Long-Range Inhibitor-Induced Conformational Regulation of Human IRE1α Endoribonuclease Activity

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ABSTRACT

Activation of the inositol-requiring enzyme-1 alpha (IRE1α) protein caused by endoplasmic reticulum stress results in the homodimerization of the N-terminal endoplasmic reticulum luminal domains, autophosphorylation of the cytoplasmic kinase domains, and conformational changes to the cytoplasmic endoribonuclease (RNase) domains, which render them functional and can lead to the splicing of X-box binding protein 1 (XBP 1) mRNA. Herein, we report the first crystal structures of the cytoplasmic portion of a human phosphorylated IRE1α dimer in complex with (3R,4S)-2,7-diazaspiro(4.5)decane-7-carboxamide, a novel, IRE1α-selective kinase inhibitor, and staurosporine, a broad spectrum kinase inhibitor. (3R,4S)-2,7-diazaspiro(4.5)decane-7-carboxamide inhibits both the kinase and RNase activities of IRE1α. The inhibitor interacts with the catalytic residues Lys599 and Glu612 and displaces the kinase activation loop to the DFG-out conformation. Inactivation of IRE1α RNase activity appears to be caused by a conformational change, whereby the α-helix is displaced, resulting in the rearrangement of the kinase domain-dimer interface and a rotation of the RNase domains away from each other. In contrast, staurosporine binds at the ATP-binding site of IRE1α, resulting in a dimer consistent with RNase active yeast Ire1 dimers. Activation of IRE1α RNase activity appears to be promoted by a network of hydrogen bond interactions between highly conserved residues across the RNase dimer interface that place key catalytic residues poised for reaction. These data implicate that the intermolecular interactions between conserved residues in the RNase domain are required for activity, and that the disruption of these interactions can be achieved pharmacologically by small molecule kinase domain inhibitors.

Introduction

Cellular stresses, such as accumulation of unfolded proteins, hypoxia, glucose deprivation, depletion of endoplasmic reticulum (ER) calcium levels, and changes in ER redox status activate the unfolded protein response (UPR), an intracellular signal transduction network involved in restoring protein homeostasis [reviewed by Walter and Ron (2011)]. To alleviate these types of stress responses, the UPR responds by halting protein translation, activating transcription of UPR-associated target genes, and degrading misfolded proteins (Harding, et al., 2002; Ron, 2002; Feldman et al., 2005). UPR signaling also regulates cell survival by modulating apoptosis and autophagy and can induce cell death under prolonged ER stress if the misfolded protein burden is too high (Ma and Hendershot, 2004; Rouschop et al., 2010; Woehlbier and Hetz, 2011).

Three key ER membrane proteins have been identified as primary effectors of the UPR: protein kinase R-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1) αβ, and activating transcription factor 6 (Schroder and Kaufman, 2005). IRE1α is a transmembrane protein that functions both as an ER stress sensing receptor via its N-terminal ER luminal domain and as a signal transducer via its cytoplasmic C-terminal kinase and endoribonuclease (RNase) domains (Tirasophon et al., 1998). Upon sensing ER stress, the extracellular portion of the IRE1α protein will homodimerize, allowing for transautophosphorylation, which, in turn, induces a conformational change, resulting in IRE1α activation.
in activation of the RNase domains (Ali et al., 2011). Phosphorylation within the kinase activation loop is an essential step for RNase activation (Prisci et al., 2014). Mammalian IRE1α excises a 26-base pair intron from the mRNA of X-box binding protein 1 (XBP 1), which causes a translational frame shift downstream of the splice site to produce XBP 1s, the active form of the transcription factor (Yoshida et al., 2001; Calfon et al., 2002; Lee et al., 2002). XBP 1 is responsible for the activation of key UPR target genes, including molecular chaperones and components of the ER-associated protein degradation machinery (Lee et al., 2003). Activation of IRE1α is also reported to result in the induction of regulated IRE1α-dependent degradation of a subset of mRNAs encoding secretory proteins or the induction of apoptosis via IRE1α signaling through its kinase domain and downstream effectors ASK1, JNK1, and Caspase-12 (Urano et al., 2000; Hollien and Weissman, 2006).

Loss of ER homeostasis (i.e., loss or hyperactivation of UPR signaling) has been attributed to a number of diseases, including cancer, diabetes, cardiovascular diseases, liver diseases, and neurodegenerative disorders, and the UPR is increasingly becoming an attractive pathway in drug discovery (Hetz et al., 2013; Maly and Papa, 2014). To this end, an increasing body of work has been performed to identify potent and selective molecules of IRE1α and better understand how these molecules bind and affect IRE1α activation mechanisms. Crystal structures of the C-terminal region of phosphorylated (active) yeast Ire1 were the first to be characterized and revealed that Ire1 forms dimers arranged in a “back-to-back” configuration, with the kinase active sites facing outward [Protein Data Bank (PDB) identity (ID) 2RIO; Lee et al., 2008; PDB ID 3F8V; Korennykh et al., 2009; PDB ID 3L0J; Wiseman et al., 2010]. This dimer arrangement also formed the basis of a rod-shaped helical structure, representing a high-order oligomeric Ire1 structure in complex with the kinase inhibitor 2-N-(3H-benzimidazol-5-yl)-4-N-(5-cyclopropyl-1H-pyrazol-3-yl)pyrimidine-2,4-diamine (APY29) (3F8V) (Korennykh et al., 2009). These yeast back-to-back structures contrast with the first reported human IRE1α dimer: a dephosphorylated C-terminal IRE1α-Mg²⁺-ADP complex, possibly representing an early state prior to phosphoryl transfer (PDB ID 3P23; Ali et al., 2011). In this structure, the kinase active sites are facing each other and are in a suitable orientation and proximity for transautophosphorylation, but the RNase domains are far from each other and inactive. A similar “face-to-face” structure was recently reported for mouse IRE1α, but this structure was phosphorylated (PDB ID 4PL3; Sanches et al., 2014). More recently, a few other dephosphorylated human crystal structures were reported. One is of a cocystal structure containing a kinase domain inhibitor bound to an IRE1α monomer (PDB ID 4U6R; Harrington et al., 2014). Here, no dimers were found with the inhibitor-bound structure, suggesting that the compound may either prevent dimerization or stabilize a monomeric IRE1α. The second report presented two back-to-back dimers of IRE1α, one in the apo form and one in an inhibitor-bound form (PDB ID 4Z7G and 4Z7H; Joshi et al., 2015). These structures are consistent with yeast IRE1α dimers, but are distinct in that the apo structure contains a twisted interface between the dimers across the RNase domains, which may represent an additional intermediate of IRE1α prior to full activation. The culmination of all these structures may depict the IRE1 protein at various levels of activation and suggest that this process is conserved evolutionarily. Here, we present a proposed structure of the final state of a human phosphorylated (active) IRE1α dimer that is co-crystallized with two kinase inhibitors that have opposing effects to the RNase activity of the protein.

Materials and Methods

IRE1α Protein Expression and Purification. The cytosolic domain of human IRE1α (NM_001433), encompassing amino acids 547–977, was cloned into pENTR/tobacco etch virus (TEV)-TOPO (Life Technologies, Carlsbad, CA) and subsequently transferred to a pDest8 (Life Technologies) vector backbone containing the N-terminal Flag epitope tag followed by the 6xHis tag and TEV cleavage site (ENLYFQG/S). Baculovirus generation was accomplished using the Bac-to-Bac baculovirus generation system (Life Technologies). Flag-His₆-TEV-IRE1α (547–977) protein expression in baculovirus-infected insect cells was accomplished following established procedures (Wasylko and Lee, 2006). Briefly, a proprietary S9 insect cell line was grown to the early log phase, infected with 1 x 10⁶ baculovirus-infected insect cells/10 l culture, and incubated at 27°C. Cell paste was harvested at 66–72 hours postinfection. A human phosphorylated or dephosphorylated IRE1α protein containing N-terminal Flag-His₆ tags and a TEV protease cleavage site between the tags and an IRE1α protein was purified from ~150 g of cells from a 10-l culture [lysed in 1.5 l of lysis buffer (50 mM Hepes, pH 7.5, 10% glycerol, and 300 mM NaCl) by the EmulsiFlex-C50 homogenizer (Avestin, Ottawa, ON, Canada)]. The protein in the clear supernatant from centrifugation at 30,000g for 30 minutes at 4°C was first captured in 20 ml of NiNTA-SF beads (Qiagen, Venlo, Netherlands) in batch mode for 4 hours at 4°C. The beads were poured into a column and washed with 20 mM imidazole in lysis buffer, and the IRE1α protein was eluted from the column by 300 mM imidazole in 50 mM Hepes, pH 7.5, and 150 mM NaCl buffer. The Ni elution pool was concentrated using a 10-kDa molecular weight cutoff filter concentrator to about 25 ml, to which 3 mg of TEV protease was added to remove the His₆ tag. This mixture was then transferred into dialysis tubing (8-kDa molecular weight cutoff) and dialyzed overnight against 3 l of MonoQ buffer A (50 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM DTT, and 1 mM EDTA), passed through a second 20-ml MonoQ column (GE Healthcare, Piscataway, NJ), and eluted with a 50–500 mM NaCl gradient over 10 column volumes. The eluted samples were analyzed by liquid chromatography–mass spectrometry, and the major peak with a mass consistent with the expected molecular weight for the protein plus three phosphates (30 mass units per phosphate) was purified in a HiLoad Superdex 200 sizing column (GE Healthcare), with a buffer of 50 mM Hepes, pH 7.5, 200 mM NaCl, 5 mM DTT, and 1 mM EDTA. The eluted protein (~2 mg protein in 1-ml aliquots) was stored at ~80°C and later used in assays and crystallization. The remaining fractions from the Mono Q column were pooled and treated with λ-phosphatase to produce the fully dephosphorylated enzyme before it was further purified and stored in the same way as the triply phosphorylated protein.

Phosphorylated IRE1α RNase Activity Assay. The nuclease enzymatic activity of phosphorylated IRE1α (pIRE1α) was measured using a dual labeled 36-mer RNA substrate that contained the IRE1α recognition sequence, with a 6-carboxyfluorescein fluorescent reporter (FAM) at the 3' end, and the Black Hole quencher-1 (BHQ-1) at the 5’ end (5’-FAM/rCrArGr/CrUrCrAr/CrGCrAr/CrUrCr/CrUrCrG/BHQ-1/3’; Integrated DNA Technologies, Coralville, IA). Upon cleavage, the release of FAM results in an increase in fluorescent signal measured at λₐₘ₈₅/λₜₘ₈₅ nm (~65/535 nm). A typical enzymatic reaction was carried out with 10 nM pIRE1α and a 500 nM substrate in a buffer containing 20 mM Hepes, pH 7.5, 5 mM MgCl₂, 10 mM NaCl, 1 mM DTT, 0.05% Tween 20, and 0.02% heat-treated casein (heated for 20 minutes at 60°C prior to use each time), and the fluorescence change was followed using an Envision plate reader (PerkinElmer, Waltham, MA).
To identify inhibitors of IRE1α nucleotide exchange, pIRE1α was screened against the GlaxoSmithKline compound collection. The RNA oligomer substrate was added to the assay plates containing 10 μM of compound. The reaction was initiated immediately by the addition of the enzyme, and the plates were centrifuged for 1 minute at 500 rpm. The final reaction mixture contained 10 nM pIRE1α, 25 nM RNA oligomer, 20 mM Hepes, pH 7.5, 5 mM MgCl2, 1 mM DTT, 0.05% Tween 20, 10 mM NaCl, and 0.02% casein. The reaction plates were incubated at room temperature for 90 minutes before the reaction was terminated with 0.015% SDS in 20 mM Hepes, pH 7.5. The plates were centrifuged for 3 minutes at 1000 rpm prior to measuring product formation using the Viewlux imager (PerkinElmer).

**Competitive Inhibition Studies.** In single compound inhibition studies, the concentrations of the substrate or noncleavable substrate (analog in which the ribose linked to the guanine at the cleavage site was replaced by deoxyribose) and GSK2850163 were varied and the enzyme concentration was kept constant at 10 nM. The modes of inhibition and the inhibition constants were determined by fitting the initial velocities to different models (competitive, uncompetitive, and non-competitive) using Grafit software (Erithacus Software). In a double inhibition experiment, the concentration of the first inhibitor was varied at several different concentrations of the second inhibitor, and the concentrations of the enzyme and substrate were kept constant at 10 and 100 nM, respectively. The reactions were monitored kinetically, and the initial reaction velocities were analyzed using the Yonetani-Theorell equation.

**Protein Crystallization and Structure Determination.** Crystals of pIRE1α (547–977) with GSK2850163 (PDB ID 4YZ9) were prepared by mixing pIRE1α with 0.5 mM GSK2850163 and incubating overnight on ice. The crystals were grown at 20°C by vapor diffusion in sitting drops containing 2 μl of protein (13 mg/ml in 50 mM Hepes, pH 7.5, 200 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.5 mM GSK2850163, and 0.25% dimethylsulfoxide) and 2 μl of reservoir solution containing polyethylene glycol (PEG) 3350 (16–22%), 100 mM Hepes, pH 7.0, and 200 mM CaCl2 acetate. The crystals were thick rods that appeared over 2–5 days and reached full size (0.05 × 0.25 × 0.3 mm) in 2 weeks. Seeding was used to improve crystal quality. The pIRE1α-GSK2850163 crystals were frozen in a solution of 20% ethylene glycol, 22% PEG 3350, and 0.2 M calcium acetate added in a stepped manner to the protein drop before mounting the crystal on the loop.

The crystals of pIRE1α with Mg2+-ADP (PDB ID 4YZW) were grown by mixing 2 μl of protein solution [10 mg/ml pIRE1α (547–977) in 50 mM Hepes, pH 7.5, 200 mM NaCl, 5 mM DTT, 1 mM EDTA, 1 mM ADP (100 mM ADP stock was pH ~7.0), and 1 mM MgCl2] with 2 μl of reservoir solution (16% PEG 3350 and 200 mM Na+ malonate, pH 6.0) in sitting drops at room temperature. Seeding was used to initiate crystal growth. Crystals appeared the next day and grew to full size in 3 weeks. For data collection, the crystals were frozen in a solution of 20% ethylene glycol, 22% PEG 3350, and 200 mM Na+ malonate, pH 6.0, and added to the protein drop before mounting the crystals on the loop.

The complex of pIRE1α (547–977) with staurosporine (STS) (PDB ID 4YZC) was prepared by mixing the protein with 0.5 mM staurosporine and incubating overnight on ice. The crystals were grown at 20°C by vapor diffusion in a sitting drop containing 2 μl of protein (13 mg/ml in 50 mM Hepes, pH 7.5, 200 mM NaCl, 5 mM DTT, and 1 mM EDTA) and 2 μl of reservoir solution containing PEG 300 (30–40%), 100 mM Hepes, pH 7.5, and 200 mM KCl. Small hexagonal plates appeared over 5–10 days and reached full size (0.05 × 0.15 × 0.15 mm) in ~20 days. The crystals were flash frozen in liquid N2 directly from the crystallization drop. All diffraction data were collected at the Advanced Photon Source, Argonne National Laboratories, Life Sciences Collaborative Access Team, Sector 21.

**Detection of XBP1 Splicing by Real-Time Polymerase Chain Reaction.** Multiple myeloma cancer cell lines were obtained from ATCC (Manassas, VA) or DSMZ (Braunschweig, Germany). Cells were cultured in the appropriate culture medium supplemented with...
after RT-PCR, reactions were run on a 3% agarose gel and visualized for 30 seconds, and 72°C for 5 minutes, with 35 cycles of amplification. Primers for human XBP 1 were forward, 5’-CTCGGTCTGGGAAAGGAGG-3’ and reverse, 5’-CGATGTTCTGGG-3’. Real-time (RT) PCR conditions were 95°C for 5 minutes, 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes, with 35 cycles of amplification. After RT-PCR, reactions were run on a 3% agarose gel and visualized using SYBR Safe DNA gel stain (Life Technologies) and a BioRad Imager (Herakles, CA).

Western Blot Analysis. RPMI 8226 cells were seeded into six-well plates at a density of 2.0 × 10⁶ cells/well in RPMI 1640 media containing 1% FCS. Cells were treated with the same conditions as described above for XBP 1 splicing detection by RT-PCR. To harvest protein lysates, cells were lysed with 60 μl of 1X cell lysis buffer (Cell Signaling Technologies, Danvers, MA) containing protease and phosphatase inhibitors. cell lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific), and samples were read on a SPECTRAmax 190 instrument ( Molecular Devices, Sunnyvale, CA). Following quantitation, 40 μg of protein was run on 4–12% Bis-Tris gels (Life Technologies), and protein was transferred onto 0.45 μm nitrocellulose membranes (Life Technologies) using the BioRad semidry transfer blotting apparatus. Membranes were blocked for 1 hour using Li-Cor Odyssey Blocking Buffer (Lincoln, NE) and then probed with the following antibodies overnight: pIRE1α S724 (1:1000,#ab48187; Abcam, Cambridge, UK), total IRE1α (1:1000, #ab37073; Abcam), and GAPDH (1:2000, #A300-639A; Bethyl Laboratories, Montgomery, TX). After washing, blots were incubated with donkey anti-rabbit IRDye-800CW secondary antibody (Li-Cor), and proteins were visualized using the Odyssey Imaging System (Li-Cor).

XBP 1 Transcriptional Activity Assay. PANC-1 cells were seeded into six-well plates at a density of 3.0 × 10⁶ cells/well in RPMI 1640 media containing 10% FCS. Cells were cotransfected with a pGL3-5× unfolded protein response element (UPRE) reporter containing five repetitions of the XBP-1 DNA binding site (a kind gift from R. Prywes, Columbia University, New York, NY) and pRL-SV40 (Promega) using the FuGENE6 transfection reagent (Roche, Indianapolis, IN). Forty-eight hours later, cells were treated with 2.5 μg/ml tunicamycin for 1 hour, followed by GSK2850163 treatment for 16 hours. Luciferase expression was measured using Dual-Glo Luciferase Assay kit (Promega) and normalized to Renilla expression levels.

Hydrogen-Deuterium Exchange. pIRE1α (547–977, 78 μM) was incubated with 250 μM GSK2850163 or 1 mM staurosporine for at least 18 hours. Two microliters of protein-inhibitor mixture was mixed with 18 μl of D2O at room temperature for 1 minute, after which 20 μl of 4 M guanidinium chloride in 1 M glycine buffer, pH 2.5, and 120 μl of formic acid were added and immediately transferred to an ice-cold bath. All subsequent treatment and analysis was done at 2–4°C. Fifty microliters of this solution was injected into a Waters Enzymegrade ethylene bridged hybrid peptide 2.1 × 30 mm column for digestion, then to a C18 column, and into an LTQ XL Orbitrap mass spectrometer (Thermo Scientific). The mass-to-charge values were calculated with XCalibur (Thermo Scientific) and compared with sequences in the Mascot database. The analysis of the hydrogen-deuterium exchange was done with HDEXaminer. Tryptic digestion of all the samples gave a sequence coverage of at least 98% of the amino acid sequence.

Preparation and Characterization of Compounds 1–24. Details are provided in the Supplemental Methods.

Results

Crystallization of the Novel Inhibitor GSK2850163 Bound to pIRE1α. Because of the relevance of the IRE1α/XBP 1 pathway in human disease, we sought to identify small molecules that would inhibit IRE1α RNase activity. GSK2850163 was discovered as a result of a high-throughput screening campaign to identify IRE1α-selective inhibitors of XBP 1 splicing. It is a highly selective inhibitor with dual activity: it inhibits IRE1α kinase activity (IC₅₀ = 20 nM) and RNase activity (IC₅₀ = 200 nM) (Fig. 1A; Table 1). In competition kinetic studies, GSK2850163 and a noncleavable RNA substrate demonstrated mutually exclusive binding to activated IRE1α (Ki = 200 ± 20 nM) (Supplemental Fig. 1). We hypothesized that this was due to binding GSK2850163 altering the preferred enzyme structure for RNA substrate binding (and vice versa) and not due to a physical overlapping of binding sites.

To investigate the mode of binding and enable structure-guided optimization of GSK2850163, we determined the crystal structure of GSK2850163 with the C-terminal portion of pIRE1α (residues 547–977; PDB ID 4YZ9) (Fig. 1B; Supplemental Fig. 2; Table 1). The structure of pIRE1α-GSK2850163 is of a back-to-back dimer, with one inhibitor molecule bound to the kinase domain of each protomer in a pocket next to the kinase αc helix, approximately 12 Å from the hinge region (Fig. 1C). GSK2850163 adopts a U-shaped conformation, with the toylko and dichlorophenyl groups facing the inside of the protein and the spirodecane core partially solvent exposed with the piperidine ring (A-ring) in a chair conformation. Two key interactions with conserved kinase catalytic residues, Ghu612 and Lys599, are observed. The urea nitrogen and the pyrroline nitrogen of GSK2850163 form a hydrogen bond interaction with the side chain of Ghu612, and the carbonyl oxygen of the urea of GSK2850163 interacts through a hydrogen bond with the side chain of Lys599.

GSK2850163 displaces the kinase activation loop of pIRE1α, such that the DFG motif is in the “out” conformation and is flipped by nearly 180°, occupying the ATP binding site (Fig. 1C). This clearly contrasts with our resolved structure of pIRE1α-ADP-Mg²⁺ (PDB ID 4YZD), where the DFG motif is found in the “in” conformation and Phe712 occupies the same pocket where the GSK2850163 binds in the pIRE1α-GSK2850163 structure (Fig. 1D; Table 1). Phe712 makes a π interaction with Tyr628 in a pocket lined by Val613, Leu616, Val625, and Leu679. Superposition of the ADP-Mg²⁺ and GSK2850163-bound pIRE1α complexes shows that GSK2850163 does not overlap with the ATP-binding site (data not shown). The structure of pIRE1α in complex with Mg²⁺-ADP forms a “face-to-face” dimer across the symmetry planes with neighboring molecules (Supplemental Fig. 3). Consistent with previously published structures, the kinase active sites are facing each other and are in a orientation and proximity favorable for transautophosphorylation [3P23 (Ali et al., 2011) and 4PL3 (Sanches et al., 2014)]. The present structure may possibly represent an early postphosphoryl-transfer dimer.
while the face-to-face structure of dephosphorylated human IRE1α may represent a state just prior to phosphoryl transfer.

**Structure Activity Relationship of GSK2850163.** To provide supportive evidence for the binding mode of GSK2850163 observed in the crystal structure, we performed structure activity relationship (SAR) studies, whereby we systematically modified the structure of the ligand and followed the effect by measuring pIRE1α RNase inhibition. First, we observed that while the S-enantiomer is inactive and its conformation does not fit in the electron density map, the R-enantiomer inhibited the RNase activity in vitro, with an IC$_{50}$ of 0.2 μM (Fig. 2A). The R-enantiomer is refined in the structure of the complex. Since the activity of the R-enantiomer is equally potent to the racemic compound and the S-enantiomer is not active, the initial SAR study was performed with the racemic analogs.

Second, the NH of urea nitrogen and the pyrrolidine nitrogen are required to make an H bond with Glu612 (2.98 Å), and any changes in the GSK2850163 (compound 1) molecule disrupting these specific H-bond interactions resulted in loss of activity. For example, replacement of the urea nitrogen (compound 2) or capping the NH with a methyl group (compound 3) caused a loss of potency (Fig. 2A). Similarly, conversion of a basic amine in the pyrrolidine ring to ASPET Journals on July 7, 2017 molpharm.aspetjournals.org Downloaded from
to a nonbasic amide yielded an inactive analog (compound 4; Fig. 2A).

Third, in the binding mode of GSK2850163 in complex with pIRE1α described here, the lipophilic groups at both ends of the molecule (the tolyl and dichlorophenyl groups) are accommodated within the lipophilic pockets observed in the core crystal structure (Supplemental Fig. 2B) and are critical for the inhibitor’s activity. All analogs with polar functionality, such as pyridines, were not active (compounds 5 and 6; Fig. 2B) or much less active (compounds 15–19; Fig. 2C). On the other hand, the lipophilic groups were well tolerated (compounds 8–14 and 20–24) in the hydrophobic pocket. Deletion of one of the chlorines to form 3- or 4-monochloro analogs (7 and 8, respectively) revealed that the 4-chloro substitution was more tolerated than the 3 position in terms of potency (Fig. 2B). Replacement of 3-chloro with other groups, such as fluoro (11), methyl (12), or methoxy (13), provided equally potent analogs, but the trifluoromethyl group (14) caused a significant loss of potency. The tolyl group at the A-ring urea preferred lipophilic substitutions, and polar functionalities (e.g., pyridines (15–17) and substituted pyridines (18 and 19)) were not tolerated. Deletion of the methyl group caused the simple phenyl analog 20 that maintained RNase activity (IC50 = 0.40 μM) (Fig. 2B). Replacement of the methyl group at the 4-position with other lipophilic groups, such as chlorine (21), methoxy (22), and fluorine (23), was well tolerated. The benzodioxole analog 24 exhibited an IC50 value of 100 nM. These SAR study results are consistent with the binding mode of GSK2850163 to pIRE1α observed in the crystal structure, and indicate that GSK2850163 functions as a kinase and RNase inhibitor of pIRE1α.

**Cellular Activity of GSK2850163.** We used a panel of eight multiple myeloma cell lines to test the effects of GSK2850163 on IRE1α RNase activity. Increased IRE1α activity has been observed in primary multiple myeloma specimens, and clinical studies have associated high levels of spliced XBP 1 mRNA with poor patient survival (Reimold et al., 2001; Nakamura et al., 2006; Bagratuni et al., 2010). To recapitulate an ER stress-induced environment in cell culture, tunicamycin, an inhibitor of N-linked glycosylation, was used (Yoshida et al., 2001). XBP 1 mRNA is found primarily in the unspliced form under basal conditions. Upon ER stress stimulation, all cells induced varying degrees of XBP 1 splicing, which could be reversed following treatment with GSK2850163 (Fig. 3B). To determine if GSK2850163 could affect the transcriptional function of XBP 1, cells expressing a reporter plasmid under the control of five tandem repeats of the UPRE motif were treated with tunicamycin, followed by increasing doses of GSK2850163 (Wang et al., 2000). The UPRE sequence contains the binding site found at the promoter of XBP 1 target genes. Induction of ER stress by tunicamycin significantly increases XBP 1 transcriptional activity in these cells, yet increasing concentrations of GSK2850163 are capable of reducing this activity (Fig. 3C). These data support our biochemical and structural evidence that GSK2850163 is an inhibitor of IRE1α, which is capable of inhibiting both kinase and RNase activities of IRE1α.

**Crystallization of Staurosporine Bound to pIRE1α.** To further understand the mechanism by which the RNase activity of IRE1α could be modulated pharmacologically with kinase inhibitors, we sought to investigate the effect of the broad-spectrum kinase inhibitor STS on IRE1α. It has been previously shown that STS inhibits IRE1α kinase activity (Ali et al., 2011) and is likely to bind to the ATP-binding site of IRE1α since it competed with the irreversible IRE1α inhibitor 4μ8C, preventing the formation of a Schiff base with the

<table>
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<th>TABLE 1</th>
<th>Data collection and refinement statistics (molecular replacement)</th>
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<td><strong>Space group</strong></td>
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<td><strong>Resolution (Å)</strong></td>
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<td><strong>Rwork/Rfree</strong></td>
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<td><strong>Completeness (%)</strong></td>
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<td><strong>Redundancy</strong></td>
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<td><strong>R.m.s. deviations</strong></td>
<td>Bond lengths (Å) 0.007, 0.008, 0.002</td>
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<td><strong>a.u., asymmetric unit.</strong></td>
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<tr>
<th>pIRE1α + GSK2850163</th>
<th>pIRE1α + ADP</th>
<th>pIRE1α + STS</th>
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<td><strong>Resolution (Å)</strong></td>
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<td><strong>Rwork/Rfree</strong></td>
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<td><strong>Completeness (%)</strong></td>
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</table>

Values in parentheses are for the highest-resolution shell. Each dataset was collected from a single crystal.
kinase active site residue Lys599 (Cross et al., 2012). We found that STS inhibited pIRE1α kinase enzymatic activity (IC₅₀ = 53 nM), but had no effect on pIRE1α RNase activity in vitro (Fig. 4A). STS exhibited a similar binding affinity toward phosphorylated IRE1α (IC₅₀ = 530 nM) and dephosphorylated IRE1α (IC₅₀ = 550 nM) in a competitive binding assay (data not shown). Interestingly, incubation of STS with dephosphorylated (inactive) IRE1α was capable of activating IRE1α RNase activity (Fig. 4B). To test the effects of STS on IRE1α RNase activity in a cellular context, RPMI 826 multiple myeloma cells were treated with STS, followed by tunicamycin, to measure XBP 1 splicing. STS treatment alone was sufficient to induce XBP 1 splicing similar to levels achieved with tunicamycin (Fig. 4C). Despite inducing XBP 1 splicing, STS did not increase IRE1α autophosphorylation. Instead, STS inhibited tunicamycin-induced autophosphorylation (Fig. 4D). STS treatment may also inhibit IRE1α autophosphorylation below basal levels, but it was difficult to measure this convincingly by Western blot analysis. These observations, both biochemical and in

![Fig. 2. Selected SAR results of compound 1 (GSK2850163). (A) Replacement of the urea nitrogen with a carbon atom (compound 2) or capping the NH with a methyl group (compound 3) caused a loss of potency. Conversion of basic amine in pyrrolidine ring to a nonbasic amide yielded an inactive analog (compound 4). (B and C) The lipophilic groups at the tolyl and dichlorophenyl groups were critical for the compound's activity. On the other hand, the polar functionalities, such as pyridines, were not tolerated.](image-url)
cells, suggest that the effects of STS on IRE1α phosphorylation and XBP 1 splicing could be due to direct binding of STS to IRE1α and not just due to general stress that could occur due to the broad-spectrum nature of the compound.

To understand the structural basis of the activation of the RNase activity of IRE1α by staurosporine, we determined the crystal structure of the human pIRE1α-STS complex (PDB ID 4YZC; Table 1). The pIRE1α-STS complex forms a back-to-back dimer, with one STS molecule bound per protomer (Fig. 4E; Supplemental Fig. 4A). STS binds in the ATP-binding site of the kinase domain and interacts with the hinge residues Glu643, Cys645, and His692 (Supplemental Fig. 4B). The activation loop containing the phosphorylated Ser residues (pSer724, pSer726, and pSer729) that was previously disordered in both the pIRE1α-GSK2850163 and pIRE1α-ADP complexes is well defined in the pIRE1α-STS complex (Supplemental Fig. 4C). pSer724 interacts with Asn750 via two H bonds, pSer726 is involved in an H-bond interaction with Arg722 (3.1 Å) and Arg728 (3.3 Å), and pS729 is interacting through an H bond with Lys716 (2.7 Å) and Arg687 (2.5 Å). A network of H bonds extends from the serines in the activation loop to the αC helix and into the active site DFG motif. Binding of STS to pIRE1α locks the kinase domain in a conformation, in which the 1) activation loop is in the DFG (711–713)-in conformation; 2) the DFG-aspartate interacts with His689 in the HRD motif in the catalytic loop; 3) the conserved Leu616 in the αC helix interacts with the DFG phenylalanine in the regulatory spine of the kinase; and 4) Arg687 of the HRD motif interacts with pSer729 (Supplemental Fig. 4C). These are the signatures of a kinase in the active conformation (Kornev and Taylor, 2010). Attempts to crystallize dephosphorylated IRE1α with STS were not successful.

**Comparison of pIRE1α Dimer Interactions and Protein Dynamics.** To test the pIRE1α dimer interactions observed in the structures, we determined the hydrogen-deuterium exchange of pIRE1α in the presence or absence of either GSK2850163 or STS in solution (Supplemental Fig. 5). Changes in the labeling pattern can be related to changes in the protein structure or dynamics resulting from a ligand-binding event (Englander et al., 2003; Percy et al., 2012). By mapping the observed labeling onto the two cocrystal structures, we observed three areas where most of the structural changes occurred. The first region is in the vicinity of the ligand-binding site (Supplemental Fig. 5A). Increased solvent exposure of the activation loop and disruption of the conserved salt bridge Arg627-Asp620 by GSK2850163 are both consistent with the displacement of the activation loop to the DFG-out conformation and the changes in the αC helix to the inactive conformation (Supplemental Fig. 5B). Second, in the presence of STS, the residues at the kinase-RNase intramolecular domain interface show increased solvent exposure (Supplemental Fig. 5C). The loosening of the intramolecular interactions is consistent with the reorientation of the pIRE1α dimer that leads to the dimerization of the RNase domains. Simultaneously, the decrease in overall labeling of the RNase domains occurs in concert with the conditions under which RNase domains dimerize (Fig. 5, A and D). Third, in the presence of bound STS, there is an increase in solvent exposure of the residues 900–916 (Supplemental Fig. 5D). These residues form a helix-loop element that interact directly with the RNA substrate and include the catalytically essential...
His910 (Dong et al., 2001; Korennykh et al., 2009). This apparent increased dynamic state of the helix-loop catalytic element may be related to the catalytic readiness favored by STS but opposed by GSK2850163.

Comparison of pIRE1α Cocrystal Structures. A comparison between the pIRE1α-GSK2850163 and pIRE1α-STS complexes shows that both structures are back-to-back dimers (Fig. 5A). Binding of GSK2850163 causes the kinase αC helix (residues 610–619) to move to the inactive conformation by an average of 4.4 ± 1.0 Å (range of 3.03–6.40 Å on superposition of the αC helix carbon atoms) as compared with the position observed in the STS complex (Fig. 5B). While the centers of mass of the kinase N domains (21.5–21.9 Å) and C domains (43.1–43.6 Å) remain constant, a nearly 20° rotation of the domains relative to one another is observed in the two structures. A differential gap of nearly 4 Å between the RNase domains in the structures of pIRE1α-GSK2850163 (29.7 Å) and pIRE1α-STS (26.1 Å) is reflected in a different set of

Fig. 4. Structure and activity of the human pIRE1α-STS complex. (A) STS inhibits pIRE1α kinase activity, but not RNase activity. (B) Titration curve showing that the nuclease activity of dephosphorylated IRE1α [IRE1α (-P)] is stimulated by STS in the RNase assay. (C) STS induces XBP 1 splicing in RPMI 8826 cells. Cells were treated with STS for 30 minutes, followed by tunicamycin (tuni) (5 μg/ml) treatment in half of the cells for 1 hour. Total RNA was harvested, and RT-PCR was performed using human-specific XBP 1 primers flanking the splice site that distinguish between unspliced (XBP 1u) and spliced (XBP 1s) XBP 1 mRNA. (D) STS inhibits tunicamycin-induced IRE1α phosphorylation. RPMI 8226 cells were treated as described in (C). Changes in IRE1α phosphorylation were detected by Western blot using a phosphospecific antibody raised against Ser 724 in the kinase activation loop. (E) pIRE1α-STS dimer (PDB ID 4YZC) in the back-to-back configuration, with STS bound in the ATP-binding site.
interactions across the RNase dimer interfaces. Hence, the RNase dimer interface in the pIRE1α-GSK2850163 complex is reduced by 58% (269 Å²) compared with the pIRE1α-STS complex (642 Å²) (Fig. 5E). In pIRE1α-GSK2850163, the RNase domains are noticeably separated, such that the network of interactions across the dimer is no longer possible. The interactions between the protomers of the pIRE1α-GSK2850163 dimer occur mostly between the kinase domains (1734 Å²), with less contact occurring between the RNase domains (269 Å²). This separation of RNase domains is consistent with the biochemical and cellular data, showing that GSK2850163 is a potent inhibitor of pIRE1α RNase activity.

In the pIRE1α-STS complex, the RNase domains form a dimer, with two sets of interdigitating H-bond interactions among His909, Asp847, and Arg905 (3.2–3.5 Å) and among Arg955, Glu836, and Asp927 (3.1–3.5 Å), which allow the key nuclease catalytic residues Tyr892, Arg905, Asn906, and His910 to be poised for action (Fig. 5D). Asp847, His909, and Arg905 are highly conserved residues across multiple species (Dong et al., 2001). These interactions across the RNase dimer interface are also analogous to those observed previously for the activation of yeast Ire1 RNase activity by quercetin (3LJ0; Wiseman et al., 2010). In the structure of pIRE1α-GSK2850163, the RNase domains are separated; thus, the

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**Fig. 5.** Conformational changes to pIRE1α upon ligand binding. (A) Increased interactions along the kinase dimer interface and decreased surface area contact of the RNase domains in pIRE1α-GSK2850163 (left panel). pIRE1α–STS structure, where the interactions are tighter between the RNase domains (right panel). A cartoon model depicts a rocking motion between inactive (left) and active (right) pIRE1α dimers. (B) Residues 610–619 that make up the αC helix move 4.4 ± 1.0 Å between pIRE1α-STS (green) and pIRE1α-GSK2850163 (salmon). The structures of the kinase domains were superimposed using Ca atoms of the residues forming the hinge region. (C) Bottom-up view of the pIRE1α-GSK2850163 RNase domains. There is one pair of residues interacting across the RNase dimer interface, which covers a contact area of 269 Å². Glu913 on chain A (green) and Arg905 on chain B (cyan) form a salt bridge, but the equivalent interaction (i.e., Glu913 of chain B and Arg905 of chain A) is not possible because the side chain of Glu913 in chain B is disordered. (D) Bottom-up view of the pIRE1α-STS RNase domains. There are two sets of interdigitating H-bond interactions. The first set involves His909, Asp847, and Arg905 (3.2–3.5 Å), and the second is between Arg905, Glu836, and Asp927 (3.1–3.5 Å). (E) Calculated buried surface area of contact for the two pIRE1α dimers and individual domains.
network of H bonds between the RNase domains observed in the active pIRE1α-STS dimer does not exist (Fig. 5C). Hence, the data from the co-crystal structures presented here suggest that the formation of interdigiting H bonds and the stabilization of the RNase dimer are critical for the active RNase conformation and its enzymatic activity.

Discussion

Recent work has implicated the UPR in a number of diseases, most notably in cancer, where UPR activation has been shown to function as a survival mechanism in cancer, promoting tumor growth, regulating angiogenesis, and facilitating adaptation to hypoxia (Ma and Hendershot, 2004; Feldman et al., 2005; Chen et al., 2014). Specifically, the IRE1α/XBP 1 pathway has shown to be overexpressed in a variety of human cancers, and activation of the pathway has been shown to be essential for the survival of highly secretory multiple myeloma cells, where the protein load is high (Feldman et al., 2005; Koong et al., 2006; Carrasco et al., 2007). These key findings, coupled with the possibility to modulate IRE1α activity via targeting two potentially druggable domains, has made IRE1α a very attractive target in drug discovery (Hetz et al., 2013; Harrington et al., 2014; Maly and Papa, 2014; Sanches et al., 2014; Joshi et al., 2015).

An increasing body of evidence indicates that IRE1α inhibitors bound to the kinase domain invariably inhibit its ATPase activity, yet some kinase inhibitors inhibit the RNase activity, while others activate its RNase activity. GSK2850163 was discovered in an attempt to identify IRE1α-selective inhibitors of XBP 1 splicing that could regulate multiple myeloma cancer cells. It is a highly selective kinase inhibitor; a panel of 284 kinases was assayed to determine the specificity of GSK2850163, and only two additional kinases were weakly inhibited by GSK2850163: Ron (IC_{50} = 4.4 μM) and FGFR1 V561M (IC_{50} = 17 μM) (Supplemental Table 1). GSK2850163 inhibits IRE1α RNase activity due to the unique way the molecule binds in the kinase domain active site. GSK2850163 binds deep in a pocket next to the kinase αC helix, approximately 12 Å from the hinge region, which is clearly distinct from the ADP binding site. The molecule adopts a U-shape conformation when bound to pIRE1α that could only be achieved with the R-stereoisomer (Supplemental Fig. 2A). Modeling of the S-stereoisomer places the difluorobenzene outside the electron density and clashes with the protein. Similarly, flipping the U-shaped molecule to the reverse orientation places the spirodacene within the electron density, but the four-bond length between the spirodacene and the tolyl group leads to a clash with Leu616, and the two-bond length is too short and causes a clash between the difluorobenzene and Lys599 instead of the H bond when in the modeled orientation. Based on this and the SAR studies performed, it is clear that only the R-stereoisomer in the modeled conformation and orientation is capable of fitting the electron density, avoiding clashes, and engaging with Glu612 and Lys599.

The GSK2850163 mode of binding differs from classic ATP-competitive inhibitors [reviewed by Dar and Shokat (2011)]. Inhibitors exemplified by APY29 (DFG-in and the αC helix in the active conformation) activate IRE1α RNase activity (Korenykh et al., 2009; Wang et al., 2012). In contrast, inhibitors like 1-(4-|8-amino-3-tert-butylimidazo(1,5-a)pyrazin-1-yl)naphthalen-1-yl)-3-[3-(trifluoromethyl)phenyl]urea (KIRA6) and several close analogs inhibit IRE1α kinase and RNase activities and possibly prevent dimer association (Wang et al., 2012; Ghosh et al., 2014). While co-crystal structures for KIRA6 are not available, a close analog was recently co-crystallized with the c-Src kinase domain, demonstrating a shift in the αC helix out to the inactive conformation (PDB ID 3QLF). Thus, it is possible that KIRA6 invokes a similar conformational change when bound to IRE1α. Similarly, another inhibitor recently described by Amgen (Thousand Oaks, CA) potently inhibited IRE1α kinase and RNase activity and was co-crystallized with a dephosphorylated IRE1α monomer (Harrington et al., 2014). The lack of IRE1α dimer structure here could be due to the compound either preventing dimerization or stabilizing a monomeric form of IRE1α. While it was previously suggested that dimerization/oligomerization occurs after autophosphorylation, recent crystal structures of dephosphorylated human IRE1α dimers demonstrate that dimerization can precede phosphorylation in both face-to-face and back-to-back configurations (Ali et al., 2011; Joshi et al., 2015). It should also be noted that this molecule shifted the αC-helix out. Hence, one commonality between this molecule, GSK2850163, and the KIRA6 analogs is that all these inhibitors shift the αC helix to an inactive conformation, which may likely be a requirement for IRE1α RNase inactivation.

Aside from kinase domain inhibitors, two other classes of IRE1α inhibitors have been characterized that target other potential drug pockets in the IRE1α protein: quercetin and salicylaldehyde-based inhibitors. Quercetin binds uniquely to yeast Ire1 in a pocket at the enzyme-dimer interface termed the Q site, thereby stabilizing Ire1 in an active conformation that augments RNase activity (Wiseman et al., 2010). While quercetin has been shown to be a broad spectrum kinase inhibitor and may potentially target the nucleotide binding site of Ire1, it did not inhibit Ire1 autophosphorylation. Independently, a considerable amount of work has been conducted to target IRE1α RNase activity directly (Maly and Papa, 2014; Sanches et al., 2014). These salicylaldehyde-based inhibitors contain a reactive electrophile that most likely covalently modifies Lys907 at the IRE1α RNase active site. It is believed that these inhibitors should not affect IRE1α autophosphorylation or dimerization. GSK2850163 is the first molecule that inhibits both kinase and RNase activity by binding to phosphorylated IRE1α and inducing a unique long-range conformational change that alters the preferred enzyme structure for RNA substrate binding.

The wealth of data on the dimeric/oligomeric state of IRE1α as a function of phosphorylation and the effects of various classes of kinase inhibitors do not fully explain the details of the conformational changes required to cause modulation of IRE1α RNase activity. The hydrogen-deuterium exchange rate, which is dependent on both structural and conformational changes, is very well suited to reveal these details and has the resolution necessary to pinpoint the required conformational changes in the solution state. Our hydrogen-deuterium exchange data are consistent with the model of RNase inhibition suggested by the pIRE1α-GSK2850163 co-crystal structure. The differences observed in labeling overlap with the changes observed in the crystal state. The critical observation of the conserved salt bridge between Arg627 and Asp620 in the kinase domain being disrupted by GSK2850163 is highly consistent with the changes in the αC helix to the out
The pIRE1α-GSK2850163 and pIRE1α-STS cocrystal structures were also compared with two recently reported human back-to-back dimers of IRE1α: one in the apo form (4Z7G) and one in the inhibitor-bound form (4Z7H). In these two structures, the RNase domains are not engaging with each other across the dimer interface and more closely resemble the pIRE1α-GSK2850163 dimer (overall dimer r.m.s. on Ca = 1.6 Å) than the pIRE1α-STS dimer (overall dimer r.m.s. on Ca = 2.7 Å). Despite this, the imidazopyridine molecule, compound 3, interacts with the hinge and the activation loop (DFG-in) in a similar fashion as staurosporine (r.m.s. on Ca = 0.9 Å) and behaves as a type I kinase inhibitor (Joshi et al., 2015). GSK2850163 and compound 3 do not occupy overlapping pockets. This structural observation is at odds with the other type I inhibitor–bound IRE1α structures. A comparison of the IRE1α apo and IRE1α-imidazopyridine complex indicates that they are nearly identical (r.m.s. on Ca = 0.6 Å). This discrepancy may be due to the way the experiments were performed, namely, that the inhibitor-bound form was resolved by soaking into the preformed apo crystals as opposed to performing cocrystallization.

The characterization of the first cocrystal structure of human phosphorylated IRE1α and its complex with a new class of kinase inhibitors has revealed a novel mode of action for the inhibition of pIRE1α kinase and RNase activity. The comparisons made between known structures across various species and with inhibitors that have differential effects on IRE1α RNase activity should provide insights into the molecular mechanisms responsible for activation and inhibition of IRE1α RNase activity. These new data should also provide a feasible path to enable the design and use of pharmacological agents that differentially affect IRE1α/XBP 1 signaling.

Coordinates for the pIRE1α-GSK2850163, pIRE1αADP-Mg2+, and pIRE1α-STS cocrystal structures have been deposited to the Protein Data Bank with the respective accession codes: PDB ID 4YZ9, 4YZD, and 4YZC.

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References


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