

## Pharmacological targeting of IRE1 in Cancer

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## **ABSTRACT**

IRE1 $\alpha$  is one of the main transducers of the Unfolded Protein Response. IRE1 $\alpha$  is playing instrumental pro-tumoral roles in several cancers and high IRE1 $\alpha$  activity has been associated with poorer prognoses. In this context, IRE1 $\alpha$  has been identified as a potentially relevant therapeutic target. Pharmacological inhibition of IRE1 $\alpha$  activity can be achieved by targeting either the kinase domain or the RNase domain. Herein, we summarize the recent advances in IRE1 $\alpha$  pharmacological targeting, we describe the identification and optimization of IRE1 $\alpha$  inhibitors as well as their mode of action and limits as anti-cancer drugs. We comment on the potential pitfalls/challenges that could be faced in the clinic and opportunities that IRE1 $\alpha$  modulating strategies may represent.

## INTRODUCTION

Cancer is the second leading cause of death globally with an estimated 18.1 million cases and 9.6 million deaths in 2018. Based on projected population aging and growth, the global burden of cancer is set to increase by more than 60% by 2040, reaching a predicted 29.4 million cases (World Health Organization). Since chemotherapies have shown their limits in many instances and that precision oncology has shown some promises, it is becoming of prime importance to identify new targets with therapeutic relevance and design new candidate drugs to impinge on their functions. Accumulating evidence points toward an emerging role of **Endoplasmic Reticulum (ER) stress**, of the **Unfolded Protein Response (UPR; see GLOSSARY)** and of proteostasis control in cancer progression [1]. The accumulation of improperly folded proteins in the ER causes ER stress, which in turn triggers the UPR as an adaptive mechanism to restore ER proteostasis and maintain the functionality of the secretory pathway. The activation of UPR leads to mRNA translation attenuation and to the upregulation of genes involved in protein folding and quality control, antioxidant responses, and lipid synthesis. However, if proteostasis restoration fails, pro-apoptotic programs are triggered in order to eliminate terminally damaged cells [2]. In the context of cancer, ER stress has been shown to play pro-tumoral roles in allowing transformed cells to cope with intrinsic stresses such as oncogene expression [3] or aneuploidy [4,5] and with extrinsic stresses such as nutrient and oxygen deprivation. Moreover, tumor cells can be subjected to extrinsic stresses caused by anticancer treatments (irradiation, chemotherapies) and it has been shown that ER stress signaling confers treatment resistance to cancer cells [6–10]. This points the UPR as an appealing target in unique or adjuvant anticancer therapeutic approaches.

## IRE1 $\alpha$ SIGNALING

IRE1 $\alpha$  (Inositol requiring enzyme 1 alpha, referred to as IRE1 hereafter) is the most evolutionary conserved ER stress sensor. IRE1 is encoded by the *ERN1* gene and it is ubiquitously expressed in mammals. It is a type I, transmembrane, ER resident protein which bears two enzymatic activities: a serine/threonine kinase and an endoribonuclease (RNase) in its cytosolic domain. Despite being considered as a conventional kinase protein, the only substrate known so far is

IRE1 itself. When improperly folded proteins accumulate in the ER, IRE1 oligomerizes and *trans*-auto phosphorylates, thereby activating its RNase activity. Thus far, the most prominent signaling output mediated by IRE1 has been identified as dependent on IRE1 RNase activity. Hence, two signaling responses are catalyzed (**Figure 1**):

- (i) The **non-conventional splicing** of the mRNA encoding X-box binding protein-1 (XBP1) to allow the expression of XBP1s (XBP1 spliced), a potent transcription factor. This occurs through the combined action of IRE1 RNase for the removal of 26 nucleotides intron and of the tRNA ligase RtcB, to ligate the 5'-OH and 2',3'-cyclic phosphate of the remaining fragments.
- (ii) The RNase-mediated cleavage of ER-bound RNA (including mRNA, miRNA, rRNA) in a process called **regulated IRE1-dependent decay** (RIDD) of RNA (the degradation being executed by cellular exonucleases following IRE1-mediated cleavage).

Beyond the signaling pathways initiated downstream of IRE1 RNase, it has been demonstrated that IRE1 was also executing its functions through scaffolding roles [11,12]. Under irremediable ER stress IRE1 assembles into a scaffold platform for the activation of both ASK1-JNK and NF- $\kappa$ B pathways. Its hyperactivation causes a decrease on RNase activity and an increase of RIDD, which induces pro-inflammatory and apoptotic factors reaching a terminal UPR[13] (**Figure 1A**). As such IRE1 operates as a multi-tasking protein that transduces signals through both catalytic and non-catalytic mechanisms. IRE1 activity is important in tumorigenesis and aggressiveness in most cancers (such as leukemia, glioblastoma, myeloma, renal, breast cancers) and has been associated with a poor prognosis [3,10,14,15]. Several types of cancers displaying high levels of IRE1/XBP1 activity have a worse prognosis than those with low activity. Besides, XBP1 promotes tumor invasion and drug resistance in cancer [8]. XBP1s was found to be overexpressed in several types of cancer and its overexpression is directly linked to tumorigenesis, suggesting proto-oncogenic activity [16]. In addition, IRE1-XBP1s was found to regulate **c-MYC** expression, contributing to oncogenicity in prostate cancer (PCa) [3,10]. In contrast to the IRE1/XBP1 axis that exhibits pro-tumorigenic signaling features, RIDD may antagonize tumor invasion and angiogenesis with no significant effect on immune cells infiltration in

glioblastoma [15]. Targets of RIDD include microRNAs, ribosomal RNAs, mRNA and ER localized mRNAs encoding membrane and secretory proteins, the latter thought to be degraded to reduce the ER load upon stress. However, how IRE1 regulation may evolve during the pathologic progression of diseases is still a matter of debate. A plethora of outstanding questions remain to be carefully addressed before fundamental discoveries can be translated into effective IRE1-targeting therapeutics to combat diseases in the clinic.

### **SMALL MOLECULES TARGETING IRE1**

Pharmacological modulation of IRE1 has been the main focus of several drug development strategies and compounds targeting either the kinase domain or the RNase domain were identified. Downstream IRE1 signaling components have also become the focus of drug discovery programs. Based on structural and biochemical analyses, several kinase domains display similarities to the IRE1 kinase [17]. The RNase domain is homologous only to that of the known anti-viral RNaseL, revealing a convergent evolution of their catalytic mechanism [18]. IRE1 and RNaseL, in addition to biochemical similarities in protein kinase domain and structural similarities in their RNase domain, share the functional consequences of their activation in initiating cellular apoptosis through JNK signaling [19]. Further advances in research narrowed the differences between the pathways. A recent report suggested a consensus cleavage site in RNaseL targets and in those mRNAs that are cleaved by IRE1 as part of the RIDD pathway [20]. The characteristics and structures of known IRE1 inhibitors are presented in **Table 1** and **Figure 2**.

Interest in targeting the IRE1 as a therapeutic strategy has prompted the search for highly selective IRE1 modulators. The characterization of the allosteric relationship between the kinase and RNase domains has allowed for the modulation of IRE1 with small molecules targeting the ATP-binding site [21–23]. Extensive crystallization efforts led to the obtention of crystal structures from yeast and human IRE1 in active and inactive forms, revealing its probable mechanism of activation through co-factor binding and **trans-autophosphorylation**, ultimately leading to the active back-to-back dimer [17]. These observations were supported by studies of mutant IRE1 proteins carrying amino acid substitutions at conserved positions in the kinase domain which

identified nucleotide binding and kinase-domain phosphorylation as necessary for IRE1 RNase activation [17,24].

**IRE1 kinase inhibitors** – Compound 3 is an optimized **type II inhibitor** and one of the earliest reported ATP-competitive inhibitors capable of decreasing RNase activity and was part of a chemical series based on the imidazo[1,5-a]pyrazin-8-amine scaffold [25]. This type of compound presents the advantage of being often more specific than those targeting active, DFG-in, kinase conformations. The imidazo[1,5-a] pyrazine motif of Compound 3 forms two hydrogen bonds with the hinge region and occupies the adenine pocket of IRE1. This competition causes movements of the DFG-motif (adopting a DFG-out conformation) contained within the activation loop. As a consequence, Compound 3 inhibits XBP1 mRNA splicing, even during ER stress [25] (**Table 1 and Figure 2A**). **Kinase-Inhibiting RNase-Attenuators (KIRAs)** were recently developed based on Compound 3 and are highly efficient ligands mono-selective towards IRE1. The first KIRA was assayed using fluorescence resonance energy transfer (FRET)-quenched XBP1 RNA mini-substrate. The most potent compound identified in this study inhibited XBP1 RNA cleavage to a similar degree as STF-083010, a cell-permeable compound that directly targets IRE1 RNase domain and disrupt IRE1-XBP1 activity [26]. KIRA compounds, as type II inhibitors, alter the oligomerization process on the DFG-motif in order to stabilize the enzyme in a helix- $\alpha$ C-out conformation, which affects both kinase and RNase activities depending on the dose used. Optimized KIRA compounds include KIRA6 [13], KIRA7 [27] and KIRA8 [28]. KIRA6 and KIRA7 were developed on the basis of Compound 3 and the three molecules share high features similarity [13], whereas KIRA8 is a sulfonamide compound displaying high selectivity [28]. They were proven to protect pancreatic  $\beta$ -cells against autoimmune diabetes, halt neurodegeneration in ALS (KIRAs 6-8)[13,23], preserve the photoreceptor degeneration induced by ER stress (KIRA6) and have anti-fibrotic effects in the lung (KIRA 7) [27]. Evident side effects after systemic administration have yet to be reported. Following the discovery of the imidazo[1,2-b]pyridazine based RNase activators, extensive targeted development and SAR studies led to a series of potent inhibitors which lock the kinase DFG motif in an unusual and inactive “DFG-up” state [29]. Compound 31 and its analogues inhibited both tunicamycin- and thapsigargin-induced IRE1-dependent splicing of XBP1 luciferase fusion mRNA in HEK293

cells. In parallel, inhibition of tunicamycin-induced production of endogenous XBP1s mRNA was demonstrated in myeloma cells using RT-qPCR at similar concentrations [29]. GSK2850163 was identified in a high throughput screen to discover new IRE1 selective inhibitors. It showed both RNase and Kinase inhibition in a dose-dependent manner in myeloma cell lines [30].

**IRE1 RNase inhibitors** – Additional modulators with a different mode of action were identified in high-throughput screens (HTS) using luciferase-based reporters in cell-based assays or recombinant proteins *in vitro* [31]. These so-called direct inhibitors share a common hydroxy-aryl-aldehyde (HAA) moiety which reacts selectively with a specific lysine (Lys907) in the RNase domain, thus forming a stable imine via **Schiff base** formation and effectively preventing ER stress-induced site-specific mRNA splicing. These include 4 $\mu$ 8C, salicylaldehydes, HNA[7], OICR573 and OICR464 [32], B-109 [33], STF-083010 [7] and the MKC analogues: MKC9989 [32], MKC8866 [32] and MKC3946 [7,34] (**Table 1 and Figure 2A**). In the case of B-109 and STF-083010, a naturally occurring hydrolysis step takes place under physiological conditions to form *in situ* the reactive HAA motif. STF-083010 was identified as an inhibitor of XBP1 mRNA splicing activated by thapsigargin, tunicamycin, glucose deprivation or severe hypoxia and was reported to block IRE1 endonuclease activity without affecting its kinase activity. STF-083010 is now in preclinical stage for multiple myeloma (MM) treatment and breast cancer displaying selective cytotoxicity towards cancer cells [26]. B-109 inhibits growth of human chronic lymphocytic leukemia (CLL) cells *in vitro* and promotes regression in a murine CLL model [33]. 4 $\mu$ 8C also inhibits RIDD mRNA degradation in Mouse Embryonic Fibroblasts cultures following Tunicamycin treatment [35]. Compared to STF-083010, 4 $\mu$ 8C was also shown to inhibit IRE1 autophosphorylation via Schiff base formation with IRE1 Lys599 located in the kinase pocket [36,37]. HNA (2-hydroxy-1-naphthaldehyde) is a small HAA molecule that showed either the same or more potent ability to inhibit the activity of IRE1 than MKC-3946 to cleave XBP1 into the active XBP1s after tunicamycin induced activation of acute myeloid leukemia cells (AML). The combination of Bortezomib (BTZ), a **proteasome** inhibitor, or AS<sub>2</sub>O<sub>3</sub> with HNA synergistically both inhibited growth of AML cells and when applied to an AML patient sample [7,38]. 4 $\mu$ 8C was tested in both cell-free assays and in cultured cells for potential activity as a reactive oxygen species (ROS)

scavenger. It was demonstrated that this molecule can effectively decrease xanthine/xanthine oxidase catalyzed superoxide production *in vitro* as well as inhibit angiotensin II-induced ROS production *in vivo*. In both cases, the concentration used is much lower than when used as a direct inhibitor of IRE1 [35]. MKC3946 is active in MM and reinforces the action of BTZ. Recent structural studies confirmed the interaction site between IRE1 Lys907 and HAA-based compounds MKC9989, MKC8866, OICR573 and OICR464. All four molecules were potent inhibitors of RNase activity *in vitro*. Salicylaldehydes were found to be selective and potent inhibitors of IRE1 endoribonuclease both *in vitro* and *in vivo* competing against the XBP1 stem-loop RNA substrate [39]. 3-methoxy-6-bromosalicylaldehyde could bind reversibly to IRE1 and block XBP1 mRNA splicing in different organs (kidney, liver, spleen) after administration of Tunicamycin [39]. It is likely that salicylaldehydes present the same binding mode with Lys907 as the other HAA-based inhibitors, but structural studies have yet to confirm this [40]. In addition to those direct modulators, other inhibitors with unconfirmed binding sites were identified. These molecules such as Toyocamycin, Doxorubicin, Quinotrierixin and Trierixin, were shown to inhibit IRE1/XBP1s activity *in vitro* and *in vivo*, despite no clear demonstration of their mode of action.

**IRE1 RNase activators** – The concept developed above is that inhibiting IRE1 activity might represent an interesting approach to weaken tumor cells adaptability and therefore make them more sensitive to their intrinsic modifications, to their challenging microenvironment or to the treatments they are exposed to. Another strategy could consist of overwhelming stress signals to cause cell death. As such APY29 or sunitinib (both **type I kinase inhibitors**) are allosteric modulators inhibiting IRE1 trans-autophosphorylation whilst promoting oligomerization and activation of its RNase activity [23,41]. Sunitinib inhibits tumor migration through remodeling of extracellular matrix and intracellular signaling pathways, such as metalloproteinases and kinases [25,37]. In particular, APY29 decreased IRE1 ubiquitylation and showed potent effects against sepsis in preclinical models of inflammation. Moreover, its interaction with Cys645 at the kinase cleft has been reported to activate IRE1 endoribonuclease activity [42]. CXC195 was first described to trigger the activation of many proteins related to ER stress including BiP, CHOP, IRE1, TRAF2, p-ASK1 and p-JNK in



bladder urothelial carcinoma cells. This induced apoptosis through stabilization of the IRE1-TRAF2-ASK1 complex [11]. CXC195 was associated with proapoptotic effects in hepatocellular carcinoma cells due to activation of IRE1 and ATF6 and inhibition of the PI3K/Akt/mTOR signaling pathway [43].-Despite there being no direct evidence of the effect of this drug on IRE1 in this model, the findings show decreasing levels of Bax, an important activator of IRE1 activity. The mechanisms by which this molecule works remain to be elucidated. Prior to the development of the imidazo[1,2-b]pyridazine based RNase inhibitors [29](*vide supra*), a limited series of ligands based on this scaffold were identified as human isoform IRE1 RNase activators [24]. Among those, compound 3 was found to enhance the endoribonuclease activity of unphosphorylated human IRE1 *in vitro* with potency ( $EC_{50}$  143 nM) similar to the inhibition of the kinase autophosphorylation activity ( $IC_{50}$  218 nM) [24]. In this context, the identification of compounds that would therefore selectively activate a specific arm of the UPR (and the IRE1 pathway in particular) is of great importance. Finally, one might propose that IRE1 activator may also boost the folding capacity of the ER, through enhanced adaptive signaling, which in turn could impact of rescuing loss-of-folding/secretory functions in protein misfolding/trafficking diseases (Table 2 and Figure 2B).

### **POTENTIAL OFF-TARGET ACTIVITIES OF IRE1-TARGETING COMPOUNDS**

The off-target activity of a compound is defined as the activity this compound could exert on an effector different from its intended biological target. It has been demonstrated that off-target effects might most commonly contribute to side effects. However, in some cases, off-target activity can be taken as an advantage for therapeutic purposes. As such the concept of poly-pharmacology has emerged to repurpose molecules that can exert activities on multiple targets thus mediating beneficial effects [44]. In this context, drug repurposing represents an exciting opportunity to highlight the potential of approved drugs and small molecules on different targets. In this context some of the compounds described in this review were not originally identified as IRE1-targeting compounds, but experiments revealed them to be effective IRE1 modulators. For instance, Toyocamycin was first isolated as an antibiotic in 1956 and extracted from *Streptomyces toyocaensis* [45]. Since it is a nucleoside- type antibiotic analogue

of adenosine it was suggested it could act as an ATP-competitive inhibitor [7,46]. Sunitinib malate (Pfizer Sutent®; ClinicalTrials.gov number NCT00428597) was first identified as an oral multi-kinase inhibitor that works by blocking multiple molecular targets implicated in the growth, proliferation and spread of cancers [47]. Two important sunitinib targets, vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) are expressed by many types of solid tumors and are thought to play a crucial role in angiogenesis. Sunitinib also inhibits other targets important to tumor growth, including KIT, FLT3 and RET[23]. Similar to Sunitinib, APY-29 was identified at first as a broad-acting kinase inhibitor. Its results on activation of UPR placed it as an allosteric modulator of IRE1 [48].

### **ADJUVANT USE OF IRE1 INHIBITORS IN CANCER**

In tumors, upregulation of UPR markers is frequently observed which could be caused by cancer specific stresses such as genomic instability, somatic mutations, oncogene expression, aneuploidy or nutrient shortage. The UPR also contributes to cancer independently of protein misfolding and ER stress was related to support survival and adaptation of cancer cells. IRE1 is emerging as a driver of migration, homing and invasion of cancer cells. Its activity modulates the adhesion of Glioblastoma multiform (GBM) cells [15], enhances metastasis of triple-negative breast cancer (TNBC) tumors to the lungs [14] and is related to chemoresistance in many types of cancers such as breast cancer, prostate cancer, multiple myeloma, leukemia and glioblastoma [3,7,10,14,34,46,49,50]. In this context, there have been many attempts to improve existing clinical treatments by co-administering IRE1 inhibitors (**Table 3**). Monotherapies with IRE1 inhibitors or in combination with antitumorigenic agents significantly reduced tumor growth of mice bearing human prostate tumors [51]. In TNBC, the breast cancer showing the worse prognosis, IRE1 RNase activity enhances the production of pro-tumorigenic factors IL-6, IL-8, CXCL1, GM-CSF, and TGFβ2 and its inhibition can sensitize TNBC cells to the chemotherapeutic of choice, paclitaxel [14]. MM is an extremely aggressive, incurable and mostly lethal disease despite advances in therapy. It arises from malignant immunoglobulin (Ig)-secreting plasma cells. Pharmacological inhibition of the IRE1–XBP1s pathway has been demonstrated as an effective and safe potential small

molecule target for MM therapy [52]. Toyocamycin [7,46] was described as a cytotoxic agent for MM cells and was shown to selectively affect IRE1 signaling but not PERK or ATF6 signaling. The adenine moiety of Toyocamycin seems to inhibit XBP1 activation and the combination of this drug with BTZ markedly induced apoptosis in BTZ-resistant MM cells. Furthermore, Anthracyclines such as Doxorubicin have been proven useful in MM and in combinations with BTZ. Doxorubicin is a chemotherapy medication used to treat many types of cancer. It acts by intercalating DNA/RNA and inhibiting Topo II. As toyocamycin, it displayed increased cytotoxicity against myeloma compared to other cancer cell lines which have lower basal IRE1-XBP1 activity [53]. Lastly, GBM is an aggressive and fatal CNS tumor in which downstream IRE1 signaling commands tumor angiogenesis, invasion and migration [15], and recently the adjuvant use of MKC8866 was found to enhance the efficacy of irradiation combined with temozolomide treatment [54].

**IRE1 SIGNALING CROSS-TALKS WITH OTHER STRESS PATHWAYS** Since IRE1 signaling has been shown to crosstalk with many cellular stress pathways. Consequently, one can expect that the modulation of IRE1 signaling could also influence other cancer-relevant pathways in malignant cells and IRE1-targeting compounds could therefore gain importance to impair the tumorigenesis. Here, we propose to describe the cross-talks between IRE1 signaling and pathways involved in the hallmarks of cancer[55] and discuss how modulating IRE1 could regulate them.

*Initiation* is the first step in the two-stage model of cancer development. Initiators cause irreversible changes (mutations) to DNA thereby increasing cancer risk. To avoid an enormous amount of DNA mutations, surveillance proteins monitor DNA integrity and activate cell cycle checkpoints and DNA repair pathways in response to DNA damage. IRE1 seems to modulate the DNA damage response through RIDD. *Promotion* is the second stage of cancer development in which IRE1 signaling is known to play instrumental roles [56]. As such the IRE1/XBP1 axis **i**) is activated in response to nutrient starvation and controls the expression of limiting enzyme of the hexosamine biosynthetic pathway (HBP) activation. This allows the production of glucose to UDP-acetylglucosamine, which serves as substrates for O- and N-linked glycosylation to promote

proteostasis [60]; **ii**) cross-talks with the hypoxia-inducible factor alpha (HIF1 $\alpha$ ) pathway, which in turn induces glycolysis and response to hypoxia [61,62]; **iii**) controls the transcription of genes coding for vascular endothelial growth factor a (VEGF-A), IL-6 and other proangiogenic signals leading to tumor neo-angiogenesis [14]; **iv**) controls the induction of tumor adaptive autophagy through the expression of Beclin-1 [66]. Alternatively, IRE1 can also exert other cancer promotion- related functions through RIDD or scaffolding activities. This was shown as **i**) RIDD activity controls the expression of SPARC mRNA in glioblastoma thus altering migration and invasion properties via the activation of the small GTPase RhoA [57,58]; **ii**) IRE1 was shown to directly bind filamin A in order to regulate the remodeling of cytoskeleton and impact in cell migration [59]. The impact of IRE1 modulation can be different in different types of cancers and studies on the precise roles of IRE1 in various cancers are essential for a relevant therapeutic intervention. For instance, studies showed that high levels of the major components of UPR, PERK, ATF6, IRE1 and both, unspliced and spliced XBP1, are observed in a variety of human tumors including brain, breast, gastric, kidney, liver, lung, and pancreatic cancers [13–15,27,36,49,60,61]. In addition, activation of IRE1 signaling led to a reduction in colorectal tumor growth and increased survival in mouse models [62], indicating that knowledge must drive the use of IRE1 targeting drugs. Hence, it appears that targeting IRE1 can have anticancer effect at both the level of the tumor cells themselves and the antitumor capacity of other stress pathways.

## **CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

The ER controls multiple cellular functions including protein folding, post-translational modifications (PTMs), fatty acid biosynthesis, detoxification, and intracellular calcium storage. As such it plays an instrumental role in tumor development and treatment response. We have described above a large panel of modulators of IRE1 activity through the targeting of its catalytic activities. In this context, one should however be cautious with the use of those pharmacological modulators as they might exert deleterious effects on normal tissues, in particular those involved in secretory functions (e.g. liver, pancreas, B cells). For instance, inhibition of PERK, another UPR sensor, by GSK2656157 in various in vivo cancer models [63] showed some efficacy on tumor growth,

however, long term administration of this compound also exerted significant side effects, including weight loss and mild diabetes, caused by damage to the pancreas, which challenged its use in the clinic. We have extensively used an IRE1 RNase inhibitor (MKC8866) in mouse orthotopic or xenograft models in immunocompetent or deficient backgrounds and thus far did not observe any deleterious side effects [14,54]. It is possible that in specific physiological conditions (e.g. infection, metabolic challenge), those inhibitors might lead to impaired responses, but this should be examined thoroughly.

IRE1 functions can also be exerted through the scaffolding of partner proteins and as such, modulating the IRE1 interactome might represent another way to reprogram IRE1 functions. The many protein-protein interactions (PPIs) involving IRE1, either with itself during the oligomerization process, with partners such as BiP, HSP47, Sigma1 Receptor, which fine-tune its activation level or Filamin A, IP3 Receptors, may represent appealing opportunities to develop PPIs inhibitors or stabilizers [64–66]. **PROTACS** and other chimeric degrader solutions [67] figure among the most trending novel therapeutic modalities as they present several advantages over small-molecule inhibition and allow for fine control over the cellular level of targeted protein and have already been applied with success to transmembrane proteins with enzymatic activities similar to IRE1 [68]. The wide range of high-affinity IRE1 ligands available, makes PROTACS a compelling option to pursue. To overcome the limitations of previously described kinase and RNase binding modulators, finding molecular starting points that bind onto other sites of the protein would be extremely valuable to develop novel allosteric inhibitors or PPI modulators. Fragment-based drug discovery approaches have proven to be particularly successful in identifying small chemical entities binding to such novel binding sites and represent another avenue worth exploring [69,70].

Until now, efforts to improve cancer treatments by exploiting IRE1 activity have been restricted to its inhibition. Indeed, the activation of IRE1 signaling under low ER stress promotes autophagy and cell survival through XBP1s mechanism. However, it has been described that under irrevocable ER stress, IRE1 oligomerizes to activate RIDD, ultimately leading to apoptosis [13]. Other death receptors like RIP1 shows the same switching mechanisms process, which depends on the context or strength of cellular processes [71,72]. Induced hyper activation of IRE1 to convert the response from promoting homeostasis to

promoting cell death could thus represent an alternative strategy in cancer treatments [13]. Furthermore, IRE1 mutants in cancer cells show defective homo-oligomerization and RNase activity, which may allow the terminal UPR to become disabled or co-opted for survival advantage [73]. Based on this knowledge and the trends observed in the field recently, the discovery of novel families of IRE1 modulators as well as drug repurposing and combined therapies appear imminent as well as pertinent. In addition to these approaches, the rapid rise and improvements demonstrated by Artificial Intelligence methods hold great promises and could accelerate significantly the drug discovery process from the design stage to the prediction of the most cost- and time-efficient synthesis pathways [74,75] (**See Outstanding questions box**).

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## GLOSSARY

**Unfolded Protein Response (UPR):** Cellular stress response related to the endoplasmic reticulum (ER) stress. The UPR is activated in response to an accumulation of unfolded or misfolded proteins in the lumen of the ER.

**Kinase Type I inhibitors:** The Type I inhibitors bind to the so called “Active Conformation” of the enzyme and are associated with the *DFG-in* conformation of this loop.

**Kinase Type II inhibitors:** Class of chemicals that trap their target kinases in an inactive, so-called *DFG-out*, state by occupying a hydrophobic pocket adjacent to the ATP binding site.

**Proteasome:** Multi-subunit assembly of proteases that selectively degrades proteins, including transcription factors that regulate the cell cycle.

**ER stress:** A condition when increased load of unfolded proteins in ER lumen causes a disturbance on the ER leading to an inability to restore its functions and induces cell death, which is usually in the form of apoptosis

**c-Myc:** Myc is a family of regulator genes and proto-oncogenes that code for transcription factors. The Myc family consists of three related human genes: c-myc, l-myc, and n-myc. c-myc was the first gene to be discovered in this family, due to homology with the viral gene v-myc.

**trans-autophosphorylation:** The phosphorylation by a protein of a residue on an identical protein. For example, phosphorylation by the other kinase within a homodimer.

**KIRAs:** Kinase-Inhibiting RNase-Attenuators. Compounds that bind to the ATP-binding site and allosterically impede the RNase activity.

**Schiff base:** A Schiff base is a compound with the general structure  $R_2C=NR'$ . They can be considered a sub-class of imines, being either secondary ketimines or secondary aldimines depending on their structure. The term is often synonymous with azomethine which refers specifically to secondary aldimines.

**PROTACS:** PROTACS are chimeric bifunctional molecules able to bind simultaneously the targeted protein and a degrader protein such as the ubiquitin E3 ligase, leading ultimately to the degradation and removal of the target from the cell



## TABLES and FIGURES LEGENDS

**Table 1. List of IRE1 inhibitors**

**Table 2. List of IRE1 activators**

**Table 3. Combined treatments of IRE1 inhibitors**

**Figure 1. IRE1 signaling and its modulation.** A. The activation of IRE1 by autophosphorylation, dimerization and oligomerization is regulated positively by many proteins (such as BiP and HSP47). Once activated, IRE1 triggers three main responses to ER stress: apoptosis through JNK pathway, activation of UPR genes through XBP1 splicing and degradation of many mRNAs by RIDD. The stabilization of the oligomeric state leads to an inflammatory/mitochondrial programmed cell death via terminal UPR. B. The inhibitors (left side) and activators (right side) of IRE1 can lead a chemotherapy sensitization of cells by IRE1 pathway. The inhibitors lead to an inactivation of IRE1 activity, inhibiting the response under ER stress. The overactivation of IRE1 leads the terminal UPR by activating an irremediable ER stress.

**Figure 2. Small molecule modulators of IRE1 $\alpha$  activity.** A. Molecular structures of the most significant IRE1 inhibitors in their chemical series, classified by their binding sites: kinase pocket binders, RNase domain ligands, and unconfirmed binding site inhibitors. B. Molecular structures of IRE1 activators. AI: ATP inhibitor; KA: Kinase activator.

**Figure 3. IRE1 targeting by small-molecule inhibitors.** A. Graphical representation of Kinase pocket inhibitors (pink) and RNase domain covalent inhibitors (green) bound to IRE1 monomeric structure. (Composite structure generated for illustrative purpose using PDB ID 4U6R in which MKC 9989 was docked using Schrodinger Maestro's CovDock; kinase domain represented in yellow; RNase domain in purple,  $\alpha$ C-helix in blue). B. X-ray crystal structure (PDB ID 4U6R) of KIRA8 bound to IRE1 kinase pocket. Key residues of the protein are shown as white stick and hydrogen interactions as yellow dotted lines. C. X-ray crystal structure (PDB ID 4PL3) of MKC 9989 covalently bound to Lys907 of IRE1 RNase domain. Key residues of the protein are shown as white stick and  $\pi$ - $\pi$  interactions as cyan dotted lines.

**Table 1. List of IRE1 inhibitors**

Drug	Kinase activity	RNase activity	RIDD	Class	EC <sub>50</sub>	Ref.
<b>Cpd 3</b>	Inhibits	Inhibits	Unknown	Type II inhibitor	3.12 μM	[25]
<b>KIRA6</b>	Inhibits	Inhibits	Inhibits	Type II inhibitor	0.17 – 0.87 μM	[13]
<b>KIRA7</b>	Inhibits	Inhibits	Inhibits	Type II inhibitor	0.11/0.22 μM	[27]
<b>KIRA8</b>	Inhibits	Inhibits	Inhibits	Type II inhibitor	0,007 μM	[28]
<b>Cpd 31</b>	Inhibits	Inhibits	Unknown	imidazo[1,2-b]pyridazin-8-amine	0,008 – 0,78 μM	[29]
<b>GSK2850163</b>	Inhibits	Inhibits	Unknown	2,7-diazaspiro[4.5]decane derivative	0,02 – 0,2μM	[30]
<b>STF-083010</b>	No effect	Inhibits	Inhibits	Inimine-based compound	25 μM	[7]
<b>HNA (2-hydroxy-1-naphthaldehyde)</b>	Unknown	Inhibits	Unknown	HAA	31 – 35 μM	[7]
<b>B-109</b>	Unknown	Inhibition of XBP1 splicing	Unknown	HAA	1.23 μM	[33]
<b>4μ8C</b>	Inhibits	Inhibits	Inhibits	HAA	0,076 μM – 6.9 μM	[37]
<b>OICR573</b>	No effect	Inhibits	Unknown	HAA	5.4 ± 1.9 μM	[32]
<b>OICR464</b>	No effect	Inhibits	Unknown	HAA	2.3 ± 0.69 μM	[32]
<b>MKC-3946</b>	No effect on auto-phosphorylation	Inhibits	Unknown	HAA	0.23 ± 0.03 μM	[7,34]
<b>MKC9989</b>	Unknown	Inhibits	Unknown	HAA	0.29 μM	[32]
<b>MKC8866</b>	Unknown	Inhibits	Inhibits	HAA	0.39 ± 0.21 μM	[32]
<b>Toyocamycin</b>	No effect	Inhibits	Unknown	Pyrolopyrimidine	0.08 – 0.18 μM	[46]
<b>Doxorubicin</b>	Unknown	Inhibits	No effect	Anthracycline antibiotic	~20 μM	[53]
<b>3-methoxy-6-</b>	Unknown	Inhibits	Unknown	Salicylaldehydes	0.41 μM	[39]

**bromosalicylaldehyde**

<b>Trierixin</b>	Inhibits	Unknown	Unknown	triene-ansamycin group	14-19 ng/mL	[76]
<b>Quino-trierixin</b>	Inhibits	Unknown	Unknown	triene-ansamycin group	0.067 $\mu$ M	[77]

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**Table 2. List of IRE1 activators**

Drug	Kinase activity	RNase activity	RIDD	Class	EC <sub>50</sub>	Ref.
<b>APY29</b>	Activates	Unknown	Unknown	Type I kinase inhibitor	0,28 $\mu$ M	[41]
<b>Sunitinib</b>	Inhibits	Activates	Unknown	Tyrosine kinase (RTK) inhibitor	3.7 $\mu$ M $\pm$ 1.2	[23]
<b>CXC195</b>	Unknown	Stabilization of IRE1-TRAF2-ASK1 complex	Unknown	Tetramethylpyrazine analog	Unknown	[43]
<b>Cpd 3</b>	Inhibits	Activates	Unknown	Imidazopyridazine	0,143 – 0,218 $\mu$ M	[24]

**Table 3. Combined treatments of IRE1 inhibitors**

Treatments	Cancer	Ref.
Compound 18 + anti-VEGF-A	TNBC*	[49]
MKC-8866 + Paclitaxel	TNBC	[14]
MKC-3946 + Bortezomib	MM*	[34]
MKC-3946 + 17AAG	MM	[34]
STF-083010 + Tamoxifen	Breast Cancer	[50]
MKC-8866 + Enzalutamide	PCa*	[3]
MKC-8866 + Abiraterone acetate	PCa	[3]
MKC-8866 + Cabazitaxel	PCa	[3]
MKC-8866 + Docetaxel	Breast Cancer	[10]
Toyocamycin + BTZ	MM	[46]
Doxorubicin + BTZ	MM	[78]
HNA + Bortezomib	AML*	[7]
HNA + AS <sub>2</sub> O <sub>3</sub>	AML	[7]
MKC8866 + Stupp	GBM	[54]

\*TNBC: triple negative breast cancer; MM: multiple myeloma; PCa: prostate cancer; AML: acute myeloid leukemia; GBM: glioblastoma multiforme

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## **OUTSTANDING QUESTIONS**

- How IRE1 acts on tumor progression in different types of cancer?
- Can we use protein structure, rational drug design and artificial intelligence to predict new molecules that modify the pathways responses?

Accepted manuscript

## Point-by-point response to the reviewer's comments

Reviewer #1: This is a nice review describing the currently available pharmacologic approaches to target IRE1 activity in different cancers. The authors accurately summarize our current understanding of the multiple signaling consequences of IRE1 activation and the importance of IRE1 signaling in many cancers, all with a focus towards defining the therapeutic potential for IRE1 inhibition. The authors are comprehensive in their description of both first and second generation compounds targeting IRE1 and their currently understood mechanisms of action. However, it would be beneficial for the authors to include a bit more discussion of the specificity of these compounds and the potential impact of these IRE1 inhibition on other stress-responsive signaling pathways (see below). Similarly, more discussion of the potential toxicity of these compounds and their possible impact on secretory tissues would also be a nice addition to this review. Overall, this review captures the current perspectives and options for modulating IRE1 signaling to target cancer and includes a detailed discussion of future directions for improving our ability to develop IRE1 inhibitors for cancer.

We thank this reviewer for his/her positive and constructive comment on our manuscript.

### Specific comments.

1. Additional discussion of the known specificity of IRE1 activating and inhibiting compounds is important. For example, APY29 and sunitinib were not originally identified as IRE1-targeting compounds. It is important for researchers employing these compounds to understand potential off-target activities of these compounds when applying them to their own research.

As recommended, we have added a section on the nature of the before mentioned compounds and the origin of their discovery. This section can be found on p9-10 of the revised manuscript.

2. Similarly, additional discussion about how IRE1 modulators influence the activity of other stress pathways in cancer would also be helpful in understanding exactly how IRE1 targeting compounds influence physiology of malignant cells.

As recommended, we have added a section on the cross-talks of IRE1 signaling pathways with other pathways controlling the cancer hallmarks. This section can be found on p11-12 of the revised manuscript.

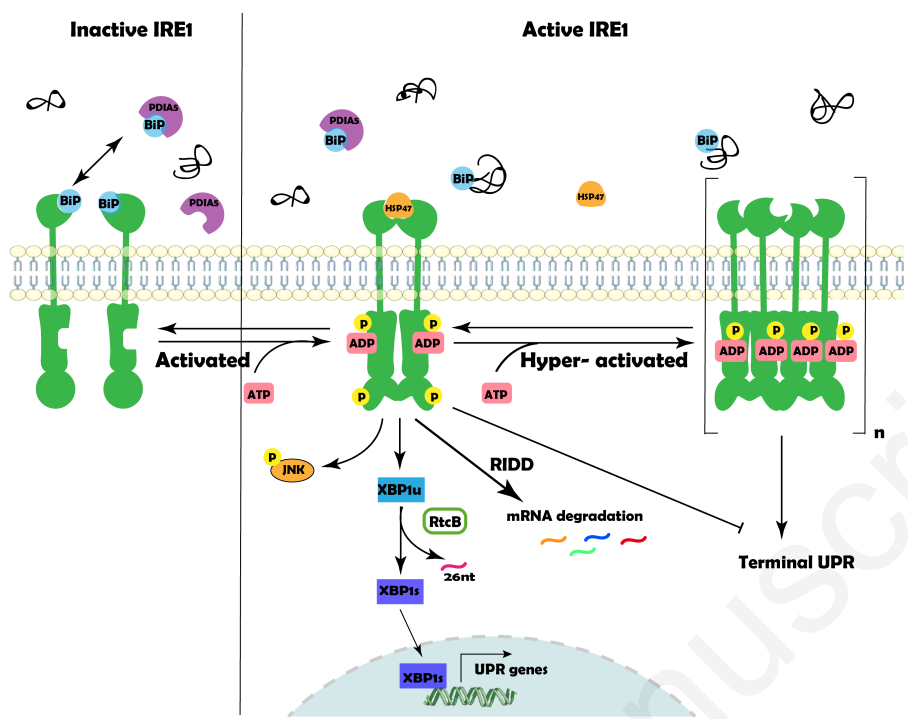
3. Lastly, more discussion of the potential for toxicity of modulating IRE1 in other tissues would be helpful for this manuscript. It is important to consider the importance of IRE1 in other tissues and how targeting IRE1 activity, even in short durations, could impact global organismal physiology.

A paragraph on the potential toxicity of IRE1-targeting compounds has been added in the Concluding remarks and perspective section and can be found on p12-13 of the revised manuscript.

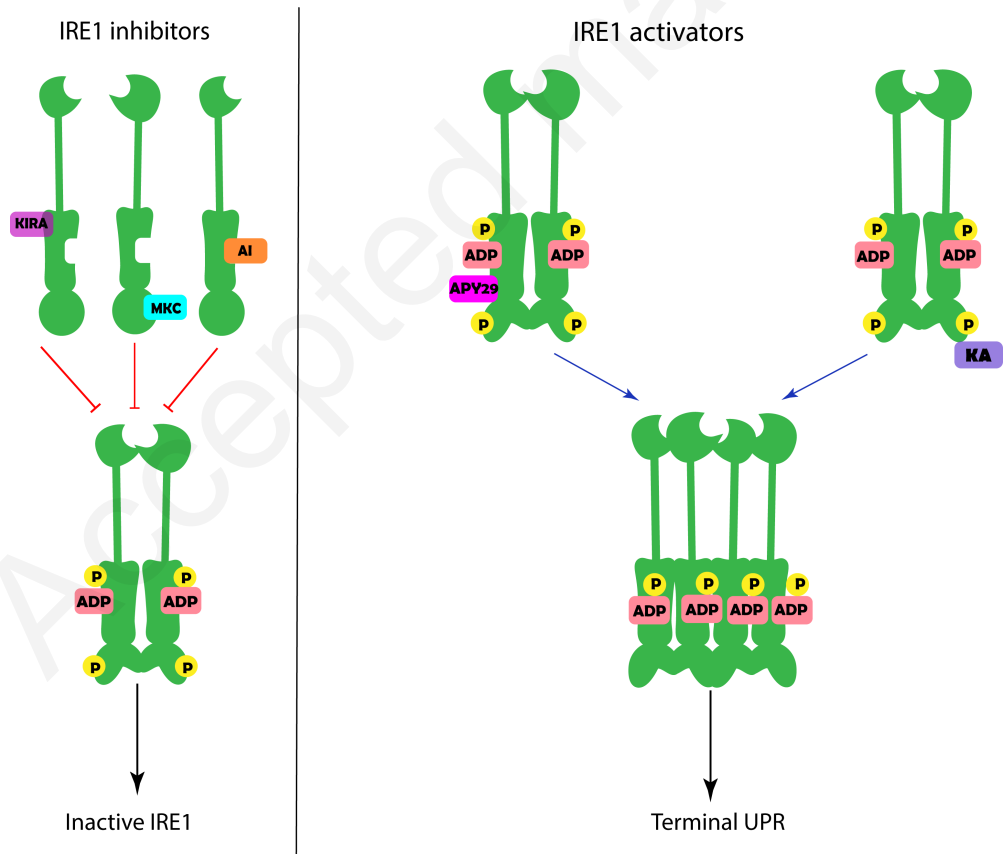
## HIGHLIGHTS

- IRE1 $\alpha$ , a type I Endoplasmic Reticulum (ER)-resident transmembrane protein, exhibits both RNase and kinase activities.
- IRE1 $\alpha$  is involved in several diseases such as cancer, immune, metabolic and neurodegenerative disorders and has thus become a relevant therapeutic target.
- Pharmacological modulation of IRE1 $\alpha$  activity, and the subsequent applications, mechanisms of action and limitations are key to better define what would be the best anticancer approach to use such strategies in clinical settings.
- We discuss the potential pitfalls/challenges and opportunities that IRE1 $\alpha$  modulating strategies may represent.

A.



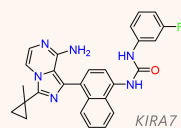
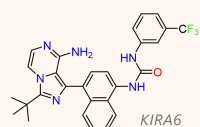
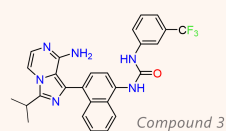
B.



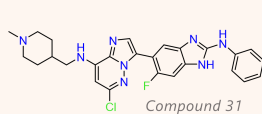
## A. RNase activity inhibitors

### Kinase pocket binders:

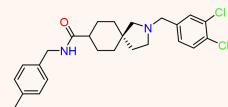
Imidazo[1,5-*a*]pyrazin-8-amine derivatives



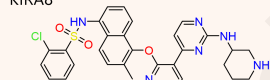
Imidazo[1,2-*b*]pyridazin-8-amine derivatives



GSK2850163

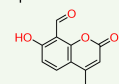


KIRA8

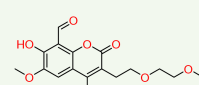


### RNase domain binders:

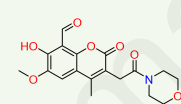
4 $\mu$ 8c



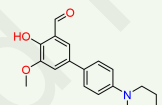
MKC9989



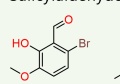
MKC8866



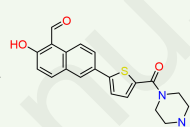
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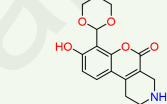
Salicylaldehyde derivatives



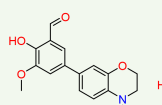
MKC3946



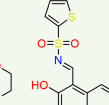
B-109



OICR464

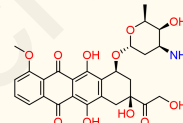


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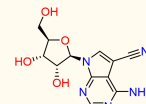


### Unconfirmed binding site:

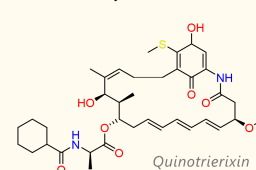
Doxorubicin



Toyocamycin

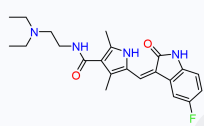


Triene-ansamycin derivatives

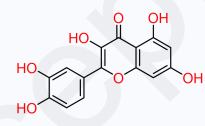


## B. RNase activity activators

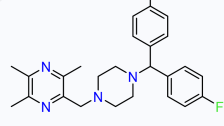
Sunitinib



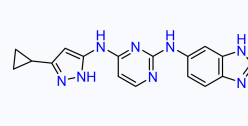
Quercetin



CXC195



APY29



Imidazo[1,2-*b*]pyridazin derivative

