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Structural and molecular bases to IRE1 activity modulation

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Abstract

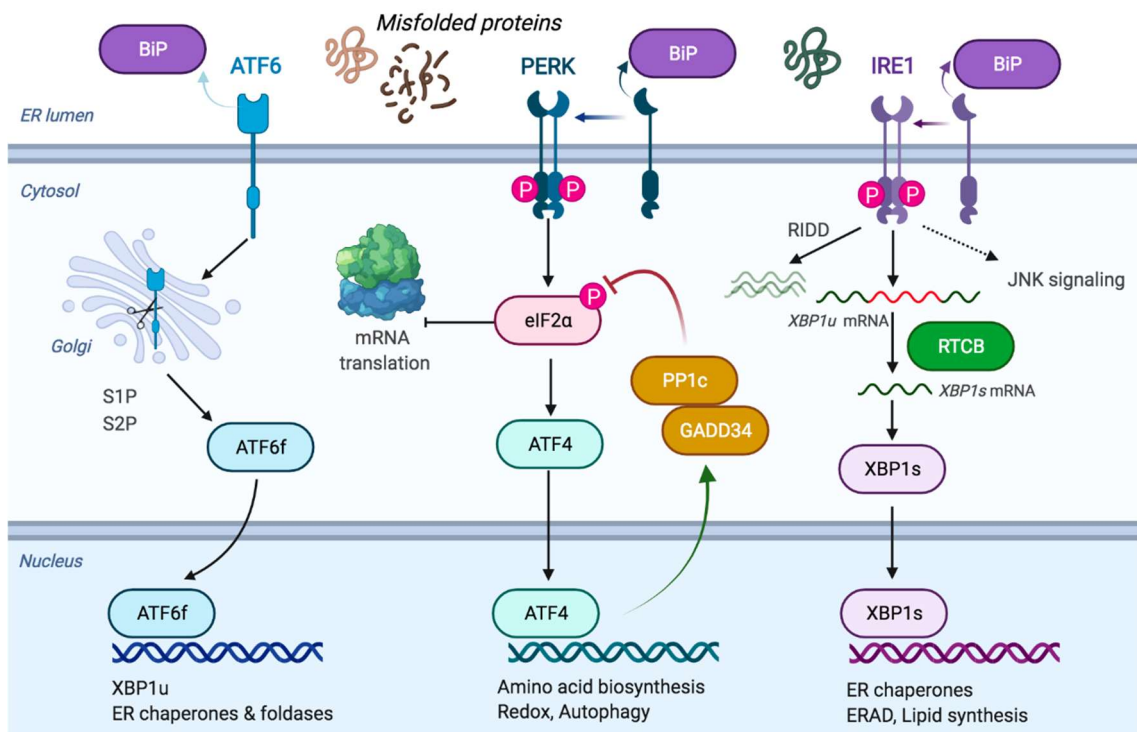
The Unfolded Protein response is an adaptive pathway triggered upon alteration of endoplasmic reticulum (ER) homeostasis. It is transduced by three major ER stress sensors, among which the Inositol Requiring Enzyme 1 (IRE1) is the most evolutionary conserved. IRE1 is an ER-resident type I transmembrane protein exhibiting an ER luminal domain that senses the protein folding status and a catalytic kinase and RNase cytosolic domain. In recent years, IRE1 has emerged as a relevant therapeutic target in various diseases including degenerative, inflammatory and metabolic pathologies and cancer. As such several drugs altering IRE1 activity were developed that target either catalytic activity and showed some efficacy in preclinical pathological mouse models. In this review, we describe the different drugs identified to target IRE1 activity as well as their mode of action from a structural perspective, thereby identifying common and different modes of action. Based on this information we discuss on how new IRE1-targeting drugs could be developed that outperform the currently available molecules.

1 **Introduction**

2 The endoplasmic reticulum (ER) is the first membranous compartment of the secretory
3 pathway in eukaryotes. It is a calcium store with concentrations 1000 to 10000 times
4 higher than in the cytosol and maintains lipid homeostasis [1]. The ER is also the gateway
5 to the secretory pathway and is instrumental for the proper folding of secretory and
6 transmembrane proteins [1]. As such, it represents a critical machinery through which
7 proteins destined to exert their functions in other cellular compartments must traffic to
8 acquire their correct conformation and post-translational modifications. It has previously
9 been demonstrated that the ER and its functionality is important for the composition of
10 the Golgi complex, the plasma membrane, lipid droplets or the phagosome
11 (autophagosome and lysosomes) [2,3]. Moreover, this compartment is in tight contact
12 with mitochondria, through the mitochondrial-associated membranes (MAMs) or with
13 endosomes or lysosomes [4,5].

14
15 When the balance between the cellular protein folding demand and the ER capacity is
16 disrupted, improperly folded secretory and transmembrane proteins accumulate in the ER,
17 a situation known as ER stress [6]. To cope with stress, the ER triggers an adaptive
18 response named the Unfolded Protein Response (UPR). The UPR is transduced by three
19 main ER resident sensors named Inositol Requiring Enzyme 1 alpha (IRE1 α , hereafter
20 referred to as IRE1), Protein Kinase RNA-like ER Kinase (PERK) and Activating
21 Transcription Factor 6 (ATF6). Activation of the UPR sensors has as principal purpose
22 to alleviate the stress and is known as the adaptive UPR. If the stress cannot be resolved,
23 the UPR instead triggers apoptosis, referred to as terminal UPR. However, the
24 mechanisms allowing the switch between adaptive and terminal UPR remain ill-
25 characterized. Among the possible candidates, the IRE1 pathway being greatly conserved
26 through evolution plays crucial roles in both physiological and pathological ER stress.
27 Mammals express two IRE1 paralogues: IRE1 α and IRE1 β . IRE1 α (named hereafter as
28 IRE1) is ubiquitously expressed and contributes to XBP1 mRNA splicing. IRE1 β
29 expression is predominant in intestinal and lung epithelia, and deletion of IRE1 β in mice
30 increases their sensitivity to experimental colitis induced by dextran sodium sulfate [7].
31 Recently, it was demonstrated that IRE1 β might negatively regulate IRE1 α signaling in
32 response to endoplasmic reticulum stress [8]. IRE1 is an ER transmembrane protein
33 activated after binding immunoglobulin protein (BiP) dissociation and/or direct binding

34 of improperly folded proteins [9,10]. IRE1 is characterized by the presence of both kinase
 35 and endoribonuclease domains in its cytosolic region. After its
 36 dimerization/oligomerization, IRE1 trans-autophosphorylates allowing the recruitment of
 37 TNF receptor-associated factor 2 (TRAF2) and the subsequent activation of the c-Jun N-
 38 terminal kinase (JNK) pathway [1]. IRE1 phosphorylation also yields a conformational
 39 change which activates the RNase domain and leads to unconventional splicing of the
 40 *XBPI* mRNA and the expression of a major UPR transcription factor XBP1s [1,6].
 41 Importantly, the ligation following the IRE1-mediated excision of the 26nt intron in the
 42 *XBPI* mRNA is catalyzed by the tRNA ligase RtcB in mammals [11–13]. Through its
 43 RNase domain, IRE1 also accounts for the cleavage of mRNA, rRNA and miRNA
 44 sequences, a process named Regulated IRE1 α -Dependent Decay (RIDD) [14,15].
 45 Interestingly, the degree of oligomerization of IRE1 may define its RNase activity
 46 towards *XBPI* mRNA splicing or RIDD, ultimately impacting on cell fate (**Figure 1**).
 47 Although the order of oligomerization required for *XBPI* mRNA splicing vs. RIDD is
 48 still debated, there is a consensus on the cytoprotective effects of XBP1s and the dual
 49 protective or cell death-inducing outputs of RIDD under ER stress [16,17].
 50



51
 52 **Figure 1.** Schematic representation of the signalling pathways composing the Unfolded
 53 Protein Response.
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55 In this model, while *XBPI* mRNA splicing is induced in the adaptive UPR (aUPR) and
56 inactivated during the terminal UPR (tUPR), RIDD displays an incremental activation
57 pattern reaching unspecific RNA degradation during tUPR. Despite our knowledge on
58 IRE1 signaling biological outputs, little is known so far on how signals emanating from
59 these two RNase activities do integrate. The existence of a multiprotein complex recruited
60 in IRE1 foci that dynamically changes composition during the course of ER stress has
61 been suggested as a possible modulation mechanism [18]. This is supported by the
62 identification of IRE1 interactors regulating *XBPI* mRNA splicing differently than RIDD
63 [19].

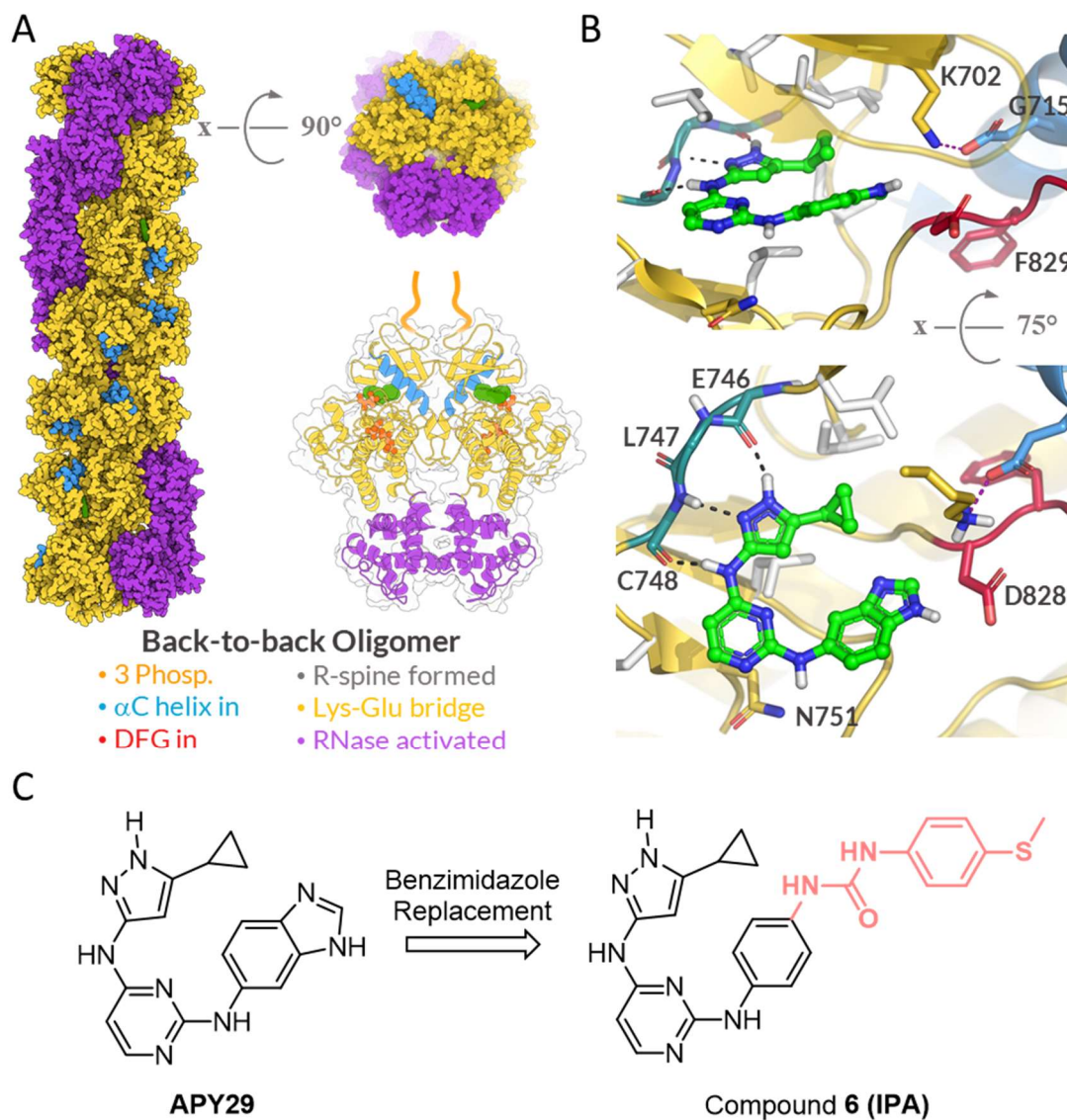
64
65 Over the past two decades, the key roles of IRE1 signaling in cellular adaptation to
66 proteotoxic stress have also been linked to many pathophysiological conditions. IRE1
67 signaling was found to play critical functions in various degenerative disorders including
68 for instance Parkinson's disease, Alzheimer's disease or Amyotrophic lateral sclerosis
69 [20–25]. It was also found to play an important role in metabolic (e.g., diabetes, NAFLD
70 [16,26] or inflammatory diseases [27,28] (e.g., Crohn's disease [29,30]). IRE1 signaling,
71 either through *XBPI* mRNA splicing or RIDD, has also been shown to contribute to tumor
72 development and to mechanisms of resistance to treatments [17]. As such, it has been
73 proven that IRE1 could act as a relevant therapeutic target in multiple myeloma [31,32],
74 acute myeloid leukemia [33], prostate [34], ovarian [35], triple negative breast [36,37],
75 gastric [38–40], liver [41,42] cancers and primary brain tumors such as glioblastoma
76 [17,43,44]. These discoveries sparked intensive efforts to identify small molecule
77 modulators of IRE1 activity to be used as therapeutic tools in various diseases. Thus far,
78 three major binding sites were identified, including the kinase pocket, the RNase pocket
79 and the dimer interface [45]. In this review, we propose to focus exclusively on
80 compounds targeting the kinase or RNase pockets, and for which structural data or
81 structural-activity relationship studies are available. IRE1 kinase inhibition was the most
82 popular strategy so far, and led to the identification of multiple chemical series with
83 different mode of actions. To better understand the molecular basis of the small molecules
84 modulating IRE1 activity presented in this review, some key concepts about kinase need
85 to be reminded. Kinase inhibitors have been classified from type I to VI based on
86 crystallographic data and key structural elements found in all kinases such as the DFG
87 (Asp-Phe-Gly) motif, R-spine, activation segment and α C helix have been used to
88 distinguish active and inactive kinases [46]. Type I, I^{1/2} and II, which are the most

89 predominant, all bind into the adenine binding pocket where they form hydrogen bonds
90 with the hinge residues that connect the small and large lobes, but differ by the protein
91 kinase conformation bound. Thus, type I inhibitor bind fully active kinases (DFG in, α C
92 in), type I^{1/2} bind to a DFG in but inactive conformation, and type II bind to a DFG out
93 inactive conformation. Type III and IV are allosteric inhibitors as they do not bind to the
94 ATP binding site, but next to it or elsewhere entirely, respectively. The type V class
95 encompasses more rare bivalent inhibitors. Finally, the recent emergence of covalent
96 compounds that target the catalytic site prompted the creation of a novel type VI class
97 [47]. The various types of IRE1 kinase inhibitors identified and developed prompted
98 significant advancement in the understanding of IRE1 activation mechanism, but did not
99 yet deliver any molecule able to enter the clinic. Regarding IRE1 RNase inhibition,
100 several compounds were identified which required covalent binding to efficiently impair
101 this catalytic activity. Although targeted covalent ligands are trending again [48], this
102 characteristic is generally considered as unfavorable due to common occurrence of off-
103 target effects/promiscuous binding. Nevertheless, one such covalent RNase inhibitor
104 MKC8866 (renamed ORIN1001) entered clinical trial in association with Paclitaxel to
105 target triple negative breast tumors (<https://clinicaltrials.gov/ct2/show/NCT03950570>).
106 The modulation of IRE1 activity by small molecules currently represents a very active
107 field of research as witnessed by the striking increase in patented inhibitors observed
108 between 2018 and 2021 from numerous companies and academic laboratories (19 patents
109 total, of which 16 were filed since 2017). In this review, beyond the extensive coverage
110 of the molecular and structural mechanisms by which IRE1 kinase and RNase targeting
111 molecules affect IRE1 functions, we will also briefly discuss on yet unexplored novel
112 modalities to pharmacologically target IRE1 (PROTAC, PPIs, FBDD, etc.)[49].
113 Collectively, the work presented below is the first of its kind as it provides a synthetic,
114 structural and molecular view of the mechanisms by which IRE1 modulators function,
115 which could provide some hints on novel strategies to identify novel and more potent
116 inhibitors.

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118 **IRE1 ATP-competitive activators**

119 *N*⁴-(1*H*-pyrazol-3-yl)pyrimidine-2,4-diamine: APY29 & IPA



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Figure 2. A. Graphical representation of the high-order oligomer structure of the *Saccharomyces cerevisiae* yeast IRE1 (yIRE1) cytosolic domain in complex with APY29 (PDB ID 3FBV) and constitutive back-to-back dimer (cartoon; ligand as green spheres; α C-helix in blue; Kinase C- & N-lobes in yellow; RNase domain in purple; Phosphorylated residues in orange). Key structural changes highlighted underneath. B. Front and top view of APY29 in the kinase pocket with key residues indicated (ligand as ball and stick with green carbon atoms; hydrogen bonds as dark gray dashed lines; salt bridge as purple dashed line; DFG motif side chains as red sticks; hinge backbone atoms as teal sticks; apolar residue as white sticks; α C-helix as blue cartoon and sticks). C. SAR optimization of APY29 by replacement of the benzimidazole moiety that led to compound 6, also known as IPA (for IRE1/PERK Activator). Structural changes highlighted in colors with bold bonds.

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134 In their pioneering work on the exploration of IRE1 higher-order activation mechanism,
135 Korennykh *et al.* reported the very first kinase site bound RNase activators, with notably
136 a unique co-crystal structure of *Saccharomyces cerevisiae* yeast IRE1 (γ IRE1) in complex
137 with a synthetic ligand [22]. Their serendipitous identification of type I kinase inhibitors
138 (Sunitinib, APY24 & APY29) capable of activating the γ IRE1 RNase activity represented
139 a major breakthrough and kickstarted the interest for IRE1 targeting kinase inhibitors. By
140 combining the use of a specific γ IRE1 construct capable of self-activating and self-
141 assembling, the authors successfully obtained an oligomeric crystal structure
142 representative of γ IRE1 activated state with their most potent activator APY29 bound
143 (Figure 2A) (PDB ID 3FBV). In this structure, the aminopyrazole core of APY29 forms
144 three hydrogen bonds with the hinge residues Glu746 and Cys748 (Glu643 and Cys645
145 in *h*IRE1), anchoring strongly the ligand in the kinase pocket (Figure 2B). Additional Van
146 der Waals interactions and probable hydrogen bonds with Asn751 and Asp828 (not
147 visible in the crystal structure) complete the interaction patterns of APY29. This observed
148 binding mode allowed to hypothesize on the superior potency of APY29 and APY24 over
149 Sunitinib, a broad inhibitor of receptor tyrosine kinases (RTKs) and an approved anti-
150 cancer drug (Sutent®). Whereas APY29 and APY24 are able to extend into the kinase
151 pocket where the ribose-phosphate moiety of ADP normally binds along with its Mg^{2+}
152 cofactor, Sunitinib opens more toward the solvent due to its different three-dimensional
153 shape. The tight crystal packing of the γ IRE1 also made possible the identification of
154 unique structural features that helped decipher the IRE1 activation mechanism. Two new
155 interfaces, not present in previous back-to-back dimer structure and dubbed IF2^c and IF3^c,
156 were identified and demonstrated to contribute to the control of IRE1 RNase activity.
157 Most notably, the α D helix and the phosphorylated activation loop (pS840, pS841 &
158 pT844) were fully visible in the IF3^c interface and revealed that the activation loop
159 extends into a partner kinase domain. Deeper analysis of this structure and its implication
160 in IRE1 activation has been reviewed elsewhere [50].

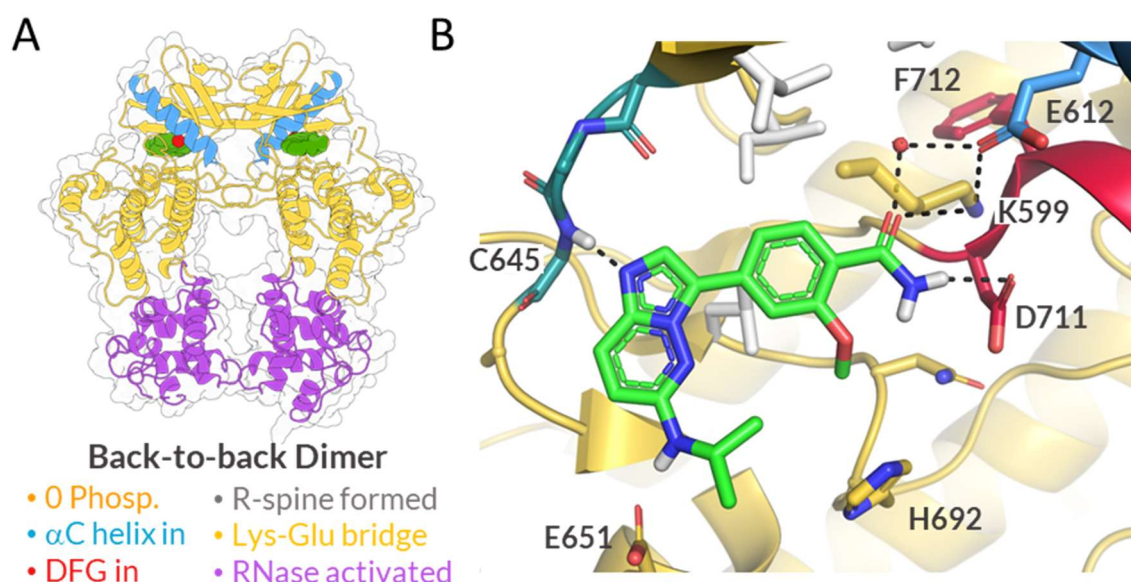
161 A few years later, the same research group presented in a second publication [51], a small
162 library of new molecules derived from APY29 and aimed at potently activating *h*IRE1
163 RNase function. The APY29 benzimidazole was replaced by a diphenyl urea moiety
164 (Figure 2C) with various substitutions, a motif successfully used in the design of other
165 kinase inhibitors [52]. The rest of the scaffold remained unchanged as it was clearly
166 identified as essential to maintain affinity and key interactions. Among the ten analogues

167 synthesized, the *para*-substituted methyl thioether compound **6** was the most potent and
 168 activated by a 900-fold factor the IRE1 RNase *in vitro*. These changes in the structure
 169 also led to a major improvement in kinase selectivity, with only 16 kinases inhibited >
 170 80% at 1 μM (total panel of 266 kinases). Compound **6** was later renamed in the paper as
 171 IRE1/PERK Activator, **IPA** (Figure 2C), as it was found to also activate the PERK
 172 signaling pathway at low concentrations, whereas inhibiting it at higher compound
 173 concentrations and the effect of combined alteration of both pathways remains to be
 174 characterized in cellular models of relevant diseases [51].

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176 *Imidazo[1,2-b]pyridazine: Compound 3*

177 In a landmark study (47), Joshi et al. reported the discovery of a second class of IRE1
 178 RNase activators and were able to obtain an unambiguous ligand-bound crystal structure
 179 (Figure 3A) (PDB ID 4Z7H), along with an apo IRE1 structure (PDB ID 4Z7G).



180

181 **Figure 3.** A. Graphical representation of the back-to-back dimer visible in the structure
 182 of *h*IRE1 in complex with compound **3** (PDB ID 4Z7H) with key structural changes
 183 highlighted underneath. B. Top view of **3** in the kinase pocket with key interaction and
 184 residues indicated. Color coding and representation as in Figure 2; red sphere represents
 185 an ordered water molecule bridging interactions with the protein.

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187 These two novel snapshots of distinct IRE1 activation stages improved the understanding
 188 of the fine structural changes happening within the kinase domain during early IRE1
 189 activation which are needed for RNase activity. The ligand, compound **3**, appears to have

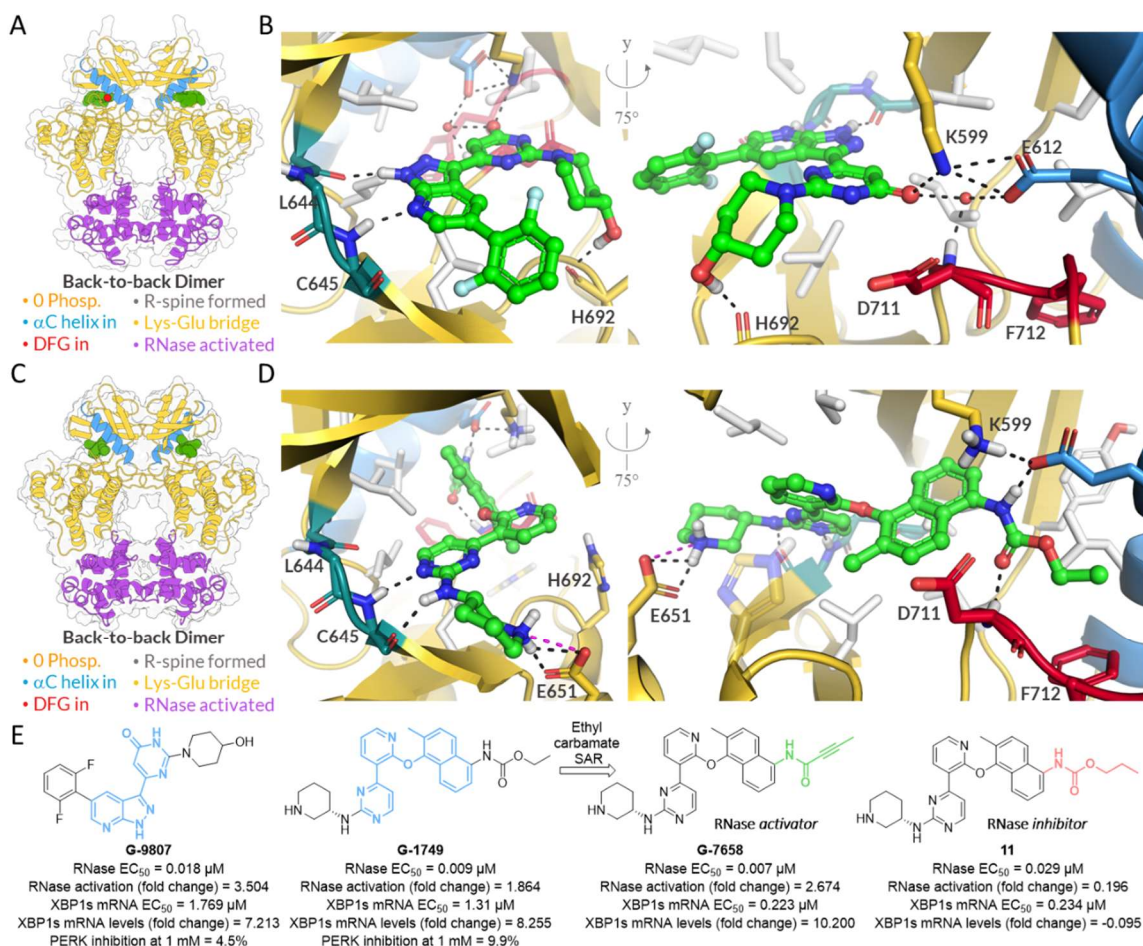
190 been identified in a high-throughput screening campaign using a DELFIA assay
191 developed by the authors [53], where it inhibited IRE1 autophosphorylation with an IC_{50}
192 of 218 nM (\pm 150 nM). Its impact on IRE1 RNase activity was then assessed in a FRET-
193 based assay using unphosphorylated IRE1 and the endoribonuclease function was
194 potently enhanced (EC_{50} = 143 nM). Similarities in its binding mode can be drawn with
195 APY29 [22], both type I kinase inhibitor residing in the adenine pocket (Figures 2B &
196 3B). As such, the usual interactions with hinge residues are present in the form of a single
197 H-bond between the backbone N-H of hinge residue Cys645 and the ligand's imidazole
198 *N*-1. Numerous Van der Waals interactions are also observed due to the nature of the
199 surrounding residues and tight enclosing of the ligand in the kinase pocket. What differs
200 from the APY29 binding mode is that the 2-methoxybenzamide reaches further towards
201 the DFG motif and Lys-Glu bridge where it makes unambiguous hydrogen bonds. In
202 particular, the amide NH_2 interacts with the Asp711 carboxylic acid group, while the
203 amide carbonyl is engaged simultaneously with Lys599 and a buried water molecule is
204 acting as a bridge to Glu612. Consequently, the conformation of the DFG motif is altered
205 and, in particular, the Phe712 side chain shifts in position to fully form the R-spine, a
206 prerequisite for RNase activation. Comparison of the ligand-bound and apo *h*IRE1
207 structures, and the APY29-oligomeric *y*IRE1 structure obtained by Korennykh *et al.* [22],
208 supported the hypothesis that these ligands activate IRE1 RNase activity through the
209 stabilization of an active conformation of the kinase site (DFG-in, α C helix in, R-spine
210 formed).

211

212 ***Naphtalenoxy-pyridyl-pyrimidine & Pyrazolo-pyridine: G-9807 & G-1749***

213 At the end of 2020, researchers from Genentech published an impactful study [54]
214 revealing the full extent of the interdomain regulation within IRE1 and how it can be
215 controlled through minor changes in ligands' molecular structures that act as a switch of
216 the bioactive conformation. The structural data obtained by Ferri *et al.* also shed light on
217 the importance of the activation loop and of its phosphorylation in regulating RNase
218 activation. In the continuation of their previous studies [31,55] and to elucidate further
219 the principles governing IRE1 RNase activation, Ferri *et al.* set out to screen the internal
220 Genentech chemical library to identify better kinase site binding ligand capable of
221 activating IRE1 RNase function. To this end, an ATP-competitive TR-FRET assay was
222 used a preliminary screen to identify kinase binders, then evaluated in IRE1-specific
223 kinetic RNA cleavage assay. The most promising hits were then tested in KMS-11

224 multiple myeloma cells, known to hyper-produce immunoglobulins in the ER and
 225 therefore be strongly dependent on the IRE1 pathway to cope with the induced ER stress.
 226 Two distinct molecules were identified, **G-9807** and **G-1749**, which present a pyrazolo-
 227 pyridine and a phenoxy-pyridyl-pyrimidine scaffold, respectively (Figure 4). Of note, G-
 228 1749 is a structurally close analogue of earlier identified RNase inhibitor KIRA8. These
 229 compounds performed extremely well in all assays, G-9807 showing a 3.50-fold increase
 230 in RNase activity *in vitro* and a 7.21-fold increase in *XBP1s* mRNA levels in cells. Similar
 231 results were found for G-1749 with a 1.86-fold and 8.26-fold increases, respectively.
 232 Kinase selectivity was also assessed and at 1 μ M, G-1749 only inhibits 6 out of the 218
 233 kinases constituting the panel (undisclosed for G-9807) and none of the compounds
 234 significantly affected PERK, even at a high concentrations (1 mM).



235
 236 **Figure 4.** Graphical representations of the back-to-back dimer visible in the structure of
 237 human IRE1 (*hIRE1*) cytosolic domain in a complex with A. compound G-9807 (PDB
 238 ID 6W3K) and C. compound G-1749 (PDB ID 6W39), with key structural changes
 239 highlighted underneath. B. Left-side and right-side views of B. G-9807 and D. G-1749 in
 240 the kinase pocket with key interaction and residues indicated. Color coding and

241 representation as in Figure 2; red sphere represents an ordered water molecule bridging
242 interactions with the protein. E. SAR study of the ethyl carbamate ‘tail’ of G-1749.
243 Modulation around the nature and size of the tail led to the but-2-ynamide-substituted
244 *RNase activator* G-7658 (PDB ID 6W3A), and 4,4-dimethylpent-2-ynamide-substituted
245 *RNase inhibitor* 11.

246

247 Using sedimentation velocity analytical ultracentrifugation experiments, the authors
248 demonstrate that G-9807 is able to stabilize a dimeric form of unphosphorylated human
249 IRE1 (*hIRE1*) cytosolic domain, similarly to previous *RNase* activators APY29 [22] and
250 compound **3** [56], whereas G-1749 does not, underlying differences in activation
251 mechanisms. Each ligand was then successfully co-crystallized in complex with
252 unphosphorylated *hIRE1* to gain structural insights into this disparity (PDB IDs 6W39 &
253 6W3K), complemented by hydrogen-deuterium exchange (HDX) experiments to study
254 the protein conformation in solution. Both the G-1749 and G-9807 crystal structures show
255 IRE1 in an activated conformation, with all the hallmark of an active kinase in complex
256 with a type I kinase inhibitor (Figures 4A & 4C). Regarding the ligands’ binding modes,
257 the usual key hydrogen bonding with the hinge residues, and in particular Cys645, are
258 observed with the pyrazolopyridine motif of G-9807 and the aminopyrimidine ring of G-
259 1749 (Figure 4B& 4D). The 4-hydroxypiperidin-1-yl of G-9807 makes an unseen before
260 hydrogen bond to the backbone carbonyl of His692. The main differences between the
261 two ligand binding modes lie in their interactions with the DFG residues and Lys-Glu
262 bridge. Whereas G-9807 interacts with Glu612 through a buried water molecule and
263 Lys599 (Figure 4B), G-1749 makes a direct hydrogen bond with Glu612 via the
264 carbamate NH. However, the true novelty was found in the structure of G-1749, where
265 the ethyl carbamate ‘tail’ of the ligand is not observed in the back pocket as seen with the
266 chlorophenyl moiety of KIRA8, but rather wedges itself in a front pocket underneath the
267 α C-helix and along the DFG residues, thus sparing the Lys-Glu bridge. As a consequence,
268 G-1749 appears to force the activation loop in an upward conformation, later confirmed
269 by the HDX analysis. This unique feature differentiates G-1749 from previously
270 published activators and from G-9807.

271 A short SAR study on this tail was thus conducted and showed that the chemical
272 substituents tolerated by the front pocket are very limited, where minor structural changes
273 such as the elongation of the alkyl chain by one carbon (compound **11**, Figure 4E) or
274 substitution of the carbamate oxygen atom by a methylene led to a complete reversal of

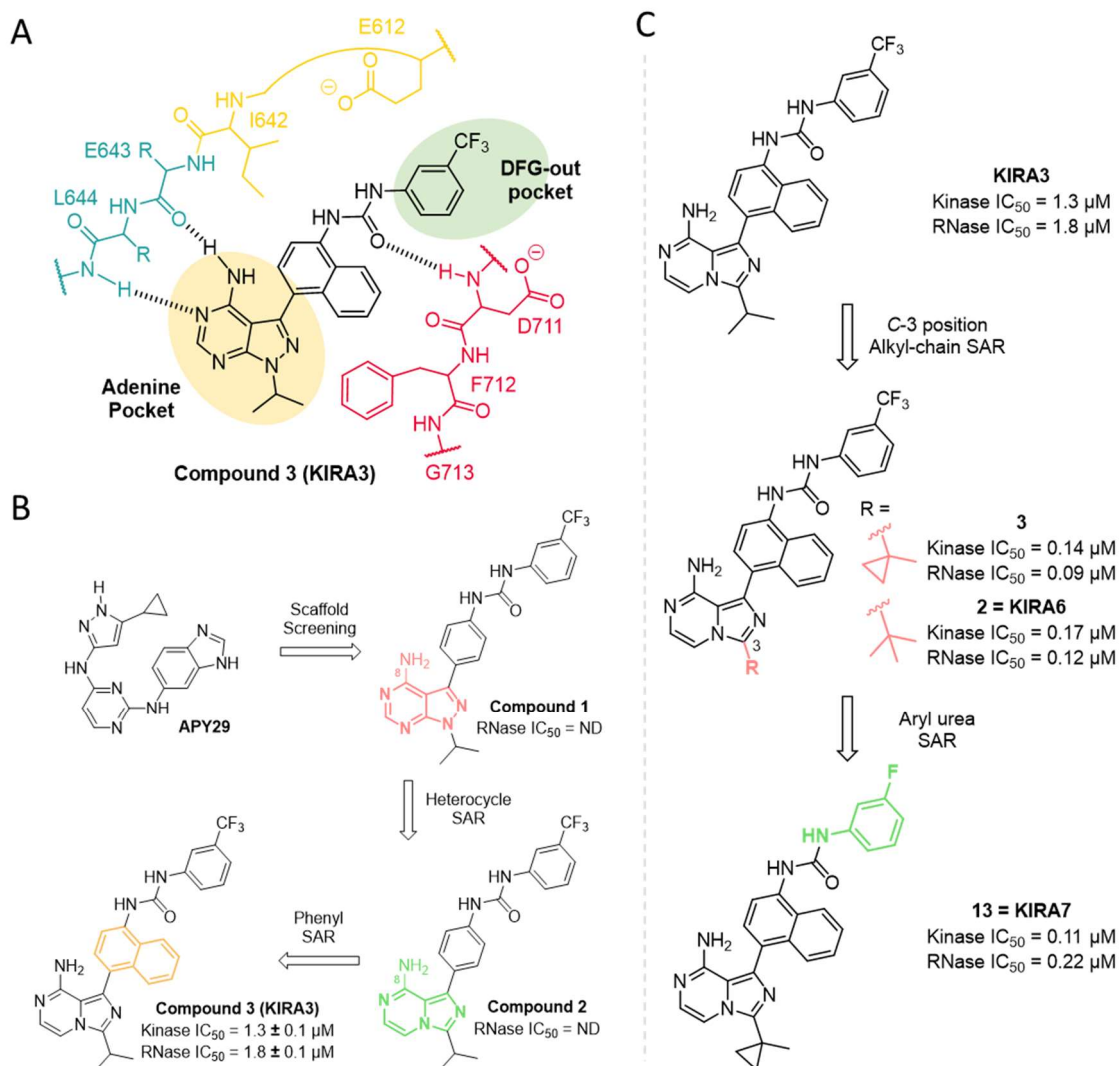
275 activity. Substituents not fitting the front pocket revert back to filling the back pocket,
276 thus breaking the Lys-Glu bridge and displacing the α C-helix and acting as a switch from
277 allosteric activation to inhibition. The most potent activator identified in the SAR study
278 was the but-2-ynamide-substituted G-7658 (Figure 4E), which was successfully co-
279 crystallized (PDB ID 6W3A) and showed near perfect overlap with G-1749. The upward
280 conformation of the activation loop upon occupation of the front pocket prompted the
281 authors to investigate the influence of its phosphorylation on ligand potency. By using
282 precise phosphorylated IRE1 constructs in a combination of techniques and assays, the
283 authors were able to demonstrate that the ability of G-1749 and its analogues to
284 allosterically activate the RNase is highly dependent on the phosphorylation state of
285 IRE1. In particular, they could pinpoint the interaction between pSer729 and Arg687 as
286 a central component to this modulation switch. This contact rigidifies the activation loop
287 in an extended and inactive configuration, thus making the conformational changes
288 needed for ligand-induced activation energetically unfavorable. To sum up, this
289 structurally driven study by Ferri *et al.* provides crucial insights into the kinase-RNase
290 interdomain regulation happening within IRE1 upon ligand-binding by allosteric
291 regulators and finally gives the tools to explore IRE1 activation strategies in a variety of
292 diseases, and in particular cancer [49].

293

294 **IRE1 ATP-competitive inhibitors**

295 ***Imidazo[1,5-a]pyrazin-8-amine based inhibitors: KIRA3, KIRA6 & KIRA7***

296 By observing the binding mode of ATP-competitive type I inhibitor APY29 and its
297 allosteric role in activating IRE1 RNase function, Wang *et al.* hypothesized that
298 development of type II inhibitors that stabilize the IRE1 kinase domain in an alternative
299 inactive conformation could result in the opposing effect on the endoribonuclease
300 activity, thereby fully inhibiting the dual activities of IRE1 [57]. Based on this hypothesis,
301 screening was carried out with a variety of type II kinase inhibitors known in the literature
302 to induce a DFG-out motif. Only one ligand, compound **1**, showed inhibitory activity in
303 the FRET-based RNase assay used for the screening. It was predicted, using Src co-crystal
304 structure, that the pyrazolopyrimidine motif of **1** could display similar interactions as
305 APY29 with the IRE1 hinge motif (Figures 5A & 5B), but its conformation in the binding
306 site has been shown to stabilize the DFG-out conformation [58].



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Figure 5. A. Proposed binding mode of compound 3 in *h*IRE1 kinase pocket in the absence of a crystal structure (color coding as in Figure 2; H-bonds as dashed dark gray lines) B. Discovery of Compound 1 from APY29 by scaffold screening and following structural optimizations through SAR studies that led to compound 3. Structural changes highlighted in colors with bold bonds C. Structural optimizations through SAR studies of the C-3 position and aryl urea that led compound 2, 3 and ultimately 13. Structural changes highlighted in colors with bold bonds.

Despite its modest activity at 20 and 60 μM concentrations, compound 1 was used as a starting point for SAR and most of the modifications (undisclosed) did not give positive results except for the replacement of the 1*H*-pyrazolo[3,4-*d*]pyrimidine scaffold by an imidazo[1,5-*a*]pyrazine core, yielding compound 2 (Figure 5B). Two hydrogen bonds are formed with the hinge region (Glu643 to Cys645); between the aromatic amine of 1 and the carbonyl of Glu643, and between a nitrogen of the pyrimidine part of the

322 imidazopyrazine core with the next peptide bond between Leu644 and Cys645. The rest
323 of the imidazopyrazine core occupies the adenine pocket. Moreover, replacing the 4-
324 anilino group with a naphthylamine group in the C-3 position provided the most potent
325 ligand **3** (Figure 5A).

326 According to the authors model, the naphthyl group can adopt an orthogonal
327 conformation with the imidazopyrazine moiety. The naphthyl rings stacks against the
328 Ile642 gatekeeper residue. The hydrophobic pocket, created by DFG-in/out movement of
329 the Phe712, is filled by the *meta*-trifluoromethylphenyl substituent. Compound **3** has an
330 inhibition capacity on the kinase activity $IC_{50} = 1.3 \mu\text{M}$ and $1.8 \mu\text{M}$ on RNase activity.
331 In line with their starting hypothesis, the authors also demonstrated through the study of
332 alkylation rates of three exposed cysteine residues (Cys572, Cys645 and Cys715), in the
333 presence or absence of ligand, that **APY29** and **3** share the same binding site, despite
334 exerting opposing effects. Unlike the other two cysteines, Cys645 is highly shielded from
335 the alkylating agent in both cases due to its direct interactions with the ligands.
336 Additionally, it was observed that the two inhibitors exert opposing effects on the
337 accessibility of Cys715, located on the activation loop of IRE1 near the C-terminal DFG-
338 motif. Compound **3** increased the rate of alkylation whereas **APY29** decreased it. In
339 consequence, the activation loop will adopt different stabilized conformations. To
340 validate their binding model, the authors generated two closely related analogues of **3**,
341 aptly named **4** and **5**, bearing key structural modifications expected to make them inactive.
342 Compound **4** was *N*-methylated on 8-position which would disrupt the hydrogen bond
343 interaction with Glu643's carbonyl. Regarding **5**, the urea connecting the naphthyl and
344 the trifluoromethylphenyl group was replaced by an amide function, intended to reduce
345 hydrogen bonding with the DFG motif and the favorable interactions with the
346 hydrophobic pocket created by its movement (Figure 5A). Consistent with their
347 predictions, both **4** and **5** showed a loss of potency in the RNase assay. With this study,
348 the authors demonstrated the possibility to inhibit RNase activity of IRE1 with type II
349 kinase inhibitors and gave keys to generate more potent compounds. Compound **3**, later
350 referred as **KIRA3** in recent literature, served as the starting point for further optimization
351 studies.

352 Four years after the initial report on the discovery of compound **3**, Feldman *et al.*
353 published a follow-up paper [59] in which a series of derivatives, named KIRAs for
354 **K**inase **I**nhibiting **R**Nase **A**ttenuators, were developed and tested to provide insight into
355 the allosteric relationship between the kinase and RNase domains (Figure 5C). In

356 particular, a SAR study was conducted to better understand how specific interactions
357 between the inhibitor and the ATP-binding site regulate IRE1 RNase activity. In the
358 absence of structural data, four series of analogues were synthesized for evaluation using
359 **KIRA3** as a benchmark. Firstly, a modification of alkyl groups R₁ was done in the C-3
360 position of the imidazopyrazine group. Compared to KIRA3, both kinase and RNase
361 activities were better for all these analogues. In particular, replacement of the isopropyl
362 group with *tert*-butyl (**2**) and cyclopropylmethyl (**3**) improves the kinase IC₅₀ from 1.3
363 μM (**1**) to 0.17 μM for **2** and 0.14 μM for **3**. Regarding RNase IC₅₀, this modification of
364 the alkyl side chain allowed a 15-fold decrease for **2** and 20-fold for **3** settling down to
365 0.12 μM and 0.09 μM, respectively. The cyclopropylmethyl was deemed the most
366 optimal alkyl substituent and retained for the continuation of the study. The second series
367 of modifications investigated the replacement of the naphthyl group with various
368 substituted phenyl rings. None of these new analogues showed improved potencies
369 compared to **3**. Only compound **7** with a 3-fluorophenyl as R₂ group maintained sub-
370 micromolar activities, but still lower by a factor of 6 and 4 in the kinase and RNase assays,
371 respectively. As a consequence, the naphthyl group was kept for further modulation.
372 Next, the aryl and urea domains were modified (series 3 and 4) to evaluate their
373 importance on the stabilization of the DFG-out motif and on the ensuing allosteric
374 inhibition of the RNase domain. These targeted modifications were motivated by the
375 assumption that the aryl-urea moiety found in all KIRAs is thought to be responsible for
376 inducing a DFG-out conformation of the kinase upon binding. The aryl group could then
377 occupy a newly created hydrophobic pocket formed by displacement of the αC-helix and
378 the Phe712 side chain. This is supported by the fact that this pharmacophore is found in
379 many type-II inhibitors [60] and is known to stabilize the inactive DFG-out conformation
380 [61]. Eleven diverse 3- and 4-substituted phenyl groups were accepted and tolerated, but
381 only the 3-fluorophenyl substituted compound **13** showed equivalent potencies to **3**; the
382 other twelve analogues wavering around 0.30/0.35 μM for kinase IC₅₀ and 0.20/0.30 μM
383 for RNase IC₅₀. The authors have also shown the possibility to incorporate a 4-
384 methylpiperazin-1-yl group, a part of a clinically approved drug imatinib, on position 4
385 of the aryl group without loss of potency. Analogues bearing substituents like benzyl,
386 heteroaryl or cycloalkyl instead of aryl were poor inhibitors, with smaller rings showing
387 the most important decreases in RNase potency, which might be due to only partial
388 displacement of the DFG motif and αC-helix. Finally, the crucial role of the urea linker
389 for allosteric inhibition of the RNase activity *via* the formation of hydrogen bonds was

390 confirmed, since masking of one the *N*-H with a methyl group induced a ~200-fold loss
391 in potency, and full loss when both hydrogens were hidden in an imidazolidinone group.
392 At the end of the study, two products were considered interesting in regards to their kinase
393 and RNase IC₅₀s, compounds **7** and **13**, later renamed **KIRA6** and **KIRA7**, respectively.
394 In cells, **KIRA6** demonstrated good allosteric inhibition of the IRE1 RNase activity and
395 promoted cell survival under ER stress [62]. **KIRA7** has been shown to decrease terminal
396 UPR signaling and prevents fibrotic effects in mice lungs [63].

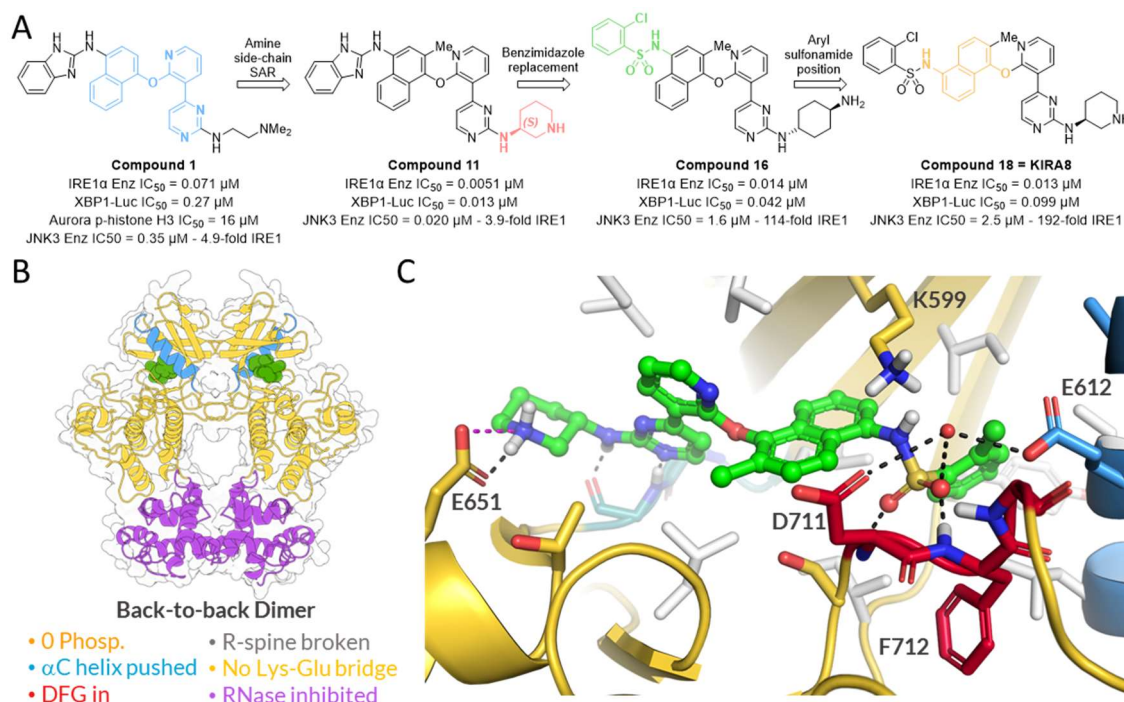
397

398 ***Pyrimidyl-pyridyloxy-naphtyl sulfonamide: KIRA8***

399 Dissatisfied with the potency and/or selectivity of the available IRE1 inhibitors at the
400 time, Harrington *et al.* [64] performed a high-throughput screening of the Amgen small-
401 molecule library to find better molecules with preclinical profile and that could be used
402 as a tool to validate that tumor cells with a high protein synthesis burden would be driven
403 to apoptosis in the absence of this survival pathway. The primary screening consisted of
404 an *in vitro* XBP1 mRNA stem-loop cleavage assay to identify IRE1 RNase inhibitors,
405 regardless of the binding site, and a cellular XBP1-luciferase assay was used as a
406 secondary readout. Selectivity against JNK3 kinase, structurally close to IRE1, was also
407 monitored. Compound **1**, a pyrimidinyl-pyridyloxy-naphtalene bearing a benzimidazole
408 moiety (Figure 6A) was identified through this HTS campaign as an IRE1 kinase and
409 RNase inhibitor (IRE1 α IC₅₀ = 71 nM, XBP1-Luc IC₅₀ = 270 nM).

410 However, **1** exhibited a moderate kinase selectivity profile with 17 kinases inhibited out
411 of 442 (at 1 μ M, percent of control (POC) < 35), among which figure the Jun and Aurora
412 kinases that could complexify the efforts to validate IRE1 as a target for oncology. The
413 authors thereafter undertook a hit-to-lead strategy on the basis of compound **1** to improve
414 selectivity and RNase inhibition. Using knowledge gained in their previous work on
415 Aurora kinases with similar analogues, a methyl group was first introduced in the *ortho*
416 position of the ether link to lock its rotation. The methylated compound **2** exhibited a 5-
417 fold increase in IRE1 RNase inhibition (IC₅₀ = 14 nM), but most importantly showed
418 increased selectivity against Aurora (IC₅₀ > 50 μ M) and JNK3 (IC₅₀ = 0.71 μ M) kinases.
419 In the meantime, the authors also modulated the amine side chain of the pyrimidine ring
420 of **1** to study its influence on inhibition and selectivity. Replacement of the basic nitrogen
421 of the tertiary amine by an aliphatic isopropyl led to a complete loss of activity and
422 selectivity. Introduction of a hydroxyl group resulted in a 2-3-fold loss in potency towards

423 IRE1 while increasing ten-fold JNK3 inhibition, resulting in a reversal of the compound's
 424 selectivity.



425
 426 **Figure 6.** A. Successive structural optimizations and key intermediates that led to
 427 compound 18 (KIRA8). Structural changes highlighted in colors with bold bonds. B.
 428 Graphical representation of the back-to-back dimer visible in the structure of *h*IRE1 in
 429 complex with compound 18 (PDB ID 6URC). Key structural changes highlighted
 430 underneath. C. Front view of compound 18 in the kinase pocket with key interaction and
 431 residues indicated. Color coding and representation as in Figure 2; red sphere represent
 432 an ordered water molecule bridging interactions with the protein.

433

434 These first results highlighted the need for basic nitrogen to maintain selectivity and good
 435 levels of IRE1 inhibition, later rationalized later by a co-crystal structure (Figure 6B &
 436 6C). Piperidines and cyclohexylamines were next investigated as cyclic surrogates.
 437 Inhibitory activity of racemic methyl-piperidine was nearly similar to 1, despite a lower
 438 selectivity against JNK3 (0.005 μ M vs 0.35 μ M for 1). Interestingly, the (*S*)
 439 stereochemistry was found to be 5-fold more active toward IRE1 than the (*R*) and to
 440 increase selectivity against JNK3. Similar observations were made in the
 441 cyclohexanediamines serie, where the cis-isomer was 4-fold less active but maintained
 442 similar selectivity against JNK3. Together, these results showed the importance of the
 443 stereochemistry, and therefore conformation, of this substituent to preserve potency.

444 Compounds **11** (Figure 6C), bearing simultaneously the (*S*)-3-aminopiperidine and
445 additional methyl on the naphthyl ring yielded excellent IRE1 RNase inhibition ($IC_{50} =$
446 $0.0051 \mu\text{M}$), but was still too active on JNK3 ($IC_{50} = 0.020 \mu\text{M}$). To mitigate the
447 selectivity issue against JNK3, efforts were next turned toward replacement of the
448 benzimidazole ring. Its replacement with a benzoxazole moiety did not have a significant
449 impact on potency or JNK3 selectivity. In contrast, introduction of a phenylsulfonamide
450 resulted in a significantly increase in selectivity (49-fold). Substitution at the ortho
451 position of this arylsulfonamide with an electron donating group (methoxy) had minimal
452 influence, whereas electron withdrawing moieties (nitrile or chloro) resulted in more
453 potent compounds in vitro and in cells. Significant selectivity improvement were also
454 observed, and in particular for the *ortho*-chlorophenyl compound **16** (114-fold, Figure
455 6C).

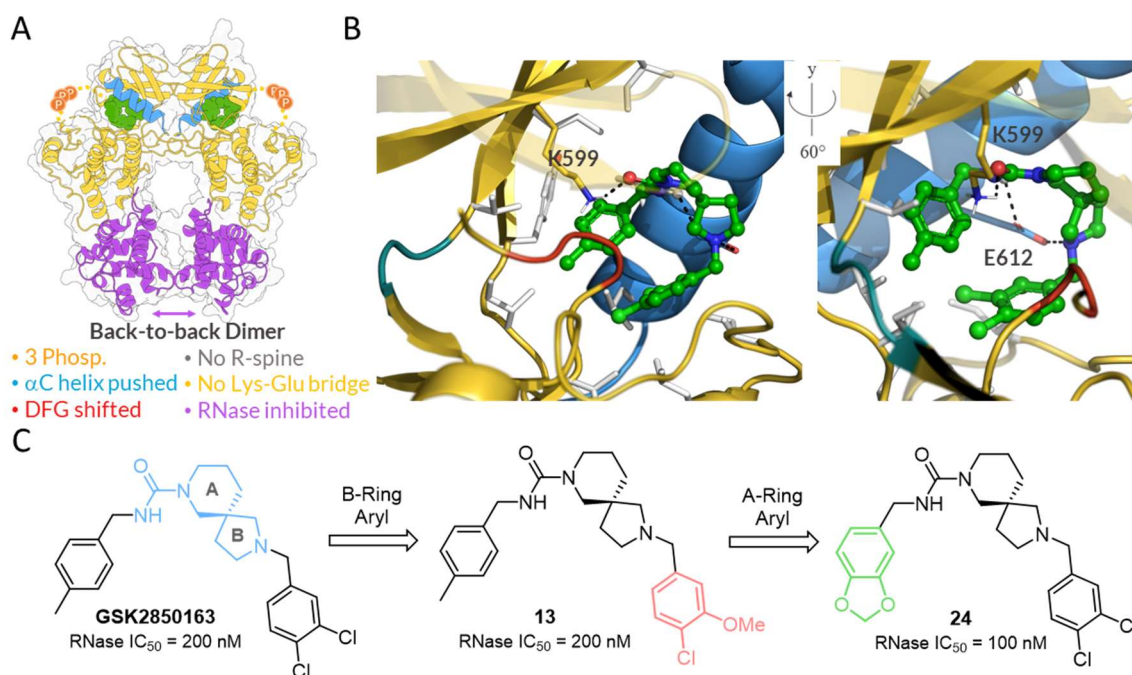
456 The obtention of a co-crystal structure of arylsulfonamide analog **16** bound to
457 dephosphorylated human IRE1 α (PDB ID 4U6R) allowed to identify some critical
458 interactions (Figure 6B). These data revealed that the *ortho*-chlorophenylsulfonamide
459 disrupt the Lys-Glu bridge, pushes outward the αC -helix by around 2 \AA , making way for
460 the aryl ring to wedges itself in a newly formed αC -helix back pocket. The sulfonamide
461 group makes two hydrogen bonds with the backbone *NH* of Asp711 and Phe712 of the
462 DFG motifs, and proximity with Lys599 (3.1 \AA) suggests that the sulfonamide *NH* is
463 deprotonated in the bound state. The crystal structure also rationalized the importance of
464 a controlled stereochemistry observed in cyclohexanediamine and piperidine substituted
465 compounds as these can make a salt bridge with Glu651 if their stereochemistry allows
466 it. Finally, observation of the binding mode of **16** provided insight for further structural
467 optimizations that ultimately led to compound **18**, a potent and most selective candidate
468 of the study with a RNase IC_{50} of $0.013 \mu\text{M}$ and a 192-fold increase in selectivity against
469 JNK3. Compound **18**, later referred as **KIRA8** in the literature, was obtained by
470 displacement the arylsulfonamide group from position 4 to 5 of the naphthyl moiety, and
471 then replacement of the *trans*-cyclohexanediamine side-chain with the (*S*)-3-
472 aminopiperidine to account for the shift in position in the kinase pocket. A crystal
473 structure of KIRA8 in complex with *h*IRE1 (PDB ID 6URC) (Figure 6B & 6C) as
474 obtained by Ferri and coworkers [54] surprisingly showed the protein in a back-to-back
475 dimer, in contrast to the structure obtained with compound **16** that showed IRE1 as a
476 monomer and was coherent with an outward swing of the αC -helix that could prevent
477 dimerization/oligomerization. According to Ferri *et al.*, the dimer observed in the KIRA8

478 structure might be an artefact of the high protein concentration in the crystallization
479 conditions. Finally, KIRA8 represented one of the first lead-like compound targeting
480 IRE1 and as such served as the basis for further structural improvements, either to study
481 the paralog-specific function of IRE1 [65] or to study IRE1 inner activation mechanisms
482 [54].

483

484 **2,8-diazaspiro[4.5]decane: GSK2850163**

485 High-throughput screening of the GlaxoSmithKline compound collection in a
486 phosphorylated IRE1 RNase activity assay led to the identification of GSK2850163, a
487 potent and selective dual inhibitor of the IRE1 kinase ($IC_{50} = 20$ nM) and RNase activities
488 ($IC_{50} = 200$ nM) presenting an original diazaspino scaffold [66]. Subsequent protein
489 crystallography efforts by the authors allowed them to obtain cocrystal structures of a
490 triply phosphorylated IRE1 in complex with the endogenous ADP-Mg²⁺ ligand (PDB ID
491 4YZD), GSK2850163 (PDB ID 4YZ9), and broad kinase inhibitor staurosporine (PDB
492 ID 4YZC), in all cases with IRE1 in a back-to-back dimer conformation (Figure 7A).



493

494 **Figure 7.** A. Graphical representation of the back-to-back dimer visible in the structure
495 of hIRE1 in complex with GSK2850163 (PDB ID 4YZ9). Key structural changes
496 highlighted underneath. B. Right-side and left-side view of GSK2850163 in the kinase
497 site with key interaction and residues indicated. Color coding and representation as in
498 Figure 2. C. Structural optimizations of the B-ring and representation of the most potent

499 analogue, compound 13, followed by A-ring modulation and obtention of 24. Structural
500 changes highlighted in colors with bold bonds.

501

502 These structures revealed GSK2850163 as a type III kinase inhibitor [46] bound in an
503 unusual fashion in the kinase pocket (Figure 7B). In contrast with other IRE1 modulators
504 binding to this domain, GSK2850163 does not make any interactions with the hinge
505 residues (Glu643; Lys644; Cys645) and actually sits ~12 Å away near the α C-helix. This
506 binding event disrupt the conserved salt bridge between the α C-helix Glu612 and active
507 site Lys599, which is needed to coordinates the α - and β -phosphate groups of the ATP
508 molecule [67], thereby locking α C-helix in an inactive ‘out’ conformation. Ligand
509 binding also pushes outward the DFG motif and disordered kinase activation loop by
510 nearly 180° (further confirmed by HDX experiments). This results in the DFG motif
511 occupying the ATP-binding site while GSK2850163 lies in the hydrophobic pocket
512 previously occupied by Phe712 of the DFG motif. There, the ligand adopts a U-shaped
513 conformation and interactions with the side chains of Glu612 and Lys599, a key catalytic
514 residue, are described. Surprisingly, the nitrogen of the pyrrolidine ring of the 2,8-
515 diazaspiro[4.5]decane is unprotonated in the original structure, despite being its most
516 likely state at physiological pH and yielding an additional salt bridge with Glu612. The
517 likely presence of a π -cation interaction between the tolyl group of GSK2850163 and the
518 amine of Lys599 is also not discussed. Of note, only the *R*-enantiomer fitted the ligand
519 electronic density and showed RNase inhibitory activity. Binding of GSK2850163 in the
520 kinase back pocket is also observed to induce conformational changes throughout IRE1,
521 resulting in an altered dimer interface and notably, an increased distance (~4 Å) between
522 the RNase domains that would, together, disrupt its mRNA splicing ability.

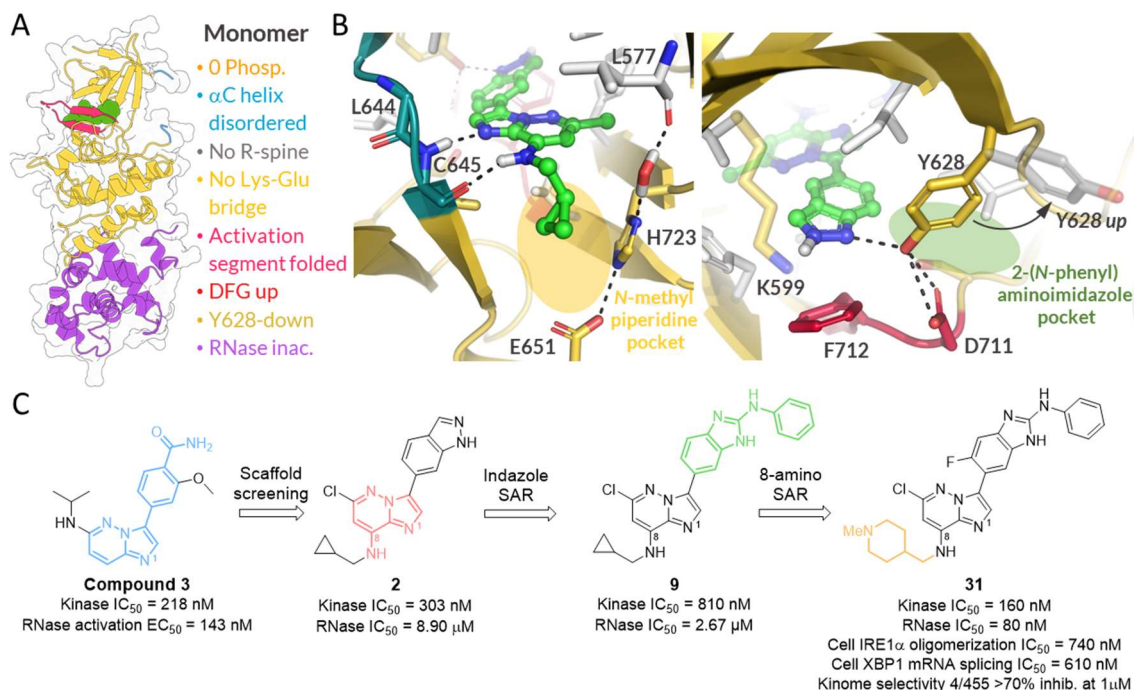
523 To support the observed binding mode and improve further the ligand, concise SAR
524 studies were carried out (Figure 7C), starting with the modulation of the NH of the urea
525 and pyrrolidine nitrogen to investigate the importance of their hydrogen bonds with the
526 carboxylic group of Glu612. Both proved to be critically important to maintain the
527 compound’s potency, with any change altering their hydrogen bonding capabilities
528 drastically reducing RNase IC_{50s}. Next, the authors focused on modulating the two aryl
529 moieties connected to each ring of the 2,8-diazaspiro[4.5]decane core, coined A and B,
530 and that are nicely accommodated by the lipophilic environment of the pocket (Figure
531 7B). Substitution of the 3,4-dichlorophenyl group connected to the pyrrolidine B-ring by

532 a more polar aromatic ring such as a pyridine was not tolerated. Interestingly, individual
533 removal of the chlorine atoms revealed that the 4-chlorobenzyl analogue has an IC₅₀ only
534 two times higher, 400 nM, highlighting the importance of the *para* position over the *meta*.
535 Based on this observation, methyl and trifluoromethyl substituents were tried on this
536 position but did not yield improvements. Modulation of the *meta* position with small
537 lipophilic functional groups (-F, -CH₃, -OCH₃) was overall well tolerated and afforded
538 equally potent ligands, with the exception of the bulkier trifluoromethyl group which
539 showed loss in potency. Similar to what was observed with the B-ring aryl, replacement
540 of the tolyl group attached to the A-ring urea by more polar pyridines led to a 30-to-60-
541 fold loss in potency. Deletion or replacement of the *p*-methyl group by small lipophilic
542 functions (-Cl, -CH₃, -OCH₃) afforded compounds with similar potency, but none better
543 than the original hit. Lastly, the authors conclude their SAR study by presenting **24**, a
544 benzo[*d*][1,3]dioxole analogue, which exhibit an RNase IC₅₀ of 100 nM, a moderate two-
545 fold improvement over GSK2850163. This study successfully identified a novel and
546 potent dual IRE1 kinase/RNase inhibitor and characterized precisely its mode of action
547 through a combination of X-ray protein crystallography and HDX experiments. Ligand
548 optimization proved to be challenging, as demonstrated by the limited success of their
549 SAR. To conclude, despite its unique binding mode among IRE1 modulators, its excellent
550 kinase selectivity, nanomolar potency, and efficacy to in multiple myeloma cell lines, this
551 class of compounds was not developed further, nor patented, by Glaxo Smith Kline for
552 undisclosed reasons.

553 ***Imidazo[1,2-*b*]pyridazin-8-amine: Compound 31***

554 Following up on their previous work on **Compound 3**, an IRE1 type I kinase
555 inhibitors/RNase activator based on a 4-(imidazo[1,2-*b*]pyridazin-3-yl)benzamide
556 scaffold, the Collins group recently reported the discovery of close structural analogues
557 based on the imidazo[1,2-*b*]pyridazine-8-amine motif and capable of inhibiting the dual
558 IRE1 activity (Figure 8) [56]. A scaffold screening on **Compound 3** led to the discovery
559 of an analog, **2**, inhibitor of both the kinase (IC₅₀ = 303 nM) and RNase functions (IC₅₀ =
560 8.90 μM), suggesting a different allosteric effect on the RNase compared to typical type I
561 IRE1 kinase inhibitors [68]. A crystal structure of **2** bound to IRE1 was obtained by
562 compound soaking in IRE1-apo crystals and showed unusual structural changes
563 throughout the IRE1 kinase domain (Figure 8A) (PDB ID 6HX1). Strong disordering of
564 the αC-helix was observed, accompanied by an unusual folding of the first half of the

565 activation segment (res. 711- 726, [69]) in an antiparallel double β -strand, thus enclosing
 566 the ligand in a tight pocket. Most notably, the DFG motif was in an original intermediate
 567 conformation, termed DFG-up by the authors, and the Tyr628 residue normally in an up
 568 conformation is pointing downward. Looking at the ligand, the imidazo[1,2-*b*]pyridazine
 569 *N*-1 and 8-NH makes hydrogen bonds with the respective functions *NH* and CO of the
 570 hinge residue Cys645 (Figure 8B). An additional H-bond between the indazole *NH* and
 571 Tyr628 is also observed.



572
 573 **Figure 8.** A. Graphical representation of the monomer visible in the structure of *hIRE1*
 574 in complex with compound **2** (PDB ID 6HX1), precursor of compound **31**. Key structural
 575 changes highlighted on the right-side. B. Left-side and right-side view of compound **2** in
 576 the kinase site. Key interactions and residues indicated as well as the likely position of
 577 the *N*-methyl piperidine (tan ellipse) and 2-(*N*-phenyl)aminoimidazole (green ellipse)
 578 moieties of **31**. Color coding and representation as in Figure 2. C. Structural optimizations
 579 of compound **3** through scaffold screening and SAR studies on the indazole moiety and
 580 aromatic amine in C-8 that led to lead compound **31**. Structural changes highlighted in
 581 colors with bold bonds.

582
 583 Moreover, this compound was screened on the large Discover X KinomeScan panel and
 584 revealed an already significant selectivity with >70% inhibition of only 26/455 wild-type
 585 and mutant kinases. To optimize the inhibition, the role of each substituent was
 586 investigated by carrying out a structure-activity relationship around **2** (Figure 8C). In

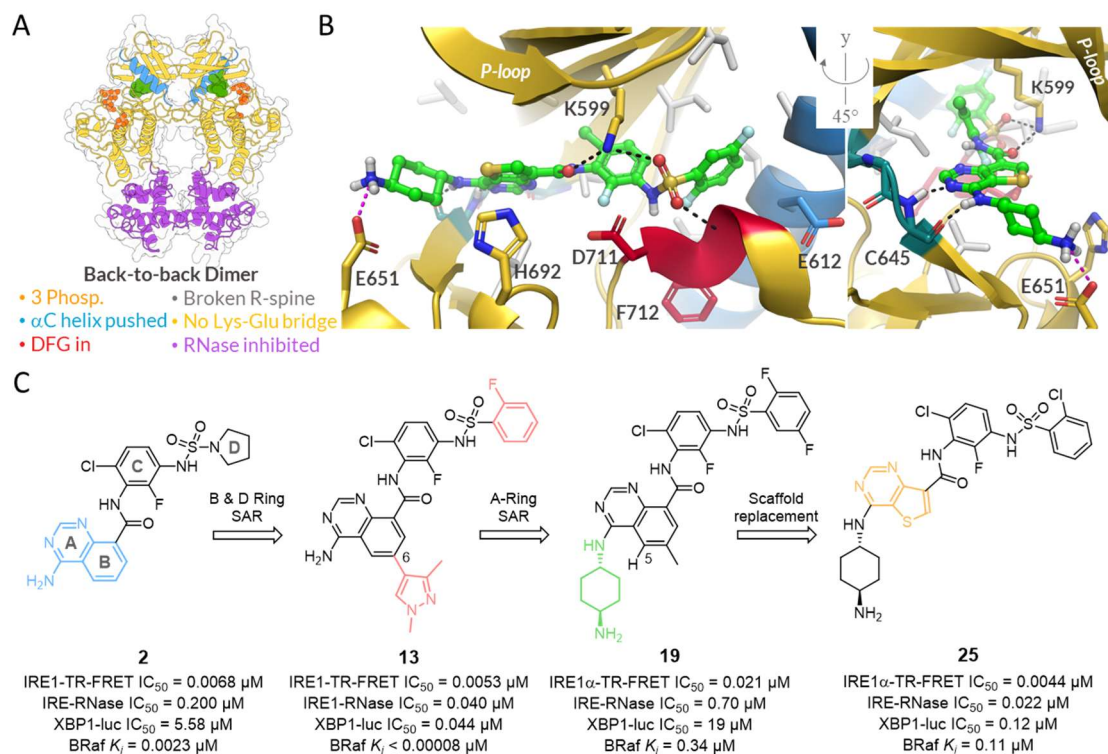
587 particular, the indazol-5-yl substituent was replaced by several heterocycle scaffolds. The
588 *N*-Methylated as well as the regioisomer (1*H*-indazol-4-yl) analogues showed the
589 importance of the 1-*H*-indazol-5-yl group to maintain the inhibitory activity. The
590 *N*-unsubstituted benzimidazole-5-yl analog exhibited a weaker kinase activity ($> 10 \mu\text{M}$)
591 and no RNase inhibition. In spite of these results, further substitution of the benzimidazole
592 at the C-2 position were explored. In particular, the (*N*-phenyl)-2-aminobenzimidazole
593 motif was envisaged as being an isostere for the diaryl urea motif which is commonly
594 found in type II kinase inhibitors [52]. Compared to compound **2**, the 2-(*N*-phenyl)amino
595 benzimidazole **9** had a similar kinase activity ($\text{IC}_{50} = 810 \text{ nM}$) but improved IRE1 RNase
596 activity ($\text{IC}_{50} = 2.67 \mu\text{M}$). *N*-Alkylation at any position of this substituted benzimidazole
597 framework (N1, N3 and NHPH) drastically reduced the potency ($>10 \mu\text{M}$), indicating that
598 the 2-(*N*-phenyl)aminoimidazole functionality is probably involved in multiple hydrogen
599 bonds. Finally, the replacement of the 8-(*N*-cyclopropylmethyl)amino substituent on the
600 imidazo[1,2-*b*]pyridazine nucleus by different 1,4-diaminocyclohexanes or cyclic amines
601 was investigated. The dual kinase-RNase inhibition was maintained or enhanced by the
602 additional presence of polar groups, but best aqueous solubility was obtained by
603 integrating the ((*N*-methylpiperidin-4-yl)methyl) amine function. One of the most active
604 compounds, **31**, exhibited nanomolar potency for biochemical inhibition of the kinase
605 ($\text{IC}_{50} = 160 \text{ nM}$) and RNase ($\text{IC}_{50} = 80 \text{ nM}$) function. The high selectivity for IRE1 over
606 other kinases was further improved ($>70\%$ inhibition of 4/455). It was speculated that this
607 high kinome selectivity of **31** could be the result of the unusual IRE1 kinase binding mode
608 displaying multiple structural disruptions. These findings further extend the knowledge
609 on IRE1 kinase site binding modulators and help pave the way towards the discovery of
610 new highly selective and cell-active inhibitors.

611

612 ***Amino-Thienopyrimidines: Compound 25***

613 From a high throughput screening of a 92K compounds library, Beveridge *et al.* recently
614 identified compound **2**, an amino-quinazoline B-Raf kinase inhibitor (B-Raf $K_i = 0.0023$
615 μM), as a promising IRE1 inhibitor [55]. It demonstrated potent ATP-competitive IRE1
616 binding affinity (IRE1-TR-FRET $\text{IC}_{50} = 0.0068 \mu\text{M}$), but modest potency on IRE1
617 endoribonuclease activity in both an enzymatic RNase inhibition assay (IRE1-RNase IC_{50}
618 $= 0.200 \mu\text{M}$) and a cellular X-box binding protein-1 splicing luciferase reporter assay
619 (XBP1-luc $\text{IC}_{50} = 5.58 \mu\text{M}$). Obtention of a co-crystal structure of **2** bound to IRE1 (PDB

620 ID 6XDF) lifted the veil on the most predominant ligand interactions within the ATP
 621 pocket and some unique structural features of the complex (Figure 9A).



622
 623 **Figure 9.** A. Graphical representation of the back-to-back dimer visible in the structure
 624 of *h*IRE1 in complex with compound 25 (PDB ID 6XDD). Key structural changes
 625 highlighted underneath. B. Front and left-side view of compound 25 in the kinase site.
 626 Key interactions and residues indicated. Color coding and representation as in Figure 2.
 627 C. Structural optimizations of compound 2 identified in a HTS campaign through SAR
 628 studies on ring A, B and D and finally scaffold replacement by a thienopyrimidine core,
 629 yielding compound 25. Structural changes highlighted in colors with bold bonds.

630
 631 These crucial interactions include classical type I/II hinge contacts with the amino-
 632 quinazoline A-ring, strong hydrogen bonding from the sulfonamide to Asp711 and
 633 Phe712 of the DFG-motif, and occupation of the α C-helix pocket by pyrrolidine D-ring,
 634 thus breaking the Glu612-Lys599 bridge and pushing outward the α C-helix (Figure 9B).
 635 Other noteworthy features were the existence of a pseudoring by intramolecular hydrogen
 636 interaction between the amide group and one nitrogen of the quinazoline core, and, in
 637 contrast to the reference ligand **KIRA8**, no ionic interaction with Glu651. Guided by
 638 these observations, the authors conducted several SAR studies on targeted position of **2**
 639 aimed at improving potency for IRE1 while reducing B-Raf kinase binding affinity.

640 While searching for D-ring analogues within an in-house chemical library to explore their
641 potential impact on α C-helix shift, the authors serendipitously discovered that a methyl
642 substitution in position C-6 on the quinazoline B-ring improved cellular *XBPI* splicing
643 inhibition by a \sim 10-fold factor. This result, combined with the close proximity of the
644 imidazole ring of His692 (\sim 4-5 Å from the quinazoline 6-position) in an unexplored polar
645 pocket encouraged the authors to target this residue. Five and six-membered heterocycles
646 (pyridines and pyrazoles) were thus introduced to interact *via* π - π stacking or hydrogen
647 bonding. The novel analogues synthesized also incorporated chloro- or fluoro-phenyl
648 sulfonamides D-rings, chosen to replace the original pyrrolidine. This first B-ring and D-
649 ring SAR study led to compound **13** (Figure 9C), showing a 125-fold improvement in the
650 XBP1 luciferase assay compared to the initial hit **2**, and for which a second crystal
651 structure was solved (PDB ID 6XDB). In this structure, the 1,3-dimethyl-1*H*-pyrazol-4-
652 yl group is engaged in a hydrogen bonding interaction with the imidazole of His692, as
653 designed, and two weaker C-H hydrogen bonds between the two methyl groups and
654 Leu577 and His579 are also observed. Unfortunately, these modifications also
655 significantly increased the binding affinity towards the B-Raf kinase.

656 To overcome this, the non-conserved residue Glu651 in IRE1 (His539 in B-Raf) was
657 targeted to gain in selectivity, a proven strategy for IRE1 inhibitors [64]. Some basic
658 amino groups were selected for generating an ionic interaction with Glu651, while
659 expected to clash with His539 in B-Raf. These novel modulations resulted in selection of
660 the *trans*-diaminocyclohexane containing compound **19** (Figure 9C) which, although
661 exhibiting reduced IRE1-TR-FRET binding potency ($IC_{50} = 0.021 \mu\text{M}$) and XBP1-luc
662 inhibition ($IC_{50} = 19 \mu\text{M}$), also showed a strong drop in B-Raf binding potency (B-Raf K_i
663 = $0.34 \mu\text{M}$). Encouraged by these results and to explain the reduced activities observed,
664 the authors turned their attention toward the effects of the *trans*-diaminocyclohexane on
665 the ligand's predicted bioactive conformation interacting with the hinge backbone.
666 Torsional analysis allowed them to identify a steric clash between the *trans*-
667 diaminocyclohexane and aromatic C-H of the quinazoline ring system. The structurally
668 related thienopyrimidine core - predicted to have a strongly reduced strain energy - was
669 selected for further investigations. A set of amino substituted thienopyrimidines were
670 synthesized and their activities were compared to their quinazoline analogues. This
671 extensive SAR study allowed, at the end, to identify compound **25**, exhibiting good IRE1-
672 XBP1 cellular potency and a \sim 50-fold decrease in B-Raf K_i potency, as a promising lead
673 for further optimization. From this last series, the authors were able to obtain a crystal

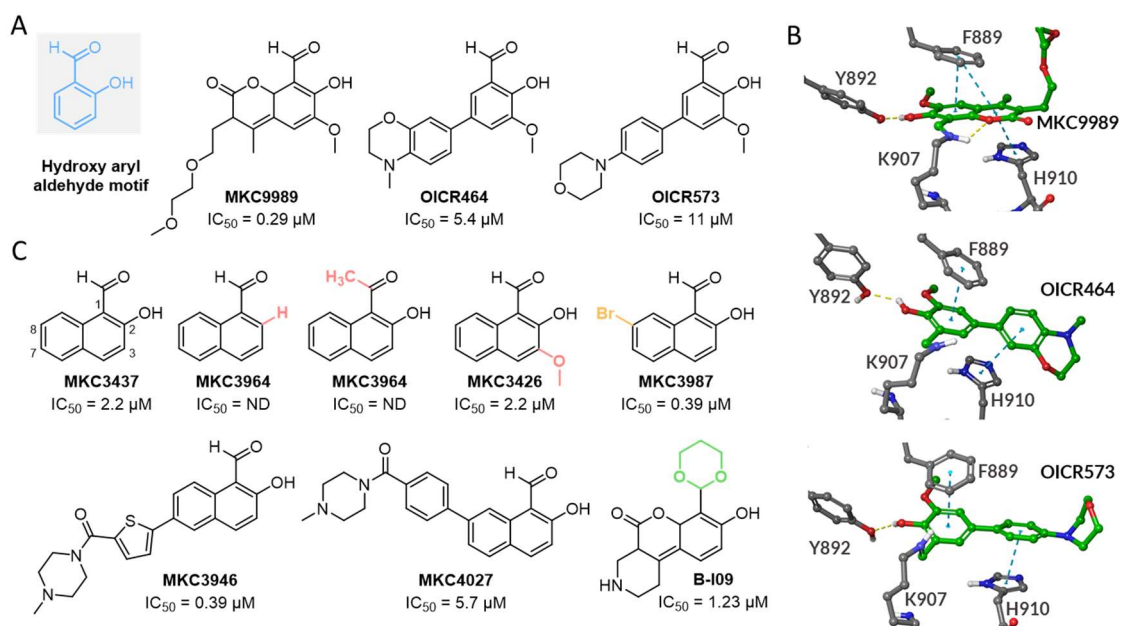
674 structure of compound **23**, a close analogue of compound **25**, in complex with IRE1
675 (Figure 9B.) (PDB ID 6XDD) which allowed to confirm the establishment of a salt bridge
676 between the *trans*-diaminocyclohexane and Glu651 as a strategy to improve IRE1
677 selectivity over B-Raf.

678

679 **IRE1 RNase domain covalent ligands**

680 ***Hydroxy aryl aldehydes: MKC, OICR, 4μ8c, B-I09***

681 An alternative class of IRE1 modulators was identified during high-throughput screening
682 attempts[70–72], that exhibit a different mode of action. This class of inhibitors shares a
683 common hydroxy aryl aldehyde (HAA) moiety (Figure 10, A.) which was later
684 demonstrated to covalently reacts with a specific nucleophile residue (Lys907) within the
685 RNase domain of IRE1, resulting in the formation of a stable imine bond through a Schiff
686 base reaction. The HAA modulators efficiently inhibit the RNase activity of IRE1 and
687 thus prevent the ER stress-induced *XBPI* mRNA cleavage. Salicylaldehyde analogues
688 were the first group of HAA inhibitors, discovered by Volkmann *et al.* in 2011 [70]. They
689 found that salicylaldehyde analogues were active in inhibiting the site-specific cleavage
690 of several mini-*XBPI* stem-loop RNAs in a dose-dependent manner. This class of
691 compounds was also capable of blocking the transcriptional up-regulation of known
692 *XBPI* targets as well as mRNAs targeted for degradation by IRE1. Kinetic analysis
693 showed that 3-ethoxy-5,6-dibromo-salicylaldehyde, a potent salicylaldehyde analog,
694 acted as a non-competitive inhibitor to the *XBPI* mRNA substrate with an $IC_{50} = 0.12$
695 μ M. Another salicylaldehyde analog, 3-methoxy-6-bromo-salicylaldehyde, heavily
696 inhibited the *XBPI* splicing in an *in vivo* assay ($IC_{50} = 0.41 \mu$ M) of acute ER stress. The
697 salicylaldehyde analogues potent for *hIRE1* were significantly less active for *yIRE1* and
698 inactive for murine IRE1. Different possibilities for the mode of action of the
699 salicylaldehyde analogues were proposed, but it could not be determined with certainty
700 whether the binding occurred near the RNase domain or at a new, distant, allosteric site.



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Figure 10. A. Molecular structures and B. binding modes of three HAA inhibitors (MKC9989, OICR464, and OICR573) identified and co-crystallized with murine IRE1 (PDB-IDs 4PL3, 4PL4, and 4PL5, respectively) [3]. The blue and yellow dashed lines in panel B. represent π - π stacking and hydrogen bond interactions, respectively. C. SAR analysis using a series of HAA analogues around a naphthalene scaffold [7,8]. IC_{50} values are related to RNase inhibition of murine IRE1.

4-methyl umbelliferone 8-carbaldehyde (**4 μ 8C**) was the first dual-ring inhibitor in the HAA class, identified by Cross *et al.* [71] among a collection of 238,287 pure compounds. An *in vivo* IC_{50} = 4.6 μ M was recorded for the compound in parallel concentration-response analyses of inhibition of XPB1 splicing. They proposed a covalent mode of action of **4 μ 8C** by the fact that the inhibitor demonstrated non-competitive inhibition. Furthermore, the significance of the aldehyde moiety in the **4 μ 8C** inhibitor was confirmed by the observation that the primary alcohol, the product of **4 μ 8C** reduction by treatment with sodium borohydride, was completely inactive. Structural activity relationship analysis of **4 μ 8C** indicated that substitution of the *ortho* hydroxyl in **4 μ 8C** and related compounds abrogated the inhibition. By tracking the **4 μ 8C** reactivity with IRE1, they could identify a highly selective residue, Lys907, in the RNase active site of which the aldehyde moiety reacts with the amine side chain and forms a stable Schiff base. Shortly after, Sanches *et al.* [72] solved three crystal structures (PDB IDs 4PL3, 4PL4 & 4PL5) of murine IRE1 in complex with three HAA inhibitors: **MKC9989**, **OICR573**, and **OICR464** (Figure 10A) that confirmed the binding site and Schiff base formation. These

724 HAA inhibitors potently inhibit the RNase activity of murine and *h*IRE1, and to a lesser
725 degree *y*IRE1 *in vitro*, with IC₅₀ values ranging from 0.23 to 44 μM, while they display
726 no or only weak effect on the auto-phosphorylating activity of IRE1 even at the highest
727 concentrations tested. They found that the potency of the three new HAA inhibitors
728 significantly depended on the pre-incubation time with IRE1, which was interpreted as
729 being linked to differences in reactivity of the aldehyde group in each HAA scaffold in
730 the Schiff base formation with Lys907. In all three solved crystal structures, IRE1 was
731 crystallized in the same face-to-face dimer form similar to *h*IRE1 [73], and not the back-
732 to-back dimer [74] or oligomer configuration adopted by *y*IRE1[22]. **MKC9989**,
733 **OICR573**, and **OICR464** all shared the same binding modes within a shallow
734 hydrophobic pocket in the RNase domain, surrounded by helices α3', α4, and α5 and
735 residues Leu886, Phe889, Tyr892, Lys907, Glu913, Leu914, and Pro915 (Figure 10B):
736 (1) a Schiff base interaction between the aldehyde moiety of the HAA and the amine side
737 chain of Lys907; (2) a strong hydrogen bond interaction between the *ortho* hydroxy group
738 of the HAAs and the side chain of Tyr892; (3) π-π stacking of the Phe889 side chain and
739 the apical aromatic ring of the HAAs; and (4) π-π stacking of the His910 side chain and
740 the basal aromatic ring of the HAAs. Mutational analysis revealed that any mutation
741 placed on the residues Phe889, Tyr892, Asn906, His910, and Lys907 abolished the
742 RNase activity of IRE1 against a single hairpin substrate. Structure activity relationship
743 analysis was also performed using a series of HAA analogues around naphthalene and
744 biphenyl scaffolds [75,76], which offers more substituent sites around the dual fused ring
745 (Figure 10C) [32,77]. Consistent with the previous SAR analysis of single aryl ring HAA
746 inhibitors [71], substitution of either aldehyde or *ortho* hydroxyl moieties, (position 1 and
747 2, respectively, Figure 10C) were not tolerated (**MKC3964** and **MKC3820**), reflecting
748 the significance of the Schiff base interaction with Lys907 and the strong hydrogen bond
749 with Tyr892. Substitution with bulky groups on positions 3 and 7, which are facing the
750 solvent, were well tolerated (**MKC3426** and **MKC3946**). Bromine substitution at
751 position 8 (**MKC3987**) was favored over hydrogen, while the bulkier group in **MKC4027**
752 was predicted to cause steric clashes and thus considered as disfavored.

753 Tang *et al.* [78] introduced a novel set of HAA inhibitors with a tricyclic chromenone
754 scaffold which were able to potently suppress the XBP1 expression and induce apoptosis.
755 They also introduced a lead inhibitor, **B-I09** (IC₅₀ = 1.23 μM), and determined its
756 pharmacokinetics and bioavailability. In **B-I09**, the aldehyde group is protected using 1,3-
757 dioxane as a putative prodrug moiety. Using LC-MS analysis they confirmed that the 1,3-

758 dioxane-protecting group remained after 2-days exposure to a FRET assay buffer and
759 was 50% intact after 1-day incubation in cell culture medium, respectively. Based on
760 these results and the bioactivity of **B-I09** compared to other derivatives, they elucidated
761 that the 1,3-dioxane-protecting moiety enhances the cellular uptake and will be
762 decomposed to expose the bioactive aldehyde group once inside the cell. Additional
763 analogous HAA inhibitors sharing different scaffolds of naphthalene [75,76], single-ring
764 and biphenyl [75], coumarin [79], and chromenone [80] were also identified and proved
765 to be potent inhibitors of IRE1 RNase activity. The mystery behind the high selectivity
766 of HAA inhibitors toward one of the specific Lysine residues (Lys907) among all 23
767 Lysine residues of IRE1 has been deciphered in two recent studies [81,82]. In a first study
768 [82], the interaction of **MKC9989** molecules with two buried Lysine residues (Lys599
769 and Lys907) and two solvent-exposed ones (Lys656 and Lys799) as representative
770 residues of IRE1 (PDB ID 4PL3), was investigated by means of molecular docking and
771 extensive MD simulations. Based on the energetic diagrams and distance analysis of the
772 MD trajectories, it was demonstrated that **MKC9989** is quite stable within the binding
773 site of the two buried Lys907 and Lys599, while the interactions with the water-exposed
774 ones are unfavorable. Since Lys599 is located within the kinase pocket, an endogenous
775 ligand like ATP is expected to strongly compete with the inhibitors in binding to Lys599,
776 which justifies their inactivity in IRE1 kinase inhibition. This was followed by a detailed
777 exploration of the selectivity of the **MKC9989** inhibitor towards residue Lys907 using
778 multiscale *in silico* techniques including Multi-Conformation Continuum Electrostatics
779 (MCCE) simulations, Quantum Mechanics/Molecular Mechanics (QM/MM)
780 calculations, covalent docking, and MD simulations [81]. According to these results,
781 Lys907 is completely deprotonated within its hydrophobic pocket, which is an essential
782 requirement for the initiation of the Schiff base reaction with the aldehyde moiety of the
783 HAA molecules. QM/MM calculations and potential energy surface analysis revealed a
784 spontaneous proton transfer (with ~0 energy barrier) from Lys907 to the adjacent Asp885,
785 consistent with the low pK_a of Lys907 calculated using the MCCE method. Using
786 covalent docking and MD simulations, it was verified that the hydrophobic pocket formed
787 by Lys907 and its neighboring residues could effectively stabilize the **MKC9989**
788 molecule by strong π - π stacking and hydrogen bonding interactions. Furthermore, Radial
789 Distribution Function (RDF) analysis confirmed that the imine bond formed during the
790 Schiff base reaction between Lys907 and **MKC9989** is inaccessible for water molecules
791 and thus the probability of imine hydrolysis is almost zero.

792

793 **Concluding remarks**

794 Here we discussed case by case the structural and molecular bases of reported IRE1 small-
795 molecule modulators and detailed the discovery and structural development of their
796 scaffolds. This overview highlights the significant advances made by the field in a
797 relatively short time (~10 years) in the comprehension of IRE1 activation mechanism and
798 pharmacological targeting of its catalytic sites. In particular, major milestones such as
799 nanomolar potency, excellent kinase selectivity, and more recently exceptional control
800 over the inter-dependent kinase-RNase relationship, have been achieved [54,55,59,64–
801 66,68]. As indicated in the introduction and although not described within this manuscript
802 for the sake of clarity and brevity, numerous new kinase-site binding inhibitors with novel
803 scaffolds were patented since 2017 by pharmaceutical industrial groups & start-ups
804 (Genentech and Paraza Pharma [83–86], Quentis therapeutics [87–94], OptiKIRA [95–
805 99]). This recent increase in publications and patent applications testifies of the dynamism
806 of the field and of the relevance of IRE1 as a therapeutic target. Hopefully, the recent rise
807 in kinase inhibitors development described above will in the near future also translate into
808 more compounds entering the clinics. As highlighted in this review, development of
809 druglike small molecules targeting IRE1 activity has been mostly restricted to its catalytic
810 sites. Nonetheless, a few other ligands and class of compounds have been reported to
811 modulate IRE1 activity through different binding site and/or mode of action [49]. In
812 particular, the naturally occurring flavonol quercetin [100] is worth mentioning as a
813 crystal structure has been solved and showed the natural product to bind at the RNase
814 domain interface of the IRE1 dimer. Targeting the RNase domain's dimer protein-protein
815 interface has recently attracted interest as it has been shown that protomer alignment
816 [101] and oligomerization state [102] can alter substrate specificity. Alternatively, we
817 believe that IRE1 is an ideal candidate to explore a range of novel modalities that arose
818 in medicinal chemistry. IRE1 is known to also exert scaffolding functions with partner
819 proteins, which could be leveraged to reprogram its functions through the modulations of
820 protein-protein interactions (PPIs) involving IRE1. The PPIs formed during the
821 oligomerization process, or with partners such as BiP, HSP47, and the Sigma1 receptor,
822 which fine-tune its activation level, Filamin A, RtcB or IP3 receptors, may represent
823 appealing opportunities to develop PPI inhibitors or stabilizers [103–105]. Other trending
824 modalities of interest are PROTACS (proteolysis targeting chimeras) and other chimeric
825 degrader solutions [106–108]. These have already been applied with success to

826 transmembrane proteins with enzymatic activities similar to IRE1 [109] and the wide
827 range of high-affinity and selective IRE1 ligands available makes PROTACS a
828 compelling option to pursue. Finally, a fragment-based drug discovery (FBDD) program
829 applied to IRE1 could help finding molecular starting points that bind to non-catalytic
830 sites of the protein, which could be extremely valuable in the development of novel
831 allosteric inhibitors or PPI modulators [110–112].

832

833 **Abbreviations**

834 ATF6; activating transcription factor 6; BiP, binding immunoglobulin protein; ER,
835 endoplasmic reticulum; HAAs, hydroxy-aryl aldehydes; HDX, hydrogen-deuterium
836 exchange; IPA, IRE1/PERK Activator; IRE1, inositol-requiring enzyme 1 alpha; PERK,
837 protein kinase R-like ER kinase; KIRAs, kinase inhibiting RNase attenuators; RIDD,
838 regulated IRE1 α -dependent decay; SAR, structure-activity relationship; UPR, unfolded
839 protein response

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841

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851

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853 The authors declare no conflicting interests. EC is founder of Thabor Therapeutics.

854

855 **ASSOCIATED CONTENTS** - 2D molecular structures and SAR schemes have been
856 drawn using ChemDraw Professional 20.0 (RRID:SCR_016768); Binding sites zoom-in
857 pictures were generated using PyMOL 2.2.0 (RRID:SCR_000305); Graphical
858 representation of IRE1 homo-multimers were generated using the Protein Imager online
859 platform [113]; Crystallographic structures were retrieved from the RSCB wwPDB
860 website (RRID:SCR_006555).

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