Small Molecule Inhibitors Targeting Heat Shock Response Pathways: Lessons from Clinical and Preclinical Studies in Cancer Therapeutics

Daniel Zhang, Dorothy Wang, and Bin Zhang

Abstract

Introduction Heat shock response (HSR) pathway is a highly conserved cellular process. HSF1 is a master transcriptional regulator responsible for the expression of several important heat shock proteins (HSP), which can effectively protect critical client proteins from misfolding and degradation, thus maintaining intracellular integrity under stressed conditions. Recent studies have demonstrated the direct connections between HSR players and tumor cell survival, validating HSR players as novel molecular targets in anticancer treatment. Small molecule screening has produced some promising HSR inhibitors for anticancer treatment. In this article, we aim to summarize the main findings from HSR inhibitors on recent clinical and preclinical studies.

Methods The authors reviewed all the relevant papers of HSR inhibitors with an emphasis on human and animal studies.

Results More than 18 unique chemical identities have been discovered with confirmed inhibition of HSR pathway. Among them, two natural products and their derivatives are currently in various phases of clinical studies. Detailed works are required to define the exact mechanisms of actions (MOA) for these compounds.

Conclusion Many hurdles in clinical application still need to be effectively addressed, such as undesirable drug toxicity and off-target effects; narrow therapeutic window; poor PK/PD profiles, etc. Recent reports on synergistic drug combination, advanced prodrug design, smart nanoparticle packaging, and RNA aptamer selection offer promising solutions to overcome these challenges. Future advancements in this fast-growing area can potentially lead to the next-generation cancer therapeutics.

D. Zhang · D. Wang · B. Zhang (✉)
Alpine Therapeutics Inc., San Diego, CA, USA
e-mail: bzhang418@gmail.com

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Abbreviations

HCS	high content screening
HSE	heat shock element
HSF1	heat shock factor 1
HSP	heat shock protein
HSR	heat shock response
HTS	high throughput screening
MOA	mechanism of action
NCT	ClinicalTrials.gov identifier number
PD	pharmacodynamics
PK	pharmacokinetics
SAR	structure-activity relationship
target ID	target identification

1 Introduction

Cancer is a malignant disease characterized by uncontrolled cell growth. Genetic and epigenetic alterations can activate oncogenes whose activities are necessary for tumor initiation and maintenance, a phenomenon called "oncogene addiction." [75]. As a result of this oncogenic transformation, cancer cells are known to exhibit "stress phenotype", such as high levels of DNA damage, aneuploidy, and reactive oxygen species [21]. These cancer cells constantly express mutated oncogenes with misfolded protein structures. Comparing to their normal counterparts, the transformed malignant cells demand a much higher expression level of molecular chaperone proteins, including HSP70, HSP90 and HSP27, etc., to preserve protein homeostasis for tumor cell survival [6]. The tumor dependency of molecular chaperone machinery creates an attractive model of cancer therapeutics by selective targeting key HSR players [17].

Heat shock factor 1 (HSF1) is the master transcription factor responsible for controlling the heat shock response [15]. HSF1 is normally maintained in an inactive monomeric state through binding to a complex containing heat shock protein (HSP) 70, HSP90 and HSP40 (Fig. 1). When cellular stress occurs by the multiple known causes of protein misfolding (such as cancer cell transformation, DNA damage, etc.), HSF1 dissociates from the chaperone complex, translocates into the nucleus, and forms active phosphorylated trimers. This activated HSF1 trimer thus binds to the heat shock element (HSE) in the Promoter regions of many heat shock proteins. As a result, activation of HSF1 leads to the mRNA induction and translation of HSP70 and other chaperone family proteins. In addition, HSF1 has a large number of target genes encoding proteins with versatile cytoprotective functions [2]. This highly conserved protective machinery is now believed to be adopted by the majority of

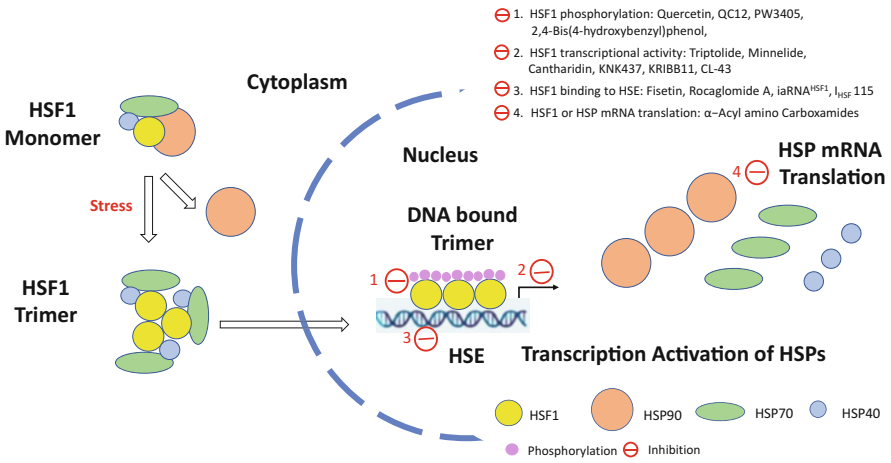


Fig. 1 Illustration of HSF1 trimerization and activation inside the cell with four types of inhibitory mechanisms of small molecules. (1) Inhibition of HSF1 phosphorylation or HSF1 dephosphorization; (2) Interference of HSF1 trimer binding to HSE; (3) Blocking HSF1 mediated transcription activation; (4) hampering mRNA translation of HSF1 and HSP. For a total of 18 compounds in this review (including two prodrugs), 14 compounds show some evidence in one type of the inhibitory mechanisms of HSF1 (listed above), although more detailed studies are essential. The MOAs for the remaining four compounds need to be determined

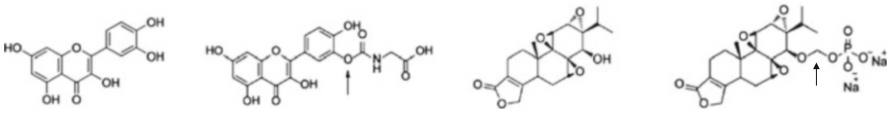
cancer cells, through which the so-called "protein folding pressure" can be mostly relieved to facilitate the survival of malignant cells [33].

Since HSF1 essentially controls the expression of all cellular HSP in tumor cells, it has been proposed to be a novel anticancer target with "non-oncogene addiction" features [68]. Dai and colleagues demonstrated that genetic HSF1 deficiency in transgenic mice inhibits tumorigenesis in a mouse skin cancer model. Similar results were also observed in genetic models containing oncogenic mutations of the RAS oncogene or inactivating mutations in the tumor suppressor p53 [14]. Other target validation results from different groups also confirmed that tumor cells have a much greater dependence on HSF1 function compared to normal cells, suggesting that cancer cells are becoming "HSF1 addicted" [43]. Thus, small molecule intervention pf tumor HSR pathway, as monotherapy or combined with other drugs, has been suggested to be a novel avenue for cancer therapeutics [15].

In recent years, efforts have been made to screen and identify small molecule HSR inhibitors [16]. Through various compound screening platforms, such as reporter based high throughput screening, image-based phenotypic screening and chemical SAR approach, more than 18 unique chemical identities or structure derivatives have been discovered with confirmed inhibition of HSR pathway (Fig. 1; [65]). According to Clinincaltrial.gov, two of the natural products, Quercetin [59] and triptolide [49] and their respective prodrugs (QC12 and Minnelide), are in various stages of clinical trial studies (Table 1 and Fig. 2). Seven of these compounds were reported to have positive antiproliferative effects in the mouse xenograft models carrying different tumor cell lines, including breast, skin, colon, hepatoma, and pancreatic cancer lines (Table 2 and Fig. 3). The remaining seven compounds

Table 1 Compounds and their prodrugs targeting HSF1 pathways with human clinical data in cancer treatment

	Chemical property	Mechanism of action (-) inhibition	Animal preclinical study	Human clinical study
Quercetin	Natural product	(-)HSF1 phosphorylation	Oncology Report 2016	Phase 1, Clinical Cancer Research 1996 (Ferry et al.)
			PLOS One 2017	Phase 2 (ID#: NCT03476330)
			Scientific Report 2016	
			Chinese J Cancer Res 2016	
			Apoptosis 2017	
			Cancinogenesis 2000	
			Cancer Prevention Research 2009	
			Molecular Cancer Therapeutics 2008	
QC12	Analog prodrug	A prodrug of quercetin		Phase 1, Ann Oncology 2001 (Mulholland et al.)
Triptolide	Natural product	(-) HSF1 transcriptional activity	Cancer Research 2007	Phase 1, Eur J Cancer 2009 (Kitzen et al.)
			Ann Thorac Surg 2015	
			Acta Pharmacologica Sinica 2015	
			BMC Cancer 2016	
			Molecular Cancer Therapeutics 2003	
			PLOS One 2012	
			Oncology Report 2014	
			Oncotarget 2016	
			Front Oncol 2019	
Minnelide	Analog prodrug	A prodrug of triptolide	Pancreatology, 2016	Phase 1 (NCT01927965), completed in 2016
			J translational Medicine 2019	Phase 1 (NCT03347994), active
			J Clinical Oncology 2016	Phase 1/1b (NCT03129139), active
			PLOS 2017	Phase 2 (MCT03117920), active.
			J Med Chem 2015	
			J. Am. Coll. Surg 2017	



Quercetin QC12 Triptolide Minnelide

Fig. 2 Chemical structures of four inhibitors of HSR pathway with available human clinical trial data. Both quercetin and triptolide are of natural origin. QC12 and Minnelide are prodrugs for quercetin and triptolide, with arrows pointing to the respective hydrolysis sites *in vivo*

Table 2 Compounds targeting HSF1 pathways with animal model data in cancer treatment

	Chemical property	Mechanism of action (−) inhibition	Animal preclinical study	Animal xenograft models
PW3405	Synthetic compound	(−) HSF1 phosphorylation	Manuscript in preparation	Mouse xenograft (pancreatic cancer)
Cantharidin	Natural product	(−) HSF1 transcriptional activity	Cell Physiol Biochem 2017	Mouse xenograft (breast cancer)
			Oncogenesis 2018	Mouse xenograft (pancreatic cancer)
			Environ Toxicol 2016	Mouse xenograft (skin cancer)
			Molecules 2017	Mouse xenograft (hepato-cellular carcinoma)
KNK437	Synthetic compound	(−) HSF1 transcriptional activity	Clinical Cancer Research 2001	Murine squamous cell cancer
			Oncogene 2019	Mouse xenograft colorectal cancer
KRIBB11	Synthetic compound	(−) HSF1 transcriptional activity	JBC 2011	Mouse xenograft (colon cancer)
			Clinical Cancer research 2018a	Mouse xenograft (myeloma)
		Direct binding to HSF1	Clinical Cancer research 2018b	Moust xenograft (myeloma)
			Oncotarget 2017	Mouse orthotopic xenograft (breast cancer)
Fisetin	Natural product	(−) HSF1 binding to HSE	Onco Rep 2017	Mouse orthotopic xenograft (hepatoma cancer)
			Cell Death Dis-ease 2019	Mouse xenograft (pancreatic cancer)
			Carcinogenesis 2015	Mouse xenograft (colon cancer)
			Int J Biol Macromol. 2019	Mouse xenograft (breast cancer)
Rocaglamide A	Natural product	(−) HSF1 binding to HSE	Science 2013	Mouse xenograft (leukemia)
			Am J Transl Res 2016	Mouse xenograft (pancreatic cancer)
			Cancer Letters 2017	Mouse xenograft (myeloma and leukemia)
CCT251236	Synthetic compound	TBD, pirin inhibition	J Med Chem 2017	Mouse xenograft (ovarian cancer)
			Clin Cancer Res 2018	Mouse xenograft (myeloma)

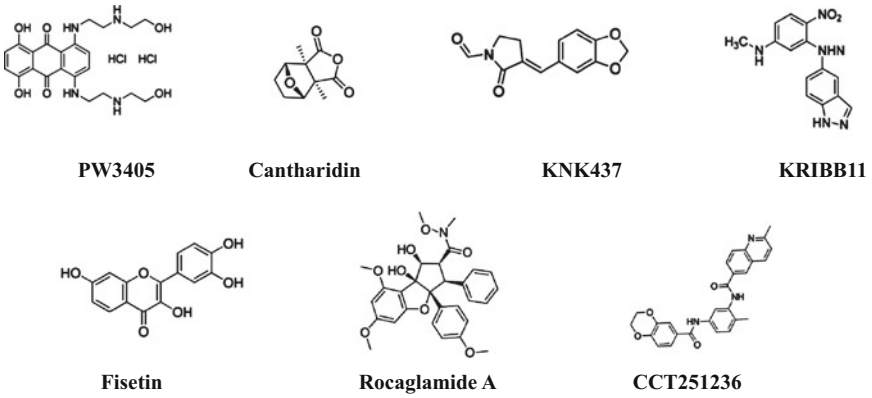


Fig. 3 Chemical structures of seven inhibitors the HSR pathway with data available in various animal xenograft models. Cantharidin, fisetin, rocaglamide A are natural compound products while PW3405, KNK437, KRIBB11, CCT251236 are synthetic compounds

Table 3 Compounds targeting HSF1 pathways with cancer cell line data available

	Chemical property	Mechanism of action (−) inhibition	Cell line study	References
2,4-Bis (4-hydroxybenzyl) phenol	Natural product	Dephosphorylation of HSF1	NCI-H460 human lung cancer line	J Nat Prod 2014
α−acyl amino Carboxamides	Synthetic compound	(−) HSF1 mRNA translation	Multiple Myeloma INA-6 line	J Med Chem 2017
Cardenolide CL-43	Natural product	(−) HSF1 transcriptional activity	HCT-116 human colon cancer line	Oncotarget 2018
$I_{HSF}115$	Synthetic compound	(−) HSP mRNA translation	A panel of 33 cancer lines	Nucleic Acid Res 2017
$iaRNA^{HSF1}$	RNA aptamer	(−) HSF1 binding to HSE	A panel of 5 cancer lines	PLOS One 2014
NZ-28	Synthetic compound	TBD	Multiple Myeloma IS line	Cancer Res 2006
			Prostate carcinoma PC-3 line	
Stresgenin B	Natural product	TBD	A panel of 6 cancer lines	J Antibiot (Tokyo) 1999
4,6-Disubstituted pyrimidine	Synthetic compound	TBD, CDK9 inhibition	Osteosarcoma U2OS line	Medchemcomm 2016

and one RNA aptamer are in the early stage of preclinical studies, with only cell line data available (Table 3 and Fig. 4). More detailed works are required to define the exact mechanisms of actions (MOA) for these compounds. As shown in Fig. 1, the inhibitory mechanisms can be primarily categorized into four types: (1) Inhibition of HSF1 phosphorylation or HSF1 dephosphorization; (2) Interference of HSF1 trimer

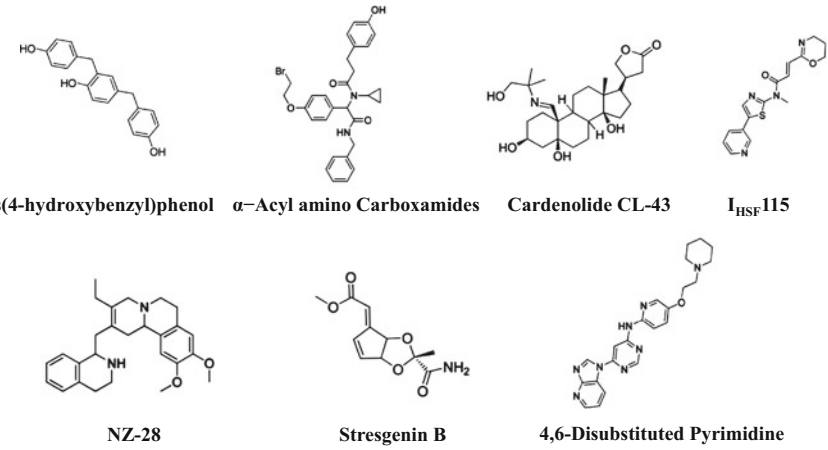


Fig. 4 Chemical structures of seven inhibitors of HSR pathway with data available only in various cancer cell lines. These inhibitors include three natural products (2,4-Bis(4-hydroxybenzyl) phenol, cardenolide CL-43, stresgenin B), four synthetic compounds (α−acyl amino carboxamides, $I_{HSF}115$, NZ-28, 4,6-disubstituted pyrimidine) and one RNA aptamer ($iaRNA^{HSF1}$)

binding to HSE; (3) Blocking HSF1 mediated transcription activation; (4) hampering mRNA translation of HSF1 and HSP. The undefined MOAs and diverse chemotypes make the clinical study of these drug candidates less predictable. In this chapter, the authors reviewed the recent progress and lessons learned from human and animal model studies. Several excellent reviews are also available on the current status of MOAs, chemical structures and screening of HSR inhibitors [16, 28, 65].

2 HSR Inhibitors Currently in Human Clinical Studies (Table 1 and Fig. 2)

2.1 Quercetin

Quercetin is one of the bioactive flavonoids found ubiquitously in fruits, vegetables and beverages [59]. Previous publications established its role as a natural antiproliferative agent against various cancer cell lines from diverse lineages and mouse xenograft models [44]. Using a gel shift assay, Nagai et al. reported that quercetin down-regulates HSF1 by the decrease of HSF1-HSE binding, rather than inhibition of HSF1 trimerization [47]. Yang and colleges used quercetin-loaded liposomes to treat rats with R3230 breast adenocarcinoma. An increase of the tumor destruction/endpoint survival *in vivo* was observed as compared to the control

treatment group [83]. Importantly, this study confirmed that HSF1 is required for quercetin-induced cancer cell death and quercetin can directly down-regulate HSF1. Moreover, quercetin was also tested in other xenograft mouse models, including hepatoma [92], ovarian cancer [39, 40], breast cancer [25, 34, 64], colon cancer [25, 54, 81]. In addition to its inhibitory role in the heat shock response pathway, these animal studies showed that quercetin can also perform its anti-cancer effects through modulating cyclins, pro-apoptotic, autophagy, PI3K/Akt and mitogen-activated protein kinase (MAPK) molecular pathways, etc.

Quercetin has been proposed as a sensitizer and protects non-cancer cells from the side effects of conventional cancer therapies [59]. The safety and potential usefulness of quercetin for cancer treatment have been documented in both animal experiments and a phase I clinical trial. The first clinical trial phase I study was done in the UK [20]. The authors investigated the pharmacokinetics of quercetin with short i.v. infusion and found 1,400 mg/m^2 as the safe bolus dose. The maximum tolerated dose was determined as 1,700 mg/m^2 three weekly. The anti-tumor effects were recorded with confirmed inhibition of lymphocyte tyrosine kinase activity. However, dimethyl sulfoxide was used as a solubilized vehicle and was unsuitable for further clinical development of quercetin.

According to clinicaltrial.gov, several clinical trials of quercetin are currently registered for the treatment of COPD, Hepatitis C and Type II diabetes, etc. Some of these trials are designed to study Quercetin as a dietary supplement for cancer prevention. For monotherapy of Quercetin, a phase II clinical trial is currently active to investigate its effects on chemoprevention for squamous cell carcinoma in patients with Fanconi anemia. Quercetin was designed to be orally administered for a maximum total daily dose of 4000 mg/day for 24 months (ClinicalTrials.gov Identifier: NCT03476330). The clinical data is expected to be available after 2023.

2.2 *QC12 (Analog Prodrug of Quercetin)*

In order to overcome the solubility issue of quercetin, a synthetic chemistry effort was undertaken to produce water-soluble derivatives of quercetin [46]. The most promising candidates of these synthetic compounds, called QC12, was selected. QC12 can be hydrolyzed *in vivo* and releases quercetin and glycine. Unfortunately, QC12 or quercetin was not detected in plasma following oral administration of QC12, confirming it was not orally bioavailable. As a result, QC12 entered the clinical phase I study with i.v. administration. The authors can detect peak QC12 concentration at >100 μM in plasma. Although quercetin was found in all patients following i.v. infusion. The relative bioavailability of quercetin is estimated to be only 20–25% released from QC12. This unsatisfactory Phase I results prevented QC12 for further clinical application [61].

2.3 Triptolide

Triptolide has been reported as one of the most potent inhibitors of heat shock response pathway [10, 49]. Westerheide et al. identified triptolide as an inhibitor of the HSF1 pathway through small molecule screening [76]. Although triptolide does not inhibit the earlier steps in the HSF1 multistep activation process, including trimer formation, hyperphosphorylation, or translocation and binding HSP70 promoter, the inhibitory effects of triptolide on HSP70 expression was reported to be at the level of transcription by interfering with the proper activity of the C-terminal transactivation domain of HSF1. Significantly reduced tumor cell viability was reported after triptolide was incubated with pancreatic cell lines, including PANC-1 and MiaPaCa-2. Triptolide also induces pancreatic cancer apoptosis via inhibition of heat shock protein 70 at mRNA level [52]

A number of mouse xenograft models were adopted to evaluate the anti-cancer effects of triptolide. Some representative examples including breast cancer [35, 36, 79], Prostate cancer [26], lung cancer [57, 58], pancreatic cancer [52], gastric cancer and melanoma [78, 82], etc. Comparing to all known HSR pathway inhibitors, triptolide exhibits a broader spectrum of anticancer activity against the common types of human cancer.

Using a semi-synthetic derivative of triptolide F60008, a phase I and PK/PD study were performed in patients with advanced solid tumors [31]. Twenty patients were enrolled, but hematological side effects were reported such as mild grade anemia. Other mild grade toxicities included constipation, fatigue, vomiting, diarrhea and nausea. Importantly, two lethal events were documented with increased caspase-3 activity and overt apoptosis in neutrophils and monocytes. PK data showed high variability between tested patients. The narrow therapeutic window and undesired water solubility largely limited the clinical application of triptolide [73, 74]. However, the prodrug of triptolide, namely Minnelide, has become an excellent candidate in the clinical study (next). In addition, a recent report on the smart drug delivery system can be an alternative solution to address toxicity issues of triptolide [78].

2.4 Minnelide (Analog Prodrug of Triptolide)

Most human clinical studies of triptolide were performed with its analog prodrug Minnelide. Minnelide is a phosphonooxymethyl prodrug with three times more water solubility than its parent molecule triptolide. Minnelide can be converted into triptolide by phosphatase *in vivo* [51]. Chugh et al. reported that Minnelide is as effective as triptolide in inhibition of pancreatic tumor cells both *in vitro* and *in vivo* [12]. In two mouse xenograft models of human colon adenocarcinoma and ovarian cancer, Minnelide was effective in reducing or eliminating tumors with a well-tolerated safety profile [51]. Banerjee and colleagues reported that Minnelide

induces cell death in a number of pancreatic cancer cell lines and reduces tumor volume in multiple xenograft mouse models, including pancreatic cancer and hepatoma [5]. Similar results were also reported on several pancreatic and melanoma xenograft models [18, 63]. Minnelide also exhibits inhibitory effects of HSP70 on the human gastric tumor xenograft mouse model, both as a single agent and in combination with chemotherapy agent CPT-11 [3]. Using an *in vivo* imaging system, Giri and colleagues reported Minnelide significantly decreased leukemic burden in multiple xenograft models of acute myeloid leukemia at doses easily achievable in patients [23]

The first open-label, phase I, safety clinical trial of Minnelide was completed in patients with advanced gastrointestinal tumors in 2016 (ID: NCT01927965). The primary objective of this study was to determine the maximum tolerated dose and the dose-limiting toxicities of Minnelide. Although no phase I data is currently available, a continued Minnelide phase II study is expected to begin for treating gastrointestinal malignancy [63]. According to Clinicaltrial.gov, there are three active clinical trials of Minnelide in human cancer treatment. A phase 1, open label, pilot study was initiated in 2018 on the pharmacokinetic and pharmacodynamic property of Minnelide with adult patients of AML (ID: NCT03347994). Minnelide capsule was also tested alone or in combination with protein-bound paclitaxel in patients with advanced solid tumor (various cancer types). This open label, phase 1 trial started in 2017 and is expected to be finished in 2021 (ID: NCT03129139). Promising response data from early stage trials has led to a phase II, international open-label trial of Minnelide in patients with refractory pancreatic cancer (ID: NCT03117920, [55]).

Interestingly, all current inhibitors of heat shock response in the clinical trial study are of natural origin or prodrug of natural compounds. Expectations are particularly high for positive results of Minnelide as monotherapy or combined treatment with other cancer drugs. Meantime, more data on animal PK/PD, toxicity, SAR analysis are becoming available for synthetic HSR inhibitors. Human clinical studies of this category of synthetic compounds are likely to be increased in the near future.

3 HSR Inhibitors Currently in Preclinical Animal Studies (Table 2 and Fig. 3)

3.1 PW3405

PW3405 was discovered as a potent heat shock response pathway inhibitor via a large-scale, unbiased, high content image-based screening in our group [89]. This synthetic compound demonstrated a nanomolar potency against HSF1 granulation after heat stress. Our study showed that the decrease of heat shock response is achieved through inhibition of HSF1 phosphorylation at the Ser326 activating site.

Thus, a potential intracellular kinase inhibitory mechanism was proposed [90]. The results from *in vivo* pancreatic cancer PC-3 xenograft models showed encouraging results with reduced tumor volume at a well-tolerated dose schedule (manuscript in preparation). Currently, PW3405 alone, or in combination with chemotherapy agents, are being investigated with several relevant mouse xenograft models. The results will be reported in due course.

3.2 Cantharidin

Cantharidin is a type of terpenoid secreted by the blister beetle Mylabris phalerata. A cell-based screening led to the discovery of this natural compound with potent activity against HSF1 [29]. Li and colleagues reported that cantharidin inhibits the growth of triple-negative breast cancer cells *in vitro* [37]. After the treatment of cantharidin, the tumor growth in MDA-MB-231 and MDA-MB-468 xenografts mice was reduced through inducing apoptosis of tumor cells. Moreover, the combination of cantharidin and antiangiogenic therapeutics presents additive antitumor effects against pancreatic cancer xenografts *in vivo* [80], although an unfavorable proangiogenic side effect was recorded. Anticancer effects of cantharidin were also reported in mouse skin cancer xenografts [38]. Using liposomal encapsulated cantharidin, increased anticancer effects were reported in a HepG2-bearing hepatocellular carcinoma xenograft model [91]. Although cantharidin has been a traditional Chinese remedy, there is no human clinical trial data recorded in clinicaltrial.gov.

3.3 KNK437

KNK437 was first reported as an inhibitor of HSF1 and HSP induction in human colon carcinoma cell [85]. The compound inhibits HSP expression at the mRNA level while it does not increase thermos-sensitivity in nontolerant cells. Since then, several animal tumor studies have been reported. In a mouse transplantable tumor model, Koishi et al. reported that KBK437 can inhibit thermotolerance via the inhibition of HSP72, thus improving the efficacy of clinical fractioned hyperthermia [32]. Another recent study showed KNK437 can inhibit colorectal cancer *in vivo*, with a significant reduction of HSP40 family member A1 expression [84]. Similar to quercetin, KNK437 may not be sufficiently potent for clinical use as high concentrations of compound are required to demonstrate inhibitory activity. The use of such high concentrations of low potency compounds increases the likelihood of off-target effects [53].

3.4 KRIBB11

Yoon and colleagues reported a synthetic compound named KRIBB11 that can directly bind to HSF1 in a western blot analysis [86]. It was proposed that the association of HSF1 and KRIBB11 can further inhibit the transcription process of heat shock proteins. In the same study, KRIBB11 can inhibit the growth of colon cancer cells in BALB/c nude mouse xenograft regression model. These results, particularly the evidence of direct HSF1 binding, promoted *in vivo* animal study of this compound. KRIBB11 was intraperitoneally administrated in a myeloma xenograft model, a significant decrease in tumor volume was observed [22]. At the molecular level, a significant reduction of HSP27 protein expression was documented in the KRIBB11 treated tumor group comparing to the control group. Using an orthotopic xenograft mouse model, KRIBB11 and AKT inhibitor MK-2206 in combination can result in the synergistic killing of breast cancer cells and inhibit tumor growth [7]. Recently, Parekh reported that KRIBB11 exhibits primary myeloma cell killing and cytotoxicity in stromal coculture, thus eliminating tumor cell protection inside the bone microenvironment in myeloma [50]. Although KRIBB11 exhibits modest *in vivo* efficacy in xenograft models as a single agent, its efficacy of HSF1 inhibition may be further improved by rational combination therapy.

3.5 Fisetin

Kim and colleagues reported their results of fisetin, a dietary flavonoid, on its inhibitory activity of HSF1 [30]. The downregulation of HSP70, HSP27 and BAG3 by fisetin significantly reduces the cellular levels of Bcl-2, Bcl-xL and Mcl-1 proteins, followed by apoptotic tumor cell death. Further analysis indicated that fisetin inhibits HSF1 by blocking the binding of HSf1 to HSP70 promoter. Treatment of colon cancer xenograft mice model with fisetin caused inhibition of HCT-116 cell growth *in vivo*. Another example is the results from a mouse liver cancer model. The orthotopically implanted tumors were inhibited by fisetin with a prolonged survival rate [39, 40]. Recently, a mouse xenograft model with luciferase expression in human pancreatic PANC-1 tumor cells was reported [27]. Bioluminescence can be emitted after the injection of luciferin intraperitoneally in a living mouse. Using this noninvasive method, tumor volume was recorded kinetically with significant size reduction in the fisetin treated group as compared to the control group. In an effort to improve the solubility and therapeutic index of fisetin, poly (lactic acid) nanoparticles loaded with fisetin were also developed. The data showed that drug-loaded nanoparticles were superior to that of free drug solution when tested against HCT116 colon cancer cells *in vitro* and antitumor test in a xenograft 4 T1 breast cancer model *in vivo* [19].

3.6 Racoglamide A

Santagata et al. adopted a well-designed reporter-based assay to screen for inhibitors of HSF1 activation. With a diversified library of 300,000 compounds from the NIH Molecular Libraries Probe Center Network, racoglamide A was identified as an inhibitor of HSF1 activation with nanomolar potency against multiple cancer cell lines [62]. Importantly, racoglamide A can significantly suppress tumor growth in an M0–91 acute myeloid leukemia xenograft mouse model with no evidence of systemic toxicity. In another study, the combination of racoglamide A and a human circularly permuted TRAIL (CPT) exhibits an efficient treatment towards mice xenografted with the CPT-resistant human acute T-cell leukemia cell line Molt-4 [77]. Furthermore, racoglamide A was reported to reduce the tumor size in a patient-derived pancreatic cancer xenograft mouse model without noticeable toxicity *in vivo* [73, 74]. Since racoglamide A is a natural Chinese herb compound with a relatively safe profile, this inhibitor or its derivatives may possibly advance to human study pending more efficacy data in animal models.

3.7 CCT251236

Using an image-based phenotypic screen, Cheeseman et al. reported the discovery of a new chemical probe, bisamide (CCT251236) as a potent inhibitor of the HSF1 stress pathway [9]. Efforts have been made to make analogs to improve solubility and bioavailability of this bisamide compound series with satisfactory mouse pharmacokinetics data. Importantly, CCT251236 displays efficacy in a human ovarian carcinoma xenograft model. In addition. CCT251236 demonstrates relatively low toxicity and was well tolerated in a mouse multidose tolerability study. The Pirin protein was proposed to have a possible role in the bisamide phenotype and the cellular effects of modulating the HSF1 pathway [9, 11]. In addition, significant anti-myeloma efficacy was observed in a myeloma xenograft mouse model after the treatment of CCT251236 through oral administration [50]. Although this group of HSR inhibitors has shown promising results in different xenograft mouse models, more animal model data, including PK/PD, MOA and biomarker studies, are essential for their advancements to the human clinical trial. Recent progress on nanoparticles and prodrugs can accelerate this translational process.

4 HSR Inhibitors in Early Preclinical Studies (Cancer Cell Lines, Table 3 and Fig. 4)

Seven compounds and one RNA aptamer were reported with antiproliferative effects in various cancer cell lines (Table 3), although publicly available data are limited for many compounds in this group. 2,4-Bis(4-hydroxybenzyl) phenol was discovered to

induce the dephosphorylation of HSF1 at Ser326 [87], a similar HSF1 inhibitory mechanism as PW3405 [89]. This compound can induce growth arrest and apoptosis of NCI-H460 human lung cancer cells. Using a smart synthetic library, Bach et al. found another HSR inhibitor, namely alpha-acyl aminocarboxamides, which can induce apoptosis in multiple myeloma cells [4]. Cardenolide CL-43, a natural compound, was identified through a heat-shock element-luciferase reporter system [48]. CL-43 can effectively inhibit the levels of all major HSP in the HCT-116 colon cancer line with no cytotoxicity observed in human fibroblasts.

An *in-silico* screening of lead-like library along with a cell-based assay led to the discovery of compound $I_{HSF}115$ [70]. This compound can bind to an isolated HSF1 DNA binding-domain fragment *in vitro* and inhibit its transcriptional activity. $I_{HSF}115$ exhibits a broad anticancer capacity in a panel of 33 cancer lines, with high sensitivity observed in multiple myeloma lines. In another study, Zaarur et al. identified NZ28 after screening of 20,000 compounds from several diversity compound libraries [88]. This compound potently inhibits the induction of HSP by heat shock, proteasome, and Hsp90 inhibitors in a variety of cell lines. An NZ28 analog, called emunin, strongly sensitizes myeloma cells to proteasome and HSP90 inhibitors as well as prostate carcinoma cells to proteasome inhibitors. Importantly, both NZ28 and emunin cause potent inhibition of HPS72 induction after heat shock in all tested cell lines.

Another phenotypic screen of 200,000 small molecules identified 4,6-disubstituted pyrimidines as a potent inhibitor of the HSF1 stress pathway [60]. Efforts on SAR and analog analysis led to the improvement of the HSF1 pathway inhibition to 14 nM potency with a U2OS human osteosarcoma tumor cell line. Interestingly, biochemical data showed high binding affinity (sub-micromolar to the single-digit micromolar range) of selected 4,6-pyrimidines to CDK9, suggesting possible roles of CDK9 in the inhibitory function of HSR. Unfortunately, this chemical series showed high clearance in mouse pharmacokinetic experiments, which were unsuitable for progression into the animal model study. Lastly, Stresgenin B was isolated as an inhibitor of the HSR pathway from a culture broth of Streptomyces sp. AS-9. This natural product showed inhibition of heat-induced reporter gene expression, including HSP70, HSP 90 and HSP110. Significant cytotoxicity against a panel of 6 cancer lines was documented with single to double-digit micromolar potency [1]

In summary, inhibitors in this category are either in their early stage of the preclinical study or have chemical liability (such as PK issues) that prevent them from entry into animal and human testing. It is also possible that the relevant animal data have not become publicly available at this point.

5 Lessons Learned from Preclinical and Clinical Studies

5.1 Improvement of Therapeutic Window by Prodrug Design and Nanoparticle Packaging

Many inhibitors in the HSR pathway show unfavorable chemical properties (low solubility and bioavailability) with a narrow therapeutic window. Treatments with

increased dosages usually lead to undesirable cytotoxicity. Recent advancements of prodrug design research make it possible to intentionally design current HSR inhibitors as prodrugs for improved therapeutic effects [56]. FDA has already approved at least 30 prodrugs, providing a promising direction for the prodrug development of HSF1 pathway inhibitors. On the other hand, some of the inhibitors are of natural origin with significant challenges in the medicinal chemistry approach and prodrug design. Nanoparticles or liposome encapsulation can be introduced for the packaging of these natural compounds with improved efficacy [72]. With the advance of cancer biomarker research and imaging technology, this type of nanomedicines can be better targeted to cancerous tissues with precision.

5.2 *Combined Application with Chemotherapy Drugs or Drugs against a Different Cancer Target*

Shevtsov et al. recently published an excellent review on combination therapy of current anticancer drugs with molecular chaperone inhibitors [66]. An optimal drug combination has been proposed to simultaneously target cytoprotective mechanisms (i.e., heat shock response pathway) and malignant proliferation drivers (oncogenes, signal transduction players, cell cycle players, etc.) Such a combination strategy can allow these drugs to act synergistically while reducing doses of individual drugs and related unfavorable side effects [42]. Several HSR inhibitors have been applied to combinational therapy with synergistic anticancer effects. For example, Xiong et al. reported that triptolide can significantly enhance the antiproliferative effects of doxorubicin in human breast cancer line MCF-7 and MDA-MB-468 [79]. In combination with curcumin, triptolide also significantly reduces tumor cell proliferation in the ovarian cancer SKOV-3 line [41]. KBIBB11 and CL-43 also exhibit similar synergistic effects when combined with AKT inhibitor [7] and conventional chemotherapy agents [48]. Thus, screening for the best combination of these drugs may be the quickest way to create a novel cancer therapy with HSR pathway inhibitors.

5.3 *Target Validation and Use of RNA Aptamer*

Due to the complex nature of HSR pathways, the underlying molecular mechanisms for the majority of HSR pathway inhibitors are not clearly identified. Only two inhibitors, namely KRIBBII and $I_{HSF}115$, demonstrate direct biochemical binding evidence to HSF1 *in vitro*. More detailed target identification studies are essential for the success of future drug development. Alternatively, recent publications on the RNA aptamer approach can open a different avenue to address this critical issue [61]. The complex tertiary folded structure of RNA aptamer makes it possible to achieve superior binding affinity and selectivity for a presumed cellular target. Currently, one aptamer drug has already been approved by the FDA [69], so more advanced projects can be initiated in this promising area [45].

5.4 *HSF1 as a Potential Biomarker for Clinical Trial Design and Prognosis Prediction*

It has been reported that elevated levels of HSF1 are generally associated with poor prognosis [8]. After analysis of over 3000 cancer patient samples, Wan and colleagues reported HSF1 overexpression as an unfavorable prognostic biomarker for a number of solid tumors, including breast cancer, hepatocellular carcinoma, non-small-cell lung cancer and pancreatic cancer, but not in osteosarcoma [71]. The recent advances in high-resolution imaging at single cell level, in combination with new molecular probes of cell types and metabolic states, will allow sensitive detection of HSF1/HSP expression of tumor samples in real time [13]. Thus, HSF1 pathway biomarkers can enable the development of tools for early cancer diagnosis, drug response prediction and therapeutic monitoring. Future clinical trial designs will particularly benefit from the better translation of basic science insights at the single cell level towards precision cancer treatment [24].

6 Conclusions

Significant progress have been made in the drug discovery and development of small molecule inhibitors against HSR pathways. Along with RNA aptamers, prodrugs, nanoparticles and combined therapy approaches, novel HSF1pathways-based cancer therapeutics will be created with tremendous clinical values.

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