an IRE1-independent process. Collectively, these findings support the notion that SCH727965 opposes the induction of XBP-1s by ER stress inducers through a fundamentally different mechanism from that of IRE1 endonuclease inhibitors.

To gain further insights into the mechanism(s) by which SCH727965 diminishes XBP-1s upregulation, J558 cells...
were exposed to SCH727965 (4 or 8 nmol/L) in the presence or absence of the transcription inhibitor actinomycin (2.5 μg/mL), after which XBP-1s expression was examined. Both SCH727965 and actinomycin, administered individually, modestly (SCH727965) or moderately (actinomycin) reduced XBP-1s expression. However, combined treatment produced considerably more pronounced reductions in XBP-1s levels (Fig. 2E, left). Similar results were obtained in 8226 multiple myeloma cells exposed to thapsigargin in conjunction with SCH727965 and actinomycin (Fig. 2E, right). Taken together, these observations argue against the possibility that SCH727965 blocks XBP-1s induction through a transcriptional mechanism.

**Downregulation of XBP-1s by SCH727965 involves a posttranslational mechanism rather than inhibition of the general translation regulatory apparatus**

Effects of SCH727965 were then examined with respect to events associated with translational regulation. Exposure of thapsigargin-treated U937 cells to SCH727965 (2–10 nmol/L) failed to modify phosphorylation of p70-S6K, eIF4E, 4EBP1, or eIF2α (Fig. 3A). Identical results were obtained in J558 cells exposed to 2 to 10 nmol/L SCH727965 alone (Fig. 3B). In addition, coadministration of the protein synthesis inhibitor cycloheximide (CHX; 5 μmol/L) with SCH727965 (8 nmol/L) produced a further reduction in XBP-1s expression (Fig. 3C). Similar results were observed in thapsigargin/SCH727965-treated 8226 multiple myeloma cells (Fig. 2E). Together, these findings argue against a translational mechanism underlying SCH727965-mediated XBP-1s downregulation.

SCH727965 diminishes nuclear accumulation of XBP-1s

Following cytoplasmic cleavage of XBP-1 by IRE1, XBP-1s translocates to the nucleus where it binds to DNA and drives the transcription of genes involved in the UPR (5), prompting examination of the effects of SCH727967 on XBP-1s nuclear disposition. As shown in Fig. 4A, J558 cells exposed to SCH727965 (5 nmol/L; 3 hours) displayed a dramatic decline in expression of XBP-1s in the nuclear fraction. In contrast, cytoplasmic expression of XBP-1s increased slightly with SCH727965 exposure, although a clear reduction in total XBP-1s expression was observed. Notably, thapsigargin markedly increased...
XBP-1s nuclear expression in 8226 cells, an effect that was abrogated by SCH727965 coadministration (Fig. 4B). Finally, confocal microscopy/immunofluorescence analysis demonstrated that J558 cells displayed clear colocalization of green fluorescence (XBP-1s) and blue fluorescence (nuclear). However, SCH727965-treated cells displayed dramatically reduced nuclear XBP-1s expression but increased cytoplasmic XBP-1s expression (Fig. 4C). Virtually identical results were observed in K562 cells exposed to thapsigargin (data not shown). These findings argue that SCH727965 attenuates XBP-1s nuclear translocation/accumulation and diminishes the total cellular expression of XBP-1s.

**CDK1 or 5 interruption plays a functional role in diminishing Grp78/XBP-1s expression and XBP-1s nuclear localization**

SCH727965 inhibits CDKs 2, 5, 1, and 9 within a similar concentration range (IC$_{50}$ of 1.0, 1.0, 3.0, and 4.0 nmol/L, respectively; ref. 16). To determine which CDKs might contribute functionally to SCH727965-mediated XBP-1s downregulation, a CDK knockdown strategy was used. First, CDK1 was knocked down via pooled shRNA plasmids in several cell lines after which responses to ER stress inducers were monitored. As shown in Fig. 5A, compared with scrambled sequence controls, shCDK1 U937 cells displayed marked reductions in basal as well as thapsigargin-stimulated Grp78 induction, accompanied by reductions in XBP-1s expression following thapsigargin exposure (Fig. 5A). Similarly, K562 cells in which CDK1 was knocked down also displayed pronounced reductions in Grp78 and XBP-1s expression following thapsigargin exposure compared with controls (Fig. 5A). Confocal microscopy/immunofluorescence analysis also demonstrated that shCDK1 U937 cells exhibited dramatically reduced nuclear XBP-1s expression following thapsigargin exposure (Supplementary Fig. S2A). In addition, J558 cells treated with the relatively specific CDK1 inhibitors RO3306, AZD5438, or JNJ-7706621 (22–24) for 6 hours exhibited significantly reduced XBP-1s expression (Fig. 5B). Together, these and the preceding shRNA knockdown studies implicate CDK1 in promoting XBP-1s nuclear accumulation and expression as well as Grp78 upregulation in leukemia and myeloma cells undergoing the UPR.

To determine whether CDK5 might also be involved in UPR regulation, K562 and Jurkat leukemia cells in which CDK5 was knocked down were used. K562/shCDK5 and Jurkat/shCDK5 cells exhibited dramatically reduced nuclear XBP-1s expression following thapsigargin exposure (Supplementary Fig. S2A), recapitulating results obtained with SCH727965 (Fig. 4C). In addition, J558 cells treated with the relatively specific CDK1 inhibitors RO3306, AZD5438, or JNJ-7706621 (22–24) for 6 hours exhibited significantly reduced XBP-1s expression (Fig. 5B). Together, these and the preceding shRNA knockdown studies implicate CDK1 in promoting XBP-1s nuclear accumulation and expression as well as Grp78 upregulation in leukemia and myeloma cells undergoing the UPR.
compared with K562/shCont cells (Supplementary Fig. S2B). Furthermore, treatment of J558 cells with PHA793887, an inhibitor of CDK2/5/9 (25) for 6 hours also displayed diminished XBP-1s expression (Fig. 5D). Collectively, these results imply that CDK5, such as CDK1, also plays an important role in regulating the IRE1 arm of the UPR. To characterize the possible involvement of CDK2 and 9 in the UPR, cells in which CDK2 or 9 were knocked down by shRNA were used. K562/shCDK9 cells exhibited clear reductions in CDK9 expression, but in sharp contrast to CDK1 or 5 knockdown cells, failed to display differences in XBP-1s or Grp78 induction following thapsigargin exposure compared with controls (Supplementary Fig. S3A). Similarly, CDK2 knockdown cells exhibited very modest or no changes in Grp78 and XBP-1s induction following thapsigargin exposure compared with its controls (Supplementary Fig. S3B). Finally, J558 cells treated with a specific CDK4/6 inhibitor (PD332991; ref. 26) or a CDK 9/2/7 inhibitor (SNS-032; ref. 27) did not display changes in Grp78 or XBP-1s expression (data not shown). Together, these findings argue against the possibility that inhibition of CDK2 or 9 by SCH727965 is responsible for attenuation of XBP-1s or Grp78 expression.

Pharmacologic or genetic disruption of XBP-1s, Grp78, CDK1, or CDK5 increases sensitivity to ER stress inducers

To investigate the functional cytoprotective contribution of the IRE1 arm of the UPR in this setting, effects of blocking the UPR by SCH727965 on the lethality of ER stress inducers (thapsigargin and tunicamycin) were examined. To this end, U937, 8226, and U266 cells were exposed to 1 to 2 nmol/L SCH727965 in the presence or absence of thapsigargin (5–100 nmol/L) for 24 hours, after which apoptosis was monitored. Exposure to single agents (SCH727965 or thapsigargin) minimally induced apoptosis, whereas combined exposure to SCH727965 and thapsigargin sharply increased apoptosis in all cell lines tested (P < 0.05 vs. single agents; Fig. 6A). Consistent with these findings, cotreatment with SCH727965 and thapsigargin markedly induced procaspase-3 activation and PARP cleavage (Fig. 6B).

To test the functional genetic contribution of IRE1, XBP-1s, and Grp78 to ER stress inducer–associated lethality, U937 and 8226 cells were transfected with a shIRE1/pLKO.1 plasmid. Both U937/shIRE1 and 8226/shIRE1 cells were significantly more sensitive to thapsigargin-induced apoptosis than their corresponding controls (P < 0.05 in each case; Fig. 6C). Similarly, U937/shXBP-1 or U937/shGrp78 knockdown cells were significantly more sensitive to thapsigargin- and tunicamycin-induced apoptosis than controls (P < 0.05; Fig. 6D and E). Finally, U937/shCDK1 or U937/shCDK5 cells were also significantly more susceptible to thapsigargin-induced apoptosis than controls (P < 0.05; Supplementary Fig. S4), recapitulating the effects of IRE1, XBP-1, or Grp78 knockdown. Together, these findings argue that disabling components of the IRE1 arm of the UPR by SCH727965 plays a significant functional role in potentiating ER stress–induced lethality.

SCH727965 inhibits tumor growth and downregulates XBP-1s in vivo

The in vivo antitumor activity of SCH727965 was then investigated in athymic nude mice. To this end, 3 × 10⁶
J558 cells were injected in the flanks of mice. After 4 days, when tumors were visible \( (n = 4) \), mice were treated with SCH727965 45 mg/kg i.p. for 5 days per week. As shown in Fig. 6F, right, mice treated with SCH727965 displayed a significant reduction in tumor size as compared with vehicle treatment \( (P < 0.05) \).
Western blot analysis conducted on excised tumor tissue revealed that SCH727965 also reduced expression of XBP-1s in vivo, consistent with in vitro observations (Fig. 6F, left).

**Discussion**

Multiple malignancies are either driven by the UPR or display basal UPR activation, which promotes their survival and/or renders them resistant to chemotherapy (9, 28, 29). The IRE1 arm of the UPR provides neoplastic cells with a mechanism to escape the lethal consequences of proteotoxic stress accompanying high-protein turnover, and as a result, has become an important target for therapeutic intervention (30). In particular, the transcription factor XBP-1s is required for plasma cell differentiation and survival, has been implicated in multiple myeloma pathogenesis, and is highly expressed in multiple myeloma cells and other cancers, where it is associated with poor survival (9, 31). It therefore represents a logical IRE1 pathway target in multiple myeloma and potentially other malignancies (9, 28). These considerations have prompted intense efforts to develop XBP-1 inhibitors, including agents that act as IRE1 RNase inhibitors that block XBP-1 splicing, for example, STF-083010, MK0186893, and MKC-3946 (12–14). Indeed, such agents have been shown to induce multiple myeloma cell death and potentiate the activity of anti-myeloma agents that trigger ER stress (12–14). However, the high concentrations (e.g., 10–100 μmol/L) of these agents that are generally required to inhibit XBP-1s expression could limit their potential. In addition, the chaperone protein Grp78 is overexpressed in many cancers and suppresses apoptosis through multiple mechanisms (10). Consequently, in diverse tumor types including, leukemia, lymphoma, and epithelial cancers, Grp78 overexpression confers resistance to a wide variety of chemotherapeutic agents (10, 11). However, direct inhibitors of Grp78 are not currently available, and proteins like Grp78 are notoriously difficult to target, for example, by small-molecule inhibitors. The present findings suggest that certain CDK inhibitors, for example, SCH727965, may provide an alternative strategy capable of disabling the cytoprotective XBP-1/Grp78 components of the UPR in neoplastic cells. It should be noted that a previous study reported that the CDK inhibitor flavopiridol induced atypical ER stress and autophagic responses in primary chronic lymphoid leukemia (CLL) cell samples (32). Differences between these and the present results may reflect multiple factors, including cell type–specific responses, or, alternatively, the 3-log higher drug concentrations used in the prior study (32).

The results presented here suggest that in contrast to the actions of IRE endonuclease inhibitors such as STF-083010, which prevent formation of the spliced form of XBP-1 (12–14), CDK inhibitors such as SCH727965 act through a fundamentally different mechanism, that is, prevention of XBP-1s nuclear localization/accumulation and accompanying downregulation. Specifically, the present findings argue that SCH727965, by inhibiting XBP-1s accumulation and nuclear localization, may in fact increase IRE1 activation, manifested by an increase in IRE1 autophosphorylation, dimerization, and JNK activation (21, 33), responses potentially reflecting a compensatory upstream feedback mechanism. Notably, inhibition of XBP-1s expression induced downregulation of several established downstream targets such as Erd4 and Grp78 (7). Results of studies involving protein (CHX) and transcription (actinomycin) inhibitors, as well as RT-PCR, argue against the possibility of transcriptional or translational mechanisms of downregulation, or interference with XBP-1s splicing. In contrast, proteasome inhibition essentially blocked downregulation of XBP-1s expression in cells exposed to SCH727965, implicating a posttranslational process, for example, proteasomal degradation, in this phenomenon. On the basis of these findings, a possible explanation for these observations is that prevention of XBP-1s nuclear transport traps this protein in the cytoplasm where it is vulnerable to ubiquitination and proteasomal elimination (34). Additional studies will be required to confirm or refute this hypothesis.

The finding that inhibition of Grp78 and XBP-1s occurred in cells in which CDK1 or 5 was knocked down, suggests a specific and previously unrecognized role for these CDKs in the IRE1 arm of the UPR. Despite the critical involvement of XBP-1s in UPR responses, current understanding of the regulation of XBP-1s activity is relatively limited. It is known that XBP-1s can be phosphorylated by p38 or through interactions with p85 PI3K, events that enhance its nuclear translocation (35, 36). In the present setting, SCH727965 did not downregulate phospho-Akt (ser473 or thr308) or phospho-p38 (Nguyen and Grant; unpublished data), arguing that SCH727965 does not block the UPR through inhibition of the p85 or p38 pathways. Of potential relevance, CDK5 has been reported to mediate ER stress via activation of MEKK1 and calcium channels or regulation of the nuclear translocation of PDX-1 (37, 38). In this context, it is conceivable that CDK5, either directly or through interactions with a cooperating protein, promotes XBP-1s nuclear localization.

CDK1 is unique among CDKs in that it is the only CDK sufficient for cell-cycle progression in mammalian cells (39). However, several CDK1 functions unrelated to cell-cycle regulation have recently been described, including involvement in both homologous recombination- and non-homologous end-joining–related DNA repair as well as phosphorylation of the C/EPBp transcription factor, an event that promotes leukemia cell differentiation (40). It is possible that CDK1 and 5 may also promote phosphorylation of XBP-1s and subsequent nuclear localization, as both CDK1 and 5 have shown identical substrate specificities in vitro (41). However, the possibility that CDK1/5 may regulate UPR through modulation of calcium channels cannot be excluded (41). Studies designed to identify the mechanism(s) by which CDK1/5 inhibition diminishes XBP-
that disabling CDK1 or 5 either genetically or pharmaceutically may be of therapeutic importance in the context of CDK1 and 5 playing a significant role in activation of the cytoprotective arm of the UPR. Agents that inhibit CDK9 and repress transcription, including SCH727965, have been shown to downregulate short-lived antiapoptotic proteins such as Mcl-1 in various tumor cell types including myeloma and leukemia (42, 43). Moreover, Mcl-1 is known to play a functional role in protecting cells from ER stress (44), raising the possibility that Mcl-1 downregulation might contribute to potentiation of ER stress-mediated cell death by SCH727965. However, the observation that CDK1 or 5 knockdown mimicked the ability of SCH727965 to potentiate thapsigargin and tunicamycin lethality argues that disruption of the IRE1/XBP-1s axis through CDK1 or 5 inhibition plays a primary functional role in this phenomenon.

Multiple phase I trials of SCH727965 in both solid tumor as well as hematologic malignancies have been completed or are underway. In a phase I trial in patients with refractory/relapsed CLL, SCH727965 administered at doses of 5 to 14 mg/m² as a 2-hour intravenous infusion on days 1, 8, and 15 of a 28-day cycle resulted in a 45% overall response rate, including several responses in patients with poor-prognostic features or who had failed another CDK inhibitor (flavopiridol; ref. 45). In a phase II trial in patients with refractory acute myeloid or lymphoid leukemia in which SCH727965 was administered at a dose of 50 mg/m² every 21-day cycle, reductions in blasts and predicted pharmacodynamics effects in leukemic cells (e.g., Mcl-1 downregulation) were observed (46). However, responses were transient, suggesting that more prolonged exposures to this agent, for example, through continuous infusion schedules may be preferable therapeutically. Notably, peak plasma concentrations considerably in excess of those necessary for inhibition of XBP-1s or Grp78 induction (e.g., µmol/L) were achieved in this study, raising the possibility that plasma concentrations capable of inhibiting the IRE1 arm of the UPR may be pharmacologically achievable with alternative schedules.

In summary, the present studies demonstrate that CDK inhibitors such as SCH727965, which potententially inhibit CDK1 or 5, disrupt the UPR in association with attenuation of XBP-1s nuclear localization and accumulation accompanied by Grp78 downregulation. These findings also highlight a heretofore unrecognized link between the cell-cycle regulatory apparatus and IRE1-mediated responses to ER stress that offers the potential for therapeutic exploitation. More specifically, the present observations suggest that CDK1 and 5 play particularly important roles in activation of the cytoprotective IRE1 arm of the UPR, including induction of Grp78, and that disabling CDK1 or 5 either genetically or pharmacologically significantly lowers the threshold for apoptosis in multiple myeloma or leukemia cells exposed to ER stress inducers. Notably, this process seems to occur through a fundamentally different mechanism from that attributed to endonuclease inhibitors (12–14), that is, prevention of XBP-1s nuclear localization and accumulation, rather than inhibition of XBP-1s splicing. In the case of SCH727965, the observations that these events occur at concentrations (e.g., low nmol/L) substantially less than those previously required for currently available RNase inhibitors (12–14) and can be recapitulated in vivo, are of potential importance. The significance of these findings is that transformed cells may be more dependent upon a functional UPR to protect them from the lethal effects of protein overload and proteotoxic stress (47). Consequently, concurrently disrupting the activity of CDK1 and 5 by agents such as SCH727965 may disable cytoprotective UPR activation, thus rendering neoplastic cells particularly vulnerable to antineoplastic agents eliciting this response (27). In addition, the link between cell-cycle regulatory CDKs and the cellular response to ER stress could also provide new therapeutic targets (e.g., CDK1 and 5) for the treatment of high-protein turnover disorders in which an activated UPR is required to prevent neoplastic cell death, for example, multiple myeloma and lymphoma (9, 28). Finally, the connection between CDKs and the UPR also provides a theoretical basis for the development of rational combination regimens involving agents that induce the cytoprotective arm of the UPR, including proteasome inhibitors (48), Hsp90 antagonists such as 17-AAG (49), or sorafenib (50). Studies designed to test these possibilities are currently underway.
References


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Dinaciclib (SCH727965) Inhibits the Unfolded Protein Response through a CDK1- and 5-Dependent Mechanism

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