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Invited Review Article

The current status and future prospects for therapeutic targeting of KEAP1-NRF2 and β-TrCP-NRF2 interactions in cancer chemoresistance

Rohini Srivastava [a], Raquel Fernández-Ginés [b], José Antonio Encinar [c], Antonio Cuadrado [b], Geoff Wells [a,*]

[a] UCL School of Pharmacy, University College London, 29/39 Brunswick Square, London, WC1N 1AX, UK
[b] Centro de Investigación Biomédica en Red Sobre Enfermedades Neurodegenerativas (CIBERNED), Instituto de Investigación Sanitaria La Paz (IdiPaz), Department of Biochemistry and Instituto de Investigaciones Biomédicas Alberto Sols UAM-CSIC, Faculty of Medicine, Autonomous University of Madrid, Madrid, Spain
[c] Institute of Molecular and Cell Biology (IBMC), Miguel Hernández University (UMH), Avda. Universidad s/n, Elche, 03202, Spain

A B S T R A C T

Drug resistance is one of the biggest challenges in cancer treatment and limits the potential to cure patients. In many tumors, sustained activation of the protein NRF2 makes tumor cells resistant to chemo- and radiotherapy. Thus, blocking inappropriate NRF2 activity in cancers has been shown to reduce resistance in models of the disease. There is a growing scientific interest in NRF2 inhibitors. However, the compounds developed so far are not target-specific and are associated with a high degree of toxicity, hampering clinical applications. Compounds that can enhance the binding of NRF2 to its ubiquitination-facilitating regulator proteins, either KEAP1 or β-TrCP, have the potential to increase NRF2 degradation and may be of value as potential chemosensitising agents in cancer treatment. Approaches based on molecular glue-type mechanisms, in which ligands stabilise a ternary complex between a protein and its binding partner have shown to enhance β-catenin degradation by stabilising its interaction with β-TrCP. This strategy could be applied to rationally discover degradative β-TrCP-NRF2 and KEAP1-NRF2 protein-protein interaction enhancers. We are proposing a novel approach to selectively suppress NRF2 activity in tumors. It is based on recent methodology and has the potential to be a promising new addition to the arsenal of anticancer agents.

1. Introduction

Cancer continues to pose a serious threat to human health and life, and is a leading cause of mortality worldwide, accounting for nearly 10 million deaths in 2020 [1]. Statistical reports indicate that over 90% of cancer-related deaths are attributed to drug-resistance, which represents the main obstacle to achieving cures in patients [2]. Chemoresistance occurs in nearly all types of cancer and across different modes of treatment, including conventional and targeted therapy [3]. Resistance can be classified as either intrinsic or acquired, depending on the time when it is developed, and both types can significantly reduce drug efficacy. Intrinsic resistance is defined as the innate resistance which exists prior to drug administration and is mainly acquired by two key mechanisms: (a) inherent genetic mutations in tumors that decrease their sensitivity to therapy and (b) activation of intrinsic pathways that contribute to detoxification of the drug [4]. Acquired resistance, on the contrary, is induced after prolonged exposure to the anti-cancer agent, despite an initial positive response to the treatment. Its underlying causes are multifactorial, affected by both tumor biology and microenvironment [5]. The main mechanisms include: (a) decreased drug uptake, (b) enhanced drug extrusion via efflux transporters, (c) drug metabolism and inactivation, (d) improved DNA damage repair and (e) evasion of programmed cell death [6].

In recent years, an imbalance in redox homeostasis has been determined as a critical factor in the development of cancer chemoresistance [7]. Numerous studies have investigated the link between oxidative stress and cancer, elucidating the pivotal role of reactive oxygen species (ROS) in regulating tumor progression. ROS acts as a double-edged sword in cancer (Fig. 1). While a small increase in oxidative stress promotes tumorigenesis by helping cancer cells grow and survive, high

Abbreviations: **ATRA**, All-trans retinoic acid; **BCL6**, B-cell lymphoma 6; **BTB**, Broad-complex, Tramtrack and Bric-à-brac; **CDK12**, Cyclin-dependent kinase 12; **CK1α**, Casein kinase 1α; **CNC**, Cap'n'Collar; **CRBN**, Cereblon; **CyP**, Cyclophilin; **DCAF15**, DDB1 and CUL4 associated factor 15; **HIF**, Hypoxia-inducible factor; **HTT**, Huntingtin protein; **IkBα**, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; **IKZF**, Ikaros family zinc finger; **KEAP1**, Kelch-like ECH-associated protein 1; **LC3**, Microtubule-associated protein 1A/1B light chain 3; **mTOR**, Mammalian target of rapamycin; **NRF2**, Nuclear factor erythroid 2-related factor 2; **RBM39**, RNA-binding motif protein 39; **RBX1**, RING-box protein 1; **SCF**, Skp1-cullin 1-F-box; **SIAH1**, Siah E3 Ubiquitin Protein Ligase 1; **sMaf**, Small musculoaponeurotic fibrosarcoma; **TIR1**, Transport inhibitor response 1; **β-TrCP**, Beta-transducin repeat-containing protein.

* Corresponding author.
E-mail address: g.wells@ucl.ac.uk (G. Wells).

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basal ROS levels activate different cell death pathways and limit further progression [8]. Hence, unlike normal cells, chemoresistant cancer cells have evolved to upregulate their antioxidant capacity to counterbalance intrinsic or drug-induced oxidative stress. The antioxidant response that enables cancer cells to defend themselves from stress conditions, is principally mediated by the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), which is a basic leucine zipper protein and member of the human cap'n'collar (CNC) family [9].

NRF2 is widely recognised as a master regulator of cellular cyto-protective responses induced by oxidative, metabolic or xenobiotic stress [10–12]. In the nucleus, NRF2 heterodimerises with small musculoaponeurotic fibrosarcoma (sMaf) proteins through its Neh1 domain and facilitates transcription (Fig. 2). It does so by binding to an enhancer sequence termed the antioxidant response element (ARE), situated within the promoter regulatory region of a specific set of target genes that encode a network of enzymes with antioxidant and detoxifying roles [14]. It is well-known that under normal physiological conditions, the ETGE and DLG motifs in the NRF2-Neh2 regulatory domain, enable its binding to the Kelch domain of the E3 ubiquitin ligase substrate adaptor Kelch-like ECH-associated protein 1 (KEAP1) [13,14]. This targets NRF2 for degradation by the ubiquitin proteasome pathway and ensures low abundance of this protein. It was also found that NRF2 levels are negatively regulated by β-transducin repeat-containing protein (β-TrCP) when its WD40 domain binds to one of two potential serine phosphorylated motifs in the NRF2-Neh6 domain, DSGIS and DSAPGS, leading to NRF2 ubiquitination and degradation [15,16]. Moreover, glycogen synthase kinase-3β (GSK-3β), a serine threonine protein kinase, has shown to modulate the activity of one of the β-TrCP binding motifs on NRF2. It catalyzes the phosphorylation of the DSGIS site and creates a phosphodegron, onto which β-TrCP is recruited and can tightly bind [17,18].

NRF2 has a dual role in tumorigenesis [8,19]. When normal cells are exposed to electrophiles or ROS, NRF2 is transiently activated (Fig. 3). Under stress conditions, electrophilic compounds react with thiol moieties of cysteine residues on KEAP1, rendering the protein temporarily ineffective for complexation [20,21]. This allows newly synthesized NRF2 to translocate into the nucleus, where it induces transcription of its target genes that enhance detoxification and antioxidation capabilities within the cell. In this manner, NRF2 protects normal cells from various oxidative insults, including chemical-induced carcinogenesis. The pro-oncogenic functions of NRF2 are observed in tumors where NRF2 is upregulated, most notably in those of the lung, liver, head and neck, ovary and stomach [22]. In these cancer cell types, somatic mutations in either NRF2 or its regulator proteins, prevent effective repression of NRF2 levels and allow the protein to confer cytoprotection to cancer cells [23–27].

In these NRF2-addicted cancer cells, KEAP1 is either deleted or expressed at very low levels and NRF2 is overexpressed and constitutively activated. As a result, NRF2 enables tumor cells to adapt to the hostile microenvironments resulting from chemo- and radiotherapy [28–30]. Constitutive hyperactivation of NRF2 confers a survival advantage to NRF2-addicted tumors and helps them develop into more aggressive, malignant and chemoresistant cancers by upregulating their antioxidant defences to counteract oxidant species. NRF2 exerts its pro-oncogenic effects through suppression of apoptotic cell death mechanisms and by stimulating proliferation, metastasis, angiogenesis and drug resistance [31–35]. Furthermore, it prevents oxidative-induced genetic damage to cancer cells through metabolic alterations that accelerate both biotransformation and clearance of DNA-damaging agents. Cancers with high NRF2 levels are generally associated with poor prognosis because of increased resistance to anti-cancer therapy. Thus, NRF2 signalling is protective in early stages of tumorigenesis but detrimental in later stages. Therefore, enhancing NRF2 activity remains an important approach for cancer chemoprevention whereas NRF2 inhibition is desirable for cancer chemosensitisation in the treatment of drug-resistant cancers [36].

Awareness of the dichotomous role of NRF2 paved the way for discovery of compounds that can disrupt NRF2 signalling to tackle NRF2 addiction in many drug-resistant cancers, with an aim to decrease their tumorigenic capacity and antioxidant activity [5,29,37,38]. While a promising therapeutic approach, the progress in the field of NRF2 inhibitors has been fairly limited as most compounds developed lack specificity and are associated with a high degree of toxicity that hampers their progression into the clinic [39]. Hence, there is an urgent unmet medical need to develop safe and effective NRF2 inhibitors to combat therapy resistance in cancer [40]. In this review, we aim to (1) provide a brief overview of the progress in the field of NRF2 inhibitors, discussing the challenges and limitations encountered thus far, and (2) focus on summarizing the latest methods of targeted protein degradation involving proteolysis-targeting chimeras (PROTACS) and molecular glues in order to draw inspiration and apply some of these techniques to degrade NRF2 in the future.

2. Development of NRF2 inhibitors for cancer treatment

The Cancer Genome Atlas (TCGA) database identified 226 unique NRF2-mutant tumors among 10,364 cases, with gain-of-function NRF2 mutations occurring in 21 out of 33 tumor types [41–43]. Moreover, loss-of-function mutations in KEAP1 have also been found in several cancers such as lung cancers, whose aetiology is driven by environmental factors [29]. This suggests a potential benefit of NRF2 inhibitors in the treatment of chemoresistant tumors and has triggered a search for these agents. The following section provides a brief summary of the most promising NRF2 inhibitors described in the literature thus far (Fig. 4).

2.1. Natural compounds

Historically, several compounds of natural origin have been reported to inhibit NRF2 [8]. Brusatol (Fig. 4A) was one of the first natural compounds studied. This quassinoid, extracted from the plant *Brucea javanica*, sparked initial interest as it reduced the expression of an ARE-luciferase reporter and sensitized a broad spectrum of tumor cell

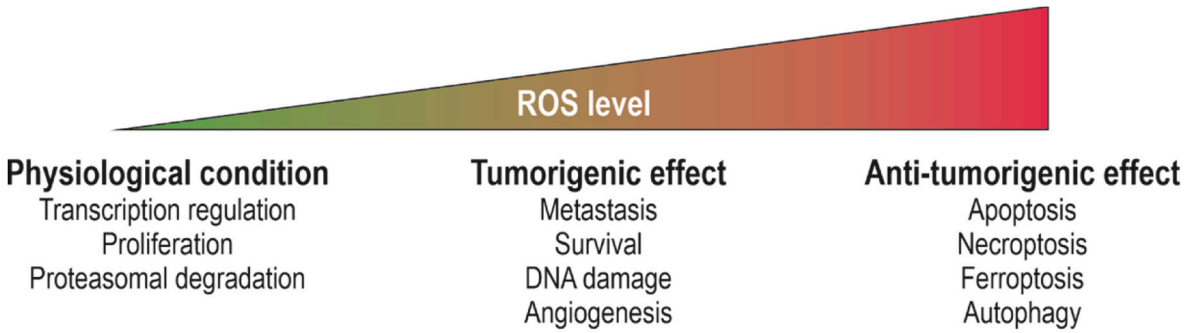

Fig. 1. ROS acts as a double-edged sword in cancer.

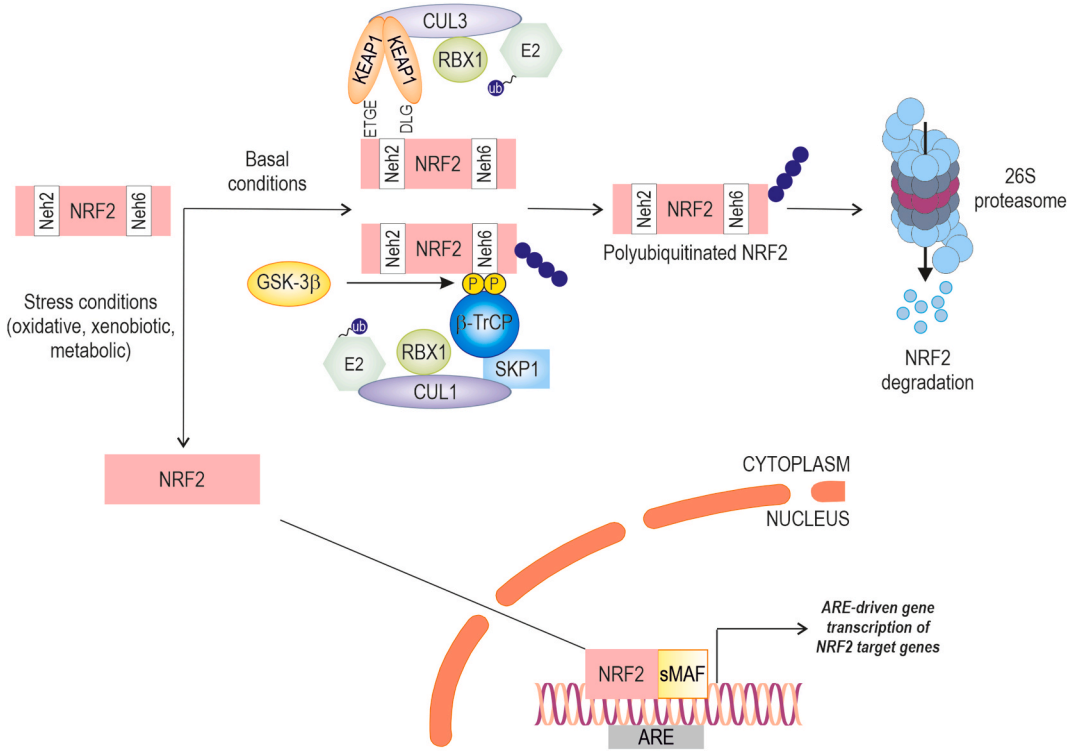

Fig. 2. NRF2 regulatory and signalling pathways. Under basal conditions, NRF2 levels are strictly controlled by KEAP1, which forms an E3 ubiquitin ligase complex with Cullin3 (Cul3) and Ring box protein-1 (RBX1), thereby promoting NRF2 ubiquitination and degradation through the proteasome pathway. NRF2 is also negatively regulated by the E3 ubiquitin ligase complex β-TrCP-SKP1-Cullin1(Cul1)-RBX1. Phosphorylation of DSGIS binding motif by GSK-3β enables recruitment and tight binding of β-TrCP complex to NRF2. NRF2 levels increase when cells are exposed to stressors such as electrophiles or ROS. NRF2 then translocates into the nucleus, forms heterodimers with sMaf proteins and binds to antioxidant response elements (AREs), activating the transcription of ARE-driven genes.

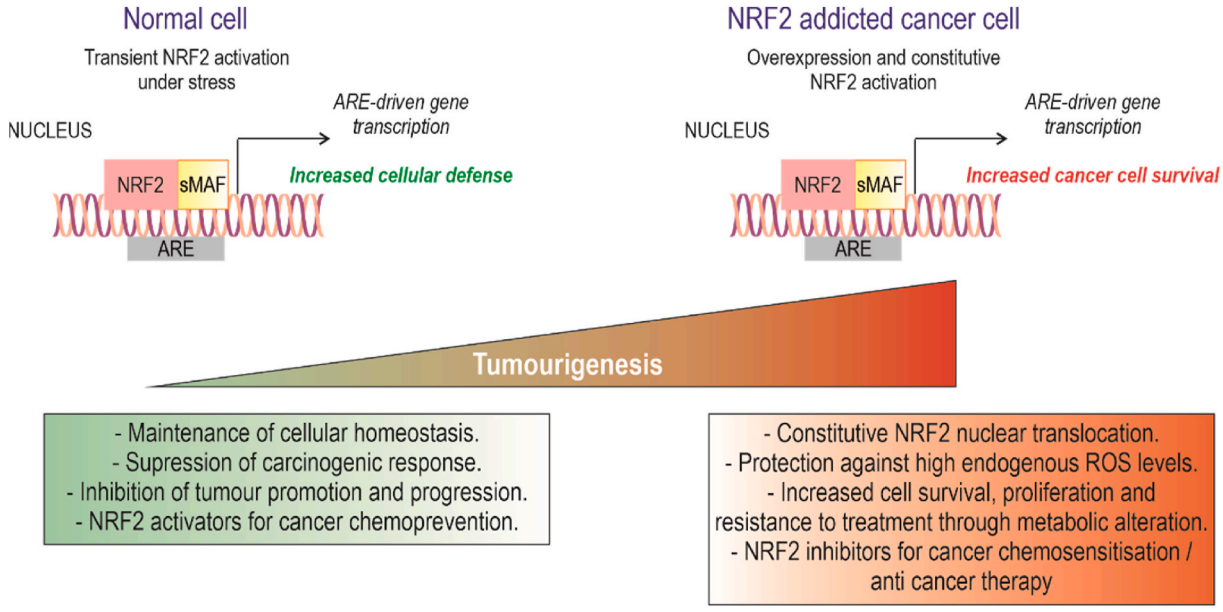

Fig. 3. Dual role of NRF2 in cancers: NRF2 confers anti-carcinogenic activity in normal cells by preventing their progression to tumor cells and cancer metastasis. It does so by eliminating ROS, carcinogens and other DNA-damaging agents. Somatic mutations in NRF2 or its regulatory proteins leads to constitutive overactivation of NRF2 which helps malignant cells adapt to high levels of endogenous ROS and avoid apoptosis. Downstream activation of cytoprotective and metabolic genes enhance cancer cell survival and growth, thereby rendering them resistant to therapy.

lines to several chemotherapeutics, including cisplatin, in culture and in xenografts [44]. Brusatol exerts antitumor effects through inhibition of NRF2 expression and transcriptional activity, resulting in suppression of cell proliferation and weakening of antioxidant defenses due to NRF2 depletion. However, brusatol is now recognised to be a general inhibitor of protein translation and synthesis [45,46]. As a result, it preferentially inhibits short-lived proteins, including but not limited to NRF2 and hence its mechanism of action is not specific. A similar concern was

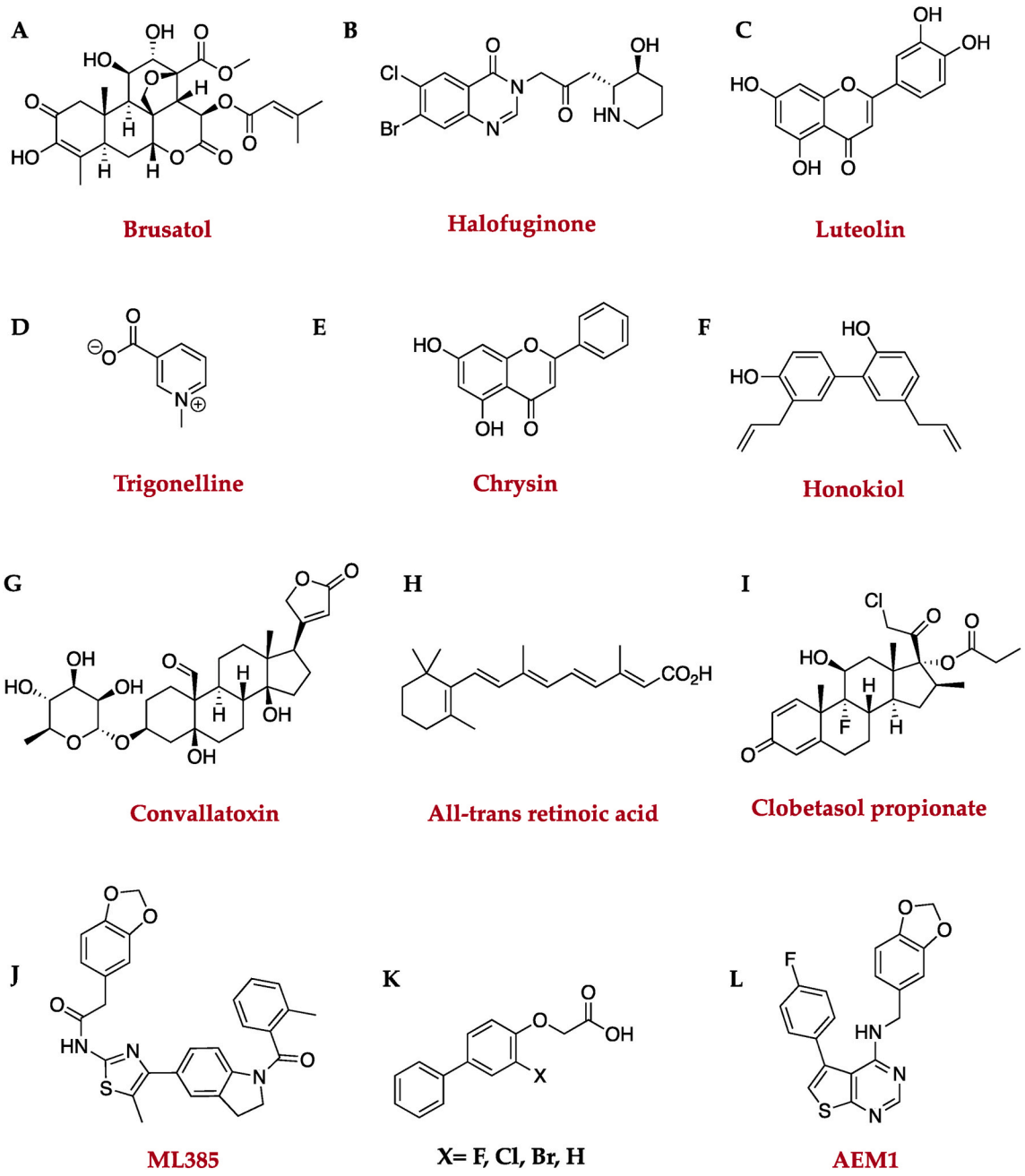

Fig. 4. Chemical structures of some representative NRF2 inhibitors. Some of the earlier compounds were: quassinoid brusatol (**A**), quinazolinone derivative halofuginone (**B**), flavonoid luteolin (**C**) and coffee alkaloid trigonelline (**D**) and nuclear receptor agonists all-trans retinoic acid (**H**) and clobetasol propionate (**I**). More recently, a thiazole-indoline compound ML385 (**J**) was found to interact with the C-terminal domain of NRF2, thereby preventing NRF2-sMaf heterodimer formation. An NRF2-Neh1 binder (**K**) identified from an NMR-based fragment screen binds at the DNA-binding interface and has shown promise in directly inhibiting NRF2-DNA interaction. AEM1 (**L**) is a benzodioxole substituted thienopyrimidine found to inhibit NRF2 transcriptional activity. Most recently discovered natural product based Nrf2 inhibitors include the flavone chrysin (**E**), lignan honokiol (**F**) and glycoside convallatoxin (**G**) among others.

raised after the identification of halofuginone (Fig. 4B), a synthetic derivative of febrifugine that has a very similar mechanism of action to brusatol [47]. Halofuginone was found to reverse the radioresistance of Lewis lung cancer cells, while improving the chemosensitivity of oesophageal and non-small cell lung cancer (NSCLC) cells to cisplatin *in vitro* and in *vivo* [48]. It induces cellular amino acid starvation that leads to repression of global protein synthesis, rapidly depleting NRF2 along with other proteins [37,39].

The flavonoids luteolin (Fig. 4C) and wogonin were reported to sensitize cells to a range of anticancer drugs by potentiating their cytotoxicity [7]. Initial investigations revealed that their mechanism of action involved accelerating the turnover of NRF2 mRNA and eliciting a strong reduction in levels of NRF2 mRNA and protein [38,49]. Further studies elucidated that wogonin promotes increased ROS production and reduces NRF2 nuclear translocation [50]. However, later work on both compounds yielded conflicting results and indicated that these flavonoids may also elicit NRF2 activation, thereby raising concerns on their value as NRF2 inhibitors [51–53]. Moreover, the coffee alkaloid trigonelline (Fig. 4D) was shown to inhibit the nuclear translocation and accumulation of NRF2 and strongly supress its activity in several pancreatic cancer cell lines [54]. Trigonelline was also found to reduce NRF2-dependent proteasomal gene expression and activity rendering

pancreatic cells more susceptible to apoptosis [55]. While the compound is emerging to be a promising molecule, its effects need to be addressed in a wider range of cell types before being taken to preclinical studies and further development.

Furthermore, apigenin, a natural bioflavonoid was found to strongly sensitize a doxorubicin-resistant hepatocellular carcinoma cell line BEL-7402/ADM to doxorubicin by downregulating the PI3K/Akt/NRF2 and miR-101/NRF2 pathways, along with inducing caspase-dependent apoptosis [56–58]. Apigenin also sensitizes pancreatic cells to chemotherapy. In addition to NRF2 inhibition, apigenin is also involved in other molecular pathways associated with vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF) and glucose transporter 1 (GLUT-1) [56,59,60].

More recently, natural product-based compounds such as procyanidins, chrysin (Fig. 4E), oridonin, honokiol (Fig. 4F), berberine and parthenolide have shown to have inhibitory activity against NRF2 in addition to a range of other targets [56,61–64]. Notably, convallatoxin (Fig. 4G) has emerged as a novel and potent NRF2 inhibitor from a screening of 644 natural compounds. This cardenolide glycoside is extracted from *Convallaria majalis* and the trunk bark of *Antiaris toxicaria* and is known for acting as a Na+/K + -ATPase inhibitor but has been recently been reconsidered in cancer research [65]. Convallatoxin's mechanism of action involves promoting GSK-3β/β-TrCP-dependent NRF2 degradation but KEAP1-independent proteolysis of NRF2. Its suppression of NRF2 is regulated at the level of proteolysis and is not transcriptionally mediated. Importantly, convallatoxin sensitized A549 cells to 5-fluorouracil-induced apoptosis, elucidating that this natural compound might be a promising chemotherapeutic adjuvant in treating NSCLC [66].

2.2. Agonists of nuclear receptors

All-*trans*-retinoic acid (ATRA) and bexarotene, agonists of the nuclear receptors retinoic acid receptor alpha (RARα) and retinoid X receptor alpha (RXRα), have shown to antagonize the expression of NRF2 target genes by inhibiting the transcriptional activity of NRF2 in an ARE-reporter cell line and in the small intestine of mice fed a diet deficient in vitamin A [67,68]. The vitamin A metabolite, ATRA (Fig. 4H), supresses activation of NRF2 pathway by inducing RARα expression, which in turn appears to form a protein complex with the Neh7 domain of NRF2 [40]. As a result, retinoids are found to prevent NRF2 binding to the ARE enhancer, which markedly reduces its ability to mediate induction of ARE-driven genes. Early studies on solid tumors have shown that ATRA sensitizes chemoresistant neuroblastoma cells to the proteasome inhibitor Bortezomib [56,69].

Upon ligand-mediated receptor activation and binding to the glucocorticoid response element, agonists of the glucocorticoid receptor, such as dexamethasone and clobetasol propionate (CP) inhibit NRF2 by blocking its transcriptional activity or preventing its nuclear translocation, respectively [36]. CP (Fig. 4I) was identified as the most potent NRF2 inhibitor from a clinical drug library screen with potential therapeutic efficacy in KEAP1 mutant lung cancer [70]. Mechanistically, CP prevents nuclear accumulation of NRF2 in a GSK3- dependent manner and promotes β-TrCP-dependent proteasomal degradation of the transcription factor. CP was introduced in a phase 2 clinical trial (NCT02368886-currently ongoing), along with the multi-kinase inhibitor regorafenib, in treating patients with refractory metastatic colorectal cancer [8]. The pharmacological value of these mechanisms of NRF2 inhibition is limited by the fact that these pathways of regulation through nuclear receptors are not specific for NRF2.

2.3. Other approaches

A quantitative high-throughput screening (HTS) of a chemical library containing 400,000 molecules and a subsequent medicinal chemistry optimisation led to the identification of ML385 as an NRF2 inhibitor [71]. This thiazole-indoline compound (Fig. 4J) was found to bind to the carboxy-terminal domain of NRF2 and interfere with its interaction with sMaf, preventing the protein heterodimer formation which is essential for activation of ARE-driven gene expression [37]. ML385 was shown to enable KEAP1-deficient NSCLC cells to overcome resistance to carboplatin and other chemotherapeutics. It substantially enhances cytotoxic effects of doxorubicin or Taxol in NSCLC [72]. While a promising strategy to supress NRF2 transcriptional activity, additional work is needed to ascertain whether ML385 is selective for the NRF2–sMaf pair or also affects other basic-region leucine zipper transcription factors involved in chemoresistance [31,46]. Moreover, this compound also elicits a reduction in levels of NRF2 protein, suggesting that additional mechanisms of NRF2 regulation might be present [29]. ML385 is the only compound whose putative mechanism of action involves direct binding to NRF2, however, no structural characterisation of the interaction has been undertaken yet [73].

A recent study utilised a fragment-based NMR screening approach to discover molecules that bind to NRF2 at the DNA binding interface but at alternative binding sites to the leucine zipper motif region, which is a common feature in many transcription factors [74]. This led to the discovery of several initial small-molecule fragment hits with binding affinities in the milli-molar range, all containing a core biphenyl phenoxy-acetic acid scaffold (Fig. 4K). Upon establishing their structure-activity relationship (SAR), the binding pose of one of the key compounds was determined by a chemical shift perturbation restrained docking simulation and can serve as a starting point for a hit-to-lead campaign in the future. Further work needs to be done to carry out structure-guided optimisation of these NRF2 hits. Lastly, AEM1, a benzodioxole substituted thienopyrimidine NRF2 inhibitor (Figure 4L), has shown to sensitize KEAP1-deficient A549 lung tumor cells to Etoposide and 5-fluorouracil [75]. However, its exact mechanism of action beyond inhibition of NRF2 transcriptional activity is unknown and does not appear to be specific for NRF2 inhibition [71].

2.4. Limitations of current NRF2 inhibitors

Inhibitors of NRF2 are being actively pursued but are not yet in clinical trials [76]. They have the potential to have a significant impact on cancer therapy, however the field is less advanced. In all cases described above, either the mechanism of inhibition is poorly understood or the compounds themselves do not have a very well-defined target, leading to lack of selectivity and often toxicity issues [39]. Despite the increasing demand for negative modulators of NRF2, selective inhibitors are yet to be developed and those under investigation are far from progressing into clinical trial evaluation.

The paucity of NRF2 inhibitors is mostly due to its lack of a well-defined three-dimensional structure, which has hampered structure based drug discovery and *in silico* analysis of compounds [77]. The current Alphafold model [78] of NRF2 (Fig. 5) has a low confidence of prediction but is consistent with it being a disordered protein. In future, further improvements to the model with additional experimental data may help to elucidate more of the secondary structure of NRF2 and aid rational design of inhibitors. The fact that NRF2 is activated and regulated through a set of protein-protein interactions (PPIs) also contributes to it being a challenging target for small molecule drug discovery [79]. It is well established that direct targeting of NRF2 with small-molecules to discover protein-DNA interaction inhibitors is difficult as it is a largely intrinsically disordered protein [74]. Hence, there are relatively few studies in the literature exploring such compounds and most have had limited success. This calls for identification of alternative therapeutic targets that lend themselves to discovery of novel compounds possessing high specificity, bioactivity and limited side-toxicity [59].

3. Protein-protein interaction enhancers

Over the last two decades, advancements in biophysical techniques

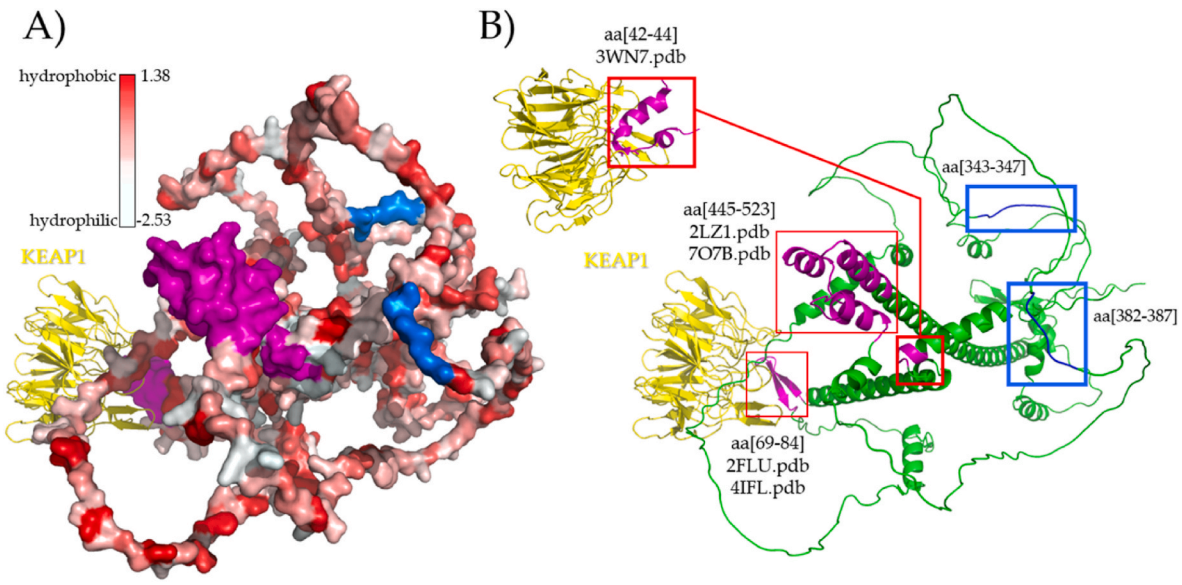

Fig. 5. Highly unpacked 3D structure of NRF2 currently predicted by Alphafold. Panel **A** shows the molecular surface of NRF2 for the 3D folding predicted by AlphaFold2. The color responds to the hydrophobicity scale of its amino acid side chains. The three regions for which experimental data on its structure are available are shown in magenta. Panel **B** shows the secondary structure (polypeptide backbone) of NRF2 in green, except for amino acids 42–44, 69–84 and 425–523 in magenta. Helix [42–44] and beta hairpin [69–84] interact with the Kelch domain of KEAP1 (yellow). The blue box locates DSGIS (343–347) and DSAPGS (382–387) phosphodegron sequences of NRF2-Neh6 domain interacting with the WD domain of β-TrCP. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

such as nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, surface plasmon resonance (SPR) and more recently the advent of cryo-electron microscopy (cryo-EM) have furthered drug discovery initiatives in structure-based drug design (SBDD), enabling expansion in the scope of the druggable genome [80–82]. Numerous drug discovery programmes now aim to exploit regulatory elements of proteins such as allosteric sites and surfaces that mediate interactions between a set of two or more proteins [83]. PPIs such as those governing

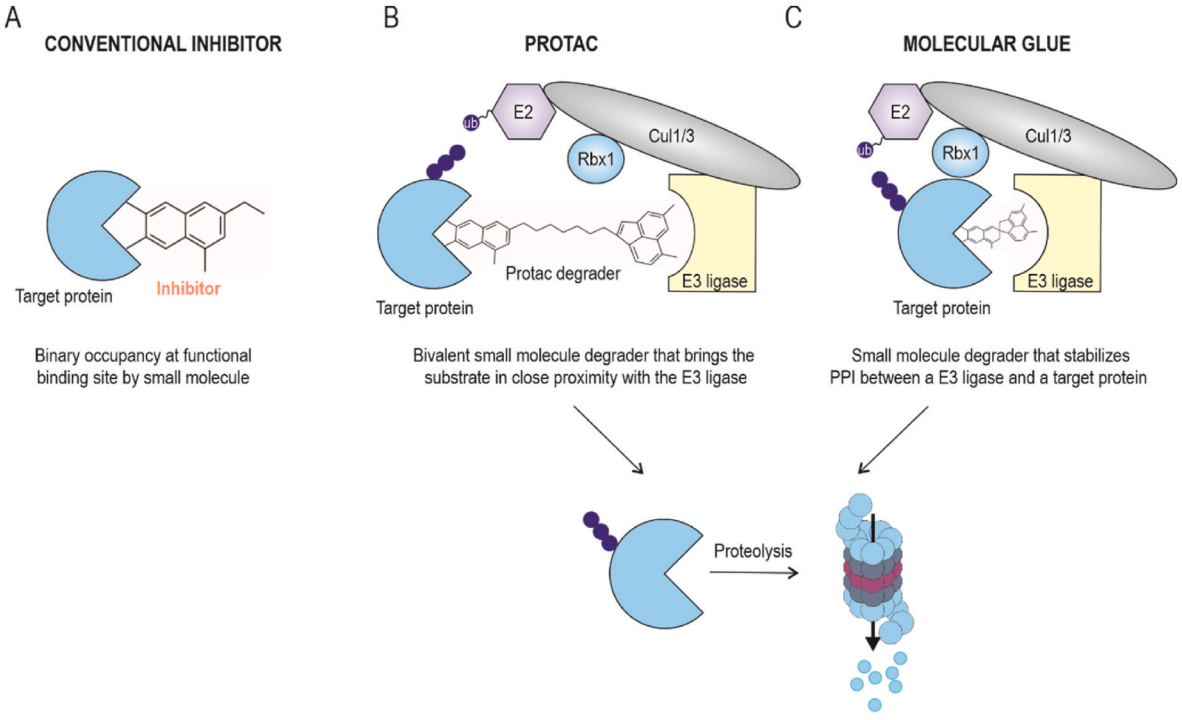

Fig. 6. Schematic of the binary and ternary therapeutic approaches. **A.** A classical inhibitor blocks a single protein interaction at a functional binding site and disrupts a particular enzymatic activity. **B.** PROTACS are bivalent degraders wherein one ligand binds to the target protein and is connected through a linker to another ligand that recruits an E3 ligase. **C.** Molecular glues are monovalent degraders that have optimised contacts to both the substrate and E3 ligase, thereby inducing a novel interaction between a pair of proteins. Both PROTACS and molecular glues are protein-protein interaction enhancers; they bring the target protein in close proximity with an E3 ligase system through ternary complex formation and allow effective degradation of the target through the ubiquitin-proteasome pathway.

the recognition of substrate proteins by E3 ubiquitin ligases are pivotal to cellular function and play a crucial role in most cell signaling pathways [84]. There is significant therapeutic potential in harnessing these systems as novel pharmaceutical targets and in developing small molecules that can modulate these interactions. Substantial research has been done to develop PPI inhibitors, especially for the KEAP1-NRF2 pair, that can act as NRF2 activators in cancer chemoprevention [85]. However, the rational design of small molecule PPI enhancers remains fairly elusive and is a field in its nascent stages [84,86].

3.1. Targeted protein degradation as an emerging therapeutic modality

Drug-induced targeted protein degradation has emerged as a powerful strategy to eliminate disease-relevant proteins that are intractable to conventional therapeutics [87]. Many of these drug targets are transcriptions factors such as NRF2, which have been traditionally described as 'undruggable' by small molecule ligands [88]. This is in large part due to their intrinsic structural disorder and lack of defined binding pockets. While targeting PPI interfaces can prove to be difficult due to the large, flat and often solvent-exposed surfaces that comprise them as well as the entropic penalty of forming a ternary complex, PPI modulators are attractive to drug development [74,89]. These target proteins typically have relatively shallow surfaces without ligandable pockets, which are difficult to access by classical small molecule drugs alone but can be bound with the help of an accessory protein (substrate adaptor) [82]. Unlike conventional inhibition driven by binary occupancy at functional binding site (Fig. 6A), PPI enhancers can target any protein binding sites as long as they position both sets of proteins in the correct orientation for the ubiquitylation machinery to work effectively and trigger downstream signalling [87]. In recent years, these agents have been viewed enthusiastically as a unique pharmacological modality to target proteins without druggable pockets.

In contrast to the conventional active site directed inhibitors that disrupt or block enzymatic activity of proteins, these degraders hijack the mechanisms cells use to destroy proteins and mobilise endogenous biology to fight disease [90]. PPI enhancers directly influence the activity or fate of target proteins by bringing them to the vicinity of regulatory proteins. They typically have an 'event-driven' pharmacology wherein long-term ternary complex formation is not required but rather they stabilise a transient interaction to enable ubiquitination to happen most efficiently on the target protein [91]. The degradation event uncouples the need for continual drug exposure. As a result, PPI enhancers typically exert their effects in sub-stoichiometric concentrations in ways not possible using classical inhibitors, essentially acting as catalysts for degradation alongside minimising the potential for off target effects [92]. Such compounds are often modelled as three-body binding equilibria and have the potential to exhibit significant cooperativity due to the specific ligand-induced molecular recognition, which is crucial to their role [83]. Moreover, chemical agents that stabilise PPIs often do not need to compete with endogenous ligands, providing some leeway in the potency required for biological activity. Degraders offer unique and high target selectivity that arises from the specific PP interface they induce in the pair of proteins [93]. Lastly, it is important to note that different molecules can induce different conformations and protein-protein interfaces. In this manner, the degrader has the ability to control the disposition of the two proteins, making it crucial to study the different interfaces and clusters of favourable conformations during drug design [94]. Despite the many advantages in exploring these novel chemotypes, many existing drug discovery and optimisation efforts still fixate on binary target engagement, in large part due to limited structural data on ternary complexes [83,95]. However, progress is gradually being made in the field.

3.2. Emerging paradigms: PROTACS and molecular glues

In recent years, significant progress has been made to target hard-to-drug proteins by employing PROTACS. These heterobifunctional molecules comprise of two small molecules connected by a linker, wherein one is bound to a target protein (substrate) and the other is bound to an E3 ubiquitin ligase, acting as a recruiter (Fig. 6B) [96]. This bivalent degrader system brings the substrate in close proximity with the ligase and promotes its ubiquitination and degradation in a proteasome-dependent manner. Although PROTACS represent an important new therapeutic modality with tremendous potential to elicit substrate degradation, they are generally associated with high molecular weight and unfavourable physicochemical and pharmaceutical properties, leading to potential challenges in drug development [84,87,97]. This approach could be employed as a potential strategy for targeted degradation of NRF2 in future but is currently limited by the lack of good ligands available to bind to the protein [71]. In contrast, the monovalent molecular-glue-type degraders are generally small molecules that more readily adhere to Lipinski's rule of five, making them attractive chemotypes for inactivating transcription factors and other challenging targets previously considered undruggable [98]. Their lower molecular mass compared to the linker-based bifunctional molecules improves many of their drug-like properties and is possibly advantageous for increased bioavailability and improved pharmacokinetic profiles.

Distinct from PROTACS, these small molecular glue-like compounds bind both the target protein and a substrate receptor of an E3 ubiquitin ligase by embedding into and stabilising a natural occurring PPI interface, thereby inducing novel interactions between the pair of proteins (Fig. 6C) [84,99]. This ultimately leads to activation or suppression of a cellular response. Thus, proteolysis of the target can occur without the need for a linker. The small molecule entity is developed to have optimised contacts for both the substrate and ligase, often resulting in high ligand efficiency, efficacy and better selectivity profiles. While PROTACS bring the pair of proteins together and mediate new PPIs, molecular glues modify the molecular surface to enable new PPIs [100]. A general feature of molecular glues is forming an enlarged composite contact surface between the protein-of-interest and its binding partner, thereby enabling direct and favourable contacts between the pair through cooperative binding.

3.3. Molecular-glue discoveries

Molecular glues are rare and represent a small subset of PPI modulators that work by stabilising PPIs. Only a handful have been documented in the literature over the past few decades and many were discovered serendipitously [99,101,102]. Several examples of molecular glues can be found in nature. These include the macrocyclic immunosuppressants cyclosporin A (CsA), FK506 and rapamycin (Fig. 7A), which were initially thought to act via conventional inhibition [103,104]. It was well-known that these natural products bind with very high affinity to their cognate immunophilin partners; cyclophilins (CyP) for CsA and FK506-binding proteins (FKBP) for FK506 and rapamycin and inhibit their common enzymatic activity [105]. More remarkably, the immunosuppressive activity of these macrolides was later discovered to arise from their ability to induce *neo*-protein-protein associations [106]. The compounds impart a gain-of-function and endow their immunophilin partners with the ability to form ternary complexes with the phosphatase calcineurin (for CsA and FK506) or with the atypical kinase mammalian target of rapamycin (mTOR) [107,108]. The inhibition of the enzymatic activities of the latter proteins accounts for the potent immunomodulative effects of CsA, FK506 and rapamycin [103]. Another key example of nature's molecular glue is the plant hormone auxin (indole-3-acetic acid, IAA), which facilitates an interaction between the SCF E3 ubiquitin ligase T1R1 and IAA transcription repressors by extending the protein interaction interface for substrate binding (Fig. 7B) [109,110]. This leads to IAA destruction by the ubiquitin-proteasome system to activate auxin-response gene expression, important for regulating plant growth and development.

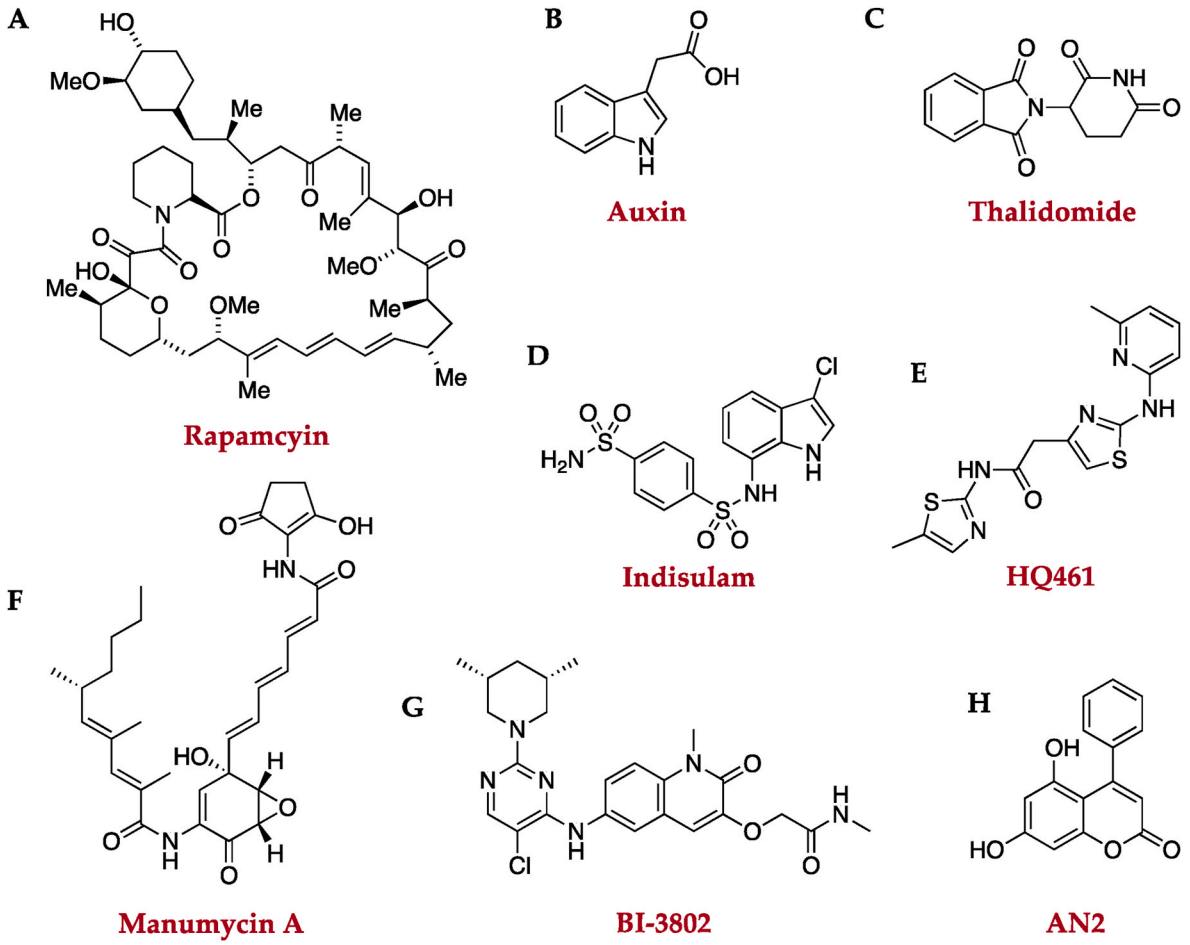

Fig. 7. Chemical structures of some representative molecular glues discovered till date. **A.** Rapamycin is a macrocyclic immunosuppressant. **B.** Thalidomide is an immunomodulatory drug. **C.** Auxin is a plant hormone. **D.** Indisulam is an anti-cancer aryl sulfonamide. **E.** HQ461 is a cyclin K degrader. **F.** Manumycin A is a polyketide. **G.** BI-3802 is a highly potent BCL6 degrader. **H.** AN2 triggers autophagic degradation of mutant huntingtin protein.

The anti-cancer agents, including the teratogenic compound thalidomide (Fig. 7C) and its second-generation derivatives pomalidomide and lenalidomide, have also been identified as molecular glues [98]. These immunomodulatory drugs (IMiDs) had been approved for the treatment of multiple myeloma due to their anti-angiogenic and anti-proliferative effects, however their mode of action remained elusive until 2010. It was then discovered that these molecules bind to Cereblon (CRBN), the substrate receptor protein of Ring E3 ligase CRL4 [111,112]. The resulting CRBN-IMiD complex is able to promote novel PPIs with non-native lymphoid transcription factors including Ikaros family zinc finger 1 (IKZF1) and Aiolos (IKZF3), as well as Casein kinase 1α (CK1α) [113–116]. This small-molecule mediated recognition event makes them *neo*-substrates to the CRBN E3 ligase, leading to their ubiquitylation and subsequent degradation by the 26S proteasome. This degradation event contributes to the clinical efficacy of IMiDs.

After elucidation of the mode of action of IMiDs, aryl sulfonamides were also uncovered to be molecular glue degraders [82]. This class of cytotoxic molecules including indisulam (Fig. 7D), tasisulam and other related anticancer sulfonamides were discovered to degrade an essential splicing factor RNA-Binding Motif Protein 39 (RBM39) by recruiting it to the E3 ligase substrate receptor DCAF15 [117–120]. This results in aberrant pre-mRNA splicing and death in a number of cancer cell lines. Most recently, several labs using different approaches have identified structurally diverse molecules that promote cyclin K degradation by causing a cyclin-dependent kinase 12 (CDK12)/cyclin K complex to directly associate to the cullin scaffolding protein DDB1 [99,102]. Most of these distinct molecules have been identified through chemogenomic screening approaches via systematic data mining. For example, the cyclin K degrader HQ461 (Fig. 7E) binds CDK12's ATP-binding pocket which seems to create an altered CDK12 surface to recruit DDB1, an adaptor protein of CRL4 [104,121,122]. This triggers the poly-ubiquitination and degradation of CDK12's partner protein Cyclin K, which eventually affects the expression of genes involved in DNA damage response.

Despite being an interesting therapeutic modality, most molecular glues have been discovered fortuitously through empirical approaches and it has limited their potential as a general strategy for therapeutic intervention [99]. However, in the past few years research in the field has been directed towards identifying new strategies in the rational discoveries of molecular glues. An emerging but relatively underexplored field are molecular glues with reactive moieties that can covalently bind to target proteins [123]. Isobe et al. investigated the molecular mechanism of natural products Asukamycin and Manumycin A (Fig. 7F), wherein both contain numerous electrophilic moieties [101]. Activity-based profiling found E3 ubiquitin ligase UBR7 to be a primary target for covalent modification by these polyketides that act as molecular glues between UBR7 and tumor suppressor TP53, which acts as a key ternary interaction partner. The transcriptional activity of the latter was enhanced by both Asukamycin and Manumycin A in a UBR7-dependent manner through a chemically induced compound-mediated protein-protein crosslink [103]. The epoxide of manumycin A is responsible for the interaction with TP53, while the unsaturated amides contribute to covalent interactions with Cys374 of UBR7.

In 2020, a new mechanism of molecular glues was elucidated, wherein these agents were found to induce target polymerisation, followed by ubiquitination and subsequent degradation [88,98]. BI-3802 (Fig. 7G) was unexpectedly discovered to induce the degradation of B-cell lymphoma 6 (BCL6), a transcription factor associated with lymphoid malignancies, during a screening of BCL6 inhibitors [124]. Cryo-EM structural analysis revealed that the solvent-exposed dimethylpiperidine group of BI-3802 interacted with the symmetric BTB-homodimer (BTB domain) of BCL6 and contributed to the formation of a ligand-protein interface, facilitating the assembly of BCL6 homodimers into filaments in a supramolecular-type structure [125]. Drug-induced specific polymerisation promoted ubiquitination and proteasomal degradation of BCL6 by the E3 ligase SIAH1. As a result of its novel BCL6-specific degradation mechanism, BI-3802 demonstrated an increased pharmacological activity compared to BCL6 inhibitors and provided a new strategy for degrader-based discovery [103].

In addition to proteasomal degradation, autophagy represents another independent pathway for protein degradation [126,127]. The latter is a bulk degradation system that works by engulfing proteins into autophagosomes for subsequent lysosomal degradation. Lu et al. developed a new strategy to discover molecular glues that can trigger autophagic degradation [128]. Using microarray-based HTS of 3375 small molecules, the team identified four compounds AN1, AN2 (Figs. 7H), 10O5 and 8F20 that can interact with both the autophagosome protein microtubule-associated protein 1A/1B light chain 3 (LC3) and the disease-causing protein mutant huntingtin protein (mHTT), but not the wild-type HTT protein. They effectively reduced mHTT levels both *in vitro* and in vivo in fly and mouse models of the disease, exhibiting potent therapeutic activity by simultaneously targeting LC3 and mHTT to promote autophagic degradation [99,123]. The detailed mechanisms of these autophagic molecular glue degraders remain to be elucidated after solving the structure of the LC3-drug- mHTT ternary complex.

3.3.1. *Rational discovery of degradative β-TrCP- β-catenin interaction stabilisers*

In 2019, Simonetta et al. reported the rational design and identification of potent molecular glues that enhance the binding of mutated oncogenic transcription factor, β-catenin, to its cognate E3 ligase β-TrCP [84]. This in turn facilitated its ubiquitination *in vitro* and induced degradation of the engineered mutant β-catenin by the 26S proteasome in a cellular system. Under normal conditions, phosphorylated β-catenin binds to β-TrCP at Ser33 and Ser37, leading to ubiquitination and degradation (Fig. 8D). However, mutations in this β-catenin phosphodegron impair its ability to effectively bind to β-TrCP, resulting in elevated levels of the protein. Aberrant high expression of β-catenin leads to various diseases including cancer [129]. The group focussed on Ser37 mutations that reduce the binding to β-TrCP by a factor of 300 and account for approximately 10% of known β-catenin mutations [67,68]. Removal of the phosphate functionality at Ser37 eliminates the electrostatic and hydrogen bonding potential of this residue that are critical in maintaining PPIs. This leads to a reduction in binding affinity, while

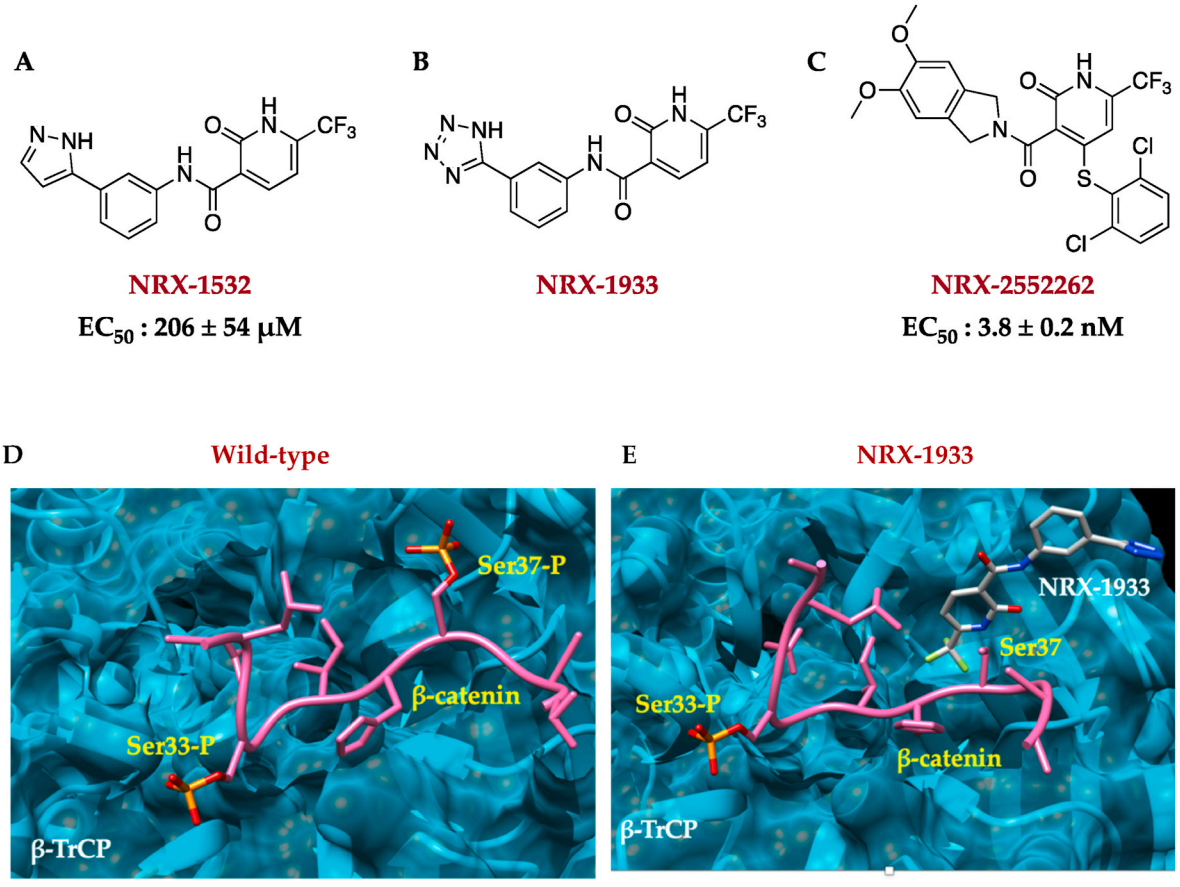

Fig. 8. Chemical and crystal structures of some representative molecular glues of the β-catenin:β-TrCP interaction identified in a 2019 study by Simonetta et al. **A.** NRX-1532 was an initial hit that re-established the pSer/Ser37 β-catenin: β-TrCP interaction with an EC50 of 206 μM. **B.** A related but more soluble tetrazole analog NRX-1933 was used to solve the crystal structure of its ternary complex wherein it binds at the β-catenin: β-TrCP interface. **C.** NRX-2552262 was the most potent compound identified and also displayed the highest levels of cooperativity in binding compared to the original hit molecule. It is an isoindoline analog with dimethoxy substitution, designed during the optimisation process. **D.** Doubly phosphorylated β-catenin binding to β-TrCP in a wild-type structure (PDB:1P22). **E.** NRX-1933 binds in the pocket left unoccupied by the β-catenin Ser37 mutation to stabilise substrate binding (PDB:6M93).

simultaneously forming a small hydrophobic pocket between the PPI interface. The team carried out a high-throughput screen of 350,000 compounds in an effort to scavenge small molecules that have the potential to restore the binding of unphosphorylated Ser37 sequence to β-TrCP. This was enabled by initially developing a fluorescence polarisation assay using a weakly binding monophosphorylated pSer/Ser37 sequence.

An initial hit NRX-1532 (Fig. 8A) re-established the pSer/Ser37 β-catenin:β-TrCP interaction with an EC50 of 206 μM [84,103]. A crystal structure of a more soluble tetrazole analog, NRX-1933 (Fig. 8B), supported its mode of action as a PPI enhancer. It was revealed that the trifluoromethylpyridone moiety occupies a small hydrophobic pocket, which is revealed by the absence of Ser37 phosphorylation (Fig. 8E). NRX-1532 was further optimised to show higher binding cooperativity and ubiquitination potency, especially against a commonly occurring S37A β-catenin mutant. This work represents an impressive example of an efficient and rational optimisation process, wherein the cooperativity was improved up to 1500-fold with an increase in potency up to 10, 000-fold, seen in NRX-252262 (Fig. 8C), an isoindoline analog with dimethoxy substitution.

This seminal work represents a rare example of a biochemical HTS approach to identify new molecular glues. Unlike in other degradative molecular glues, the glueing effects of these enhancer molecules are achieved by significant cooperativity in binding in the entire ternary complex instead of simply binding to the E3 ligase alone [84]. Improvements in cooperativity were pivotal to overcome the binding defect, restore functional ubiquitination in the mutant system and promote proteasomal degradation. The team prioritized molecular modifications that were contained at the β-TrCP:β-catenin interface rather than growing the enhancer molecules away from the protein-protein interface. This key strategy allowed concomitant improvements in both the cooperativity and potency of the enhancers. Binding affinity was improved by increasing ligand efficiency rather than increasing ligand molecular weight. This ensured that the molecular glues retained acceptable molecular weight for small-molecules with desirable drug-like physicochemical properties throughout the optimisation process.

Further investigation revealed that these enhancers are specific for the Ser37 mutant and no increase in binding to other native targets of β-TrCP was observed [98]. The enhancer molecules provide the essential binding surface between β-TrCP and β-catenin that is lost as a result of the S37A β-catenin mutation. The screening approach shows that it is possible to repair and restore the lost binding of a substrate protein to its cognate E3 ubiquitin ligase by rationally designed molecules. This study highlighted several advantages of reinforcing interactions with a native substrate of β-TrCP rather than hijacking neosubstrates. Firstly, degradability is highly likely since the natural substrate contains lysine residues that are suitable for ubiquitination [103]. More importantly, the E3 ligase can be expected to be expressed in the relevant tissue, which is an essential prerequisite for successful interaction and subsequent degradation.

3.4. Degradative molecular glues targeting β-TrCP-NRF2 and KEAP1-NRF2 interactions

Transcription factors such as β-catenin and NRF2 are implicated in the development of several diseases including many types of cancers [130–132]. However, they continue to remain a difficult-to-drug class of proteins. Substantial research over the past decade have made it evident that levels of transcription factors are regulated by a common mechanism involving post-translational modifications (PTMs) that lead to protein degradation via the ubiquitin proteasome system [24,87]. It is also clear that in most cases, dysregulation arises from mutations leading to compromised binding of transcription factors to their cognate ligases that lead to aberrant over-expression and manifestation of several cancers. The work by Simonetta et al. provides a framework to rationally design and employ small-molecule glues that can target such oncogenic transcription factors by enhancing substrate: ligase interactions to promote their degradation [84]. Ultimately, this strategy could lend itself to being useful in targeting therapeutically important transcription factors such as NRF2 and potentially even different classes of proteins previously deemed undruggable.

Employing the key principles of the pioneering study discussed above, we propose that compounds that can 'lock' NRF2 to its regulator proteins, either KEAP1 or beta-TrCP, offer potential new 'molecular glue' approaches that can speed up NRF2 degradation through stabilisation of protein-protein interactions. This novel, targeted approach would have the potential to suppress inappropriate NRF2 activity in chemoresistant tumors and sensitize them to cancer therapeutics, thereby improving drug efficacy and treatment outcomes. It is inspired by recent methodology in the literature, wherein a similar oncogenic transcription factor is successfully degraded through interactions with its regular protein and can pave the way for novel therapeutic modalities targeting NRF2 [84].

Molecular glues targeting NRF2 may need to be selectively delivered to tumors in order to enhance their effects in cancer cells and minimize the reduced adaptive response to redox and electrophilic stress in normal cells [133]. However, as NRF2 is found at low concentrations under homeostatic conditions [134], the effects of NRF2 inhibitors on non-tumor cells may be limited but remain to be evaluated. Moreover, mutations in either NRF2 or its regulatory proteins, KEAP1 or β-TrCP in some tumors could be used to design agents that specifically target those corresponding tumors [25,27,41,135]. Since NRF2 undergoes PTMs such as phosphorylation by protein kinases in cells and during activation processes, it is also important to consider these chemically modified structures for specific protein-protein interaction analysis during the drug design process [136,137].

While the KEAP1-NRF2 system is a potential target for monomeric degraders to achieve NRF2 degradation, it is possible that if tumor cells have a highly oxidative environment, KEAP1 cysteines might be modified, either by sulfhydryl oxidation or electrophilic adduct formation [20,138]. This could disrupt the ubiquitination machinery by disrupting the interaction between KEAP1 and the Cul3/RBX1 complex and potentially reduce the effectiveness of the molecular glues. However, our rationale for the KEAP1-NRF2 system is that if a molecular glue could enhance the weaker DLG-KEAP1 interaction (instead of the pre-existing strong ETGE-KEAP1 interaction), this would bias the equilibrium of the KEAP1-NRF2 complex towards a more 'closed' form [79]. The Cul3/Rbx1 system would remain functional as the molecular glues would not be behaving as cysteine-reactive electrophiles and thereby enhance NRF2 ubiquitination and degradation. On the other hand, the β-TrCP-NRF2 system appears to be a more promising target since the PPI requires phosphorylation by GSK-3β and other priming kinases [15,16]. Hence, it is possible to envision situations of only partial binding or low-affinity binding, which lends itself well to modulation by molecular glues.

Recently, a macromolecular assembly of the ubiquitination machinery involving β-TrCP (Fig. 9) has been solved with cryo-electron microscopy (PDB 6TTU), showing an intermediate of the cullin-RING E3 ligase complex [139]. This complex catalyzes the ubiquitination of target proteins and subsequent proteasomal degradation. The complex includes β-TrCP, which is the protein responsible for recognizing and binding phosphorylated target proteins and the substrate IkBa. Applying these principles to Nrf2, the "molecular glue" can stabilise the protein-protein molecular interactions between NRF2 and β-TrCP, thereby increasing the rate of NRF2 degradation. The availability of structural information enables a rational design of "glue compounds" using computational molecular docking and dynamics techniques.

4. Conclusion and future perspectives

Targeted protein degradation is emerging as a promising new

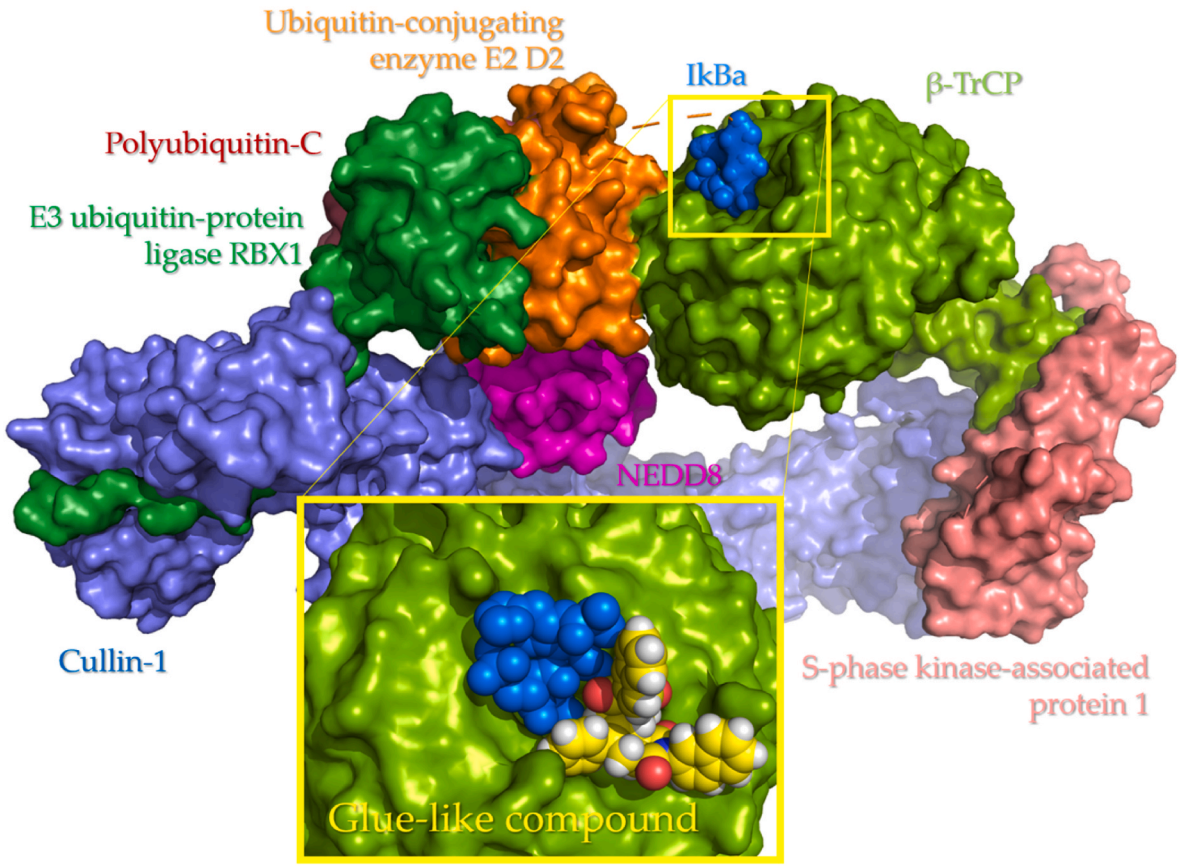

Fig. 9. Glue-compounds search strategy. Figure based on the 6TTU structure from electron microscopy, showing an intermediate of the cullin-RING E3 ligase complex including β-TrCP and substrate IkBa.

approach in drug discovery and development [126,140]. It has been clear in the past few years that molecular glues represent a new frontier in developing therapeutics for otherwise poorly ligandable targets. Despite having great potential, molecular glues remain obscure and hard to discover rationally since binding to two proteins has to be integrated into one molecule [103]. In comparison, a lot more resources have been devoted to designing PROTACS that are relatively easier to develop once there are known ligands for the protein of interest [141]. PROTACS approaches are yet to be applied to NRF2 due to the paucity of ligands for the transcription factor. However, any future discoveries of good NRF2 binders can create new avenues for research employing PROTACS as a complementary approach to molecular glues in the targeted degradation of NRF2. The suboptimal physicochemical and pharmacokinetic properties of PROTACS are partially counterbalanced by their catalytic mode of action, which lends them to be highly efficacious overall [84,103]. However, optimising PROTACS continues to remain a challenge.

On the contrary, molecular glues typically possess more favourable drug-like properties such as lower molecular weight, higher cell permeability and better oral absorption [100,142]. Thus, they offer a superior alternative approach to innovative drug discovery in the field of targeted protein degradation. A key learning from research so far is that molecular glues need a substantial amount of cooperativity to act in an efficient manner [84,143]. Most molecular glues so far have been discovered serendipitously and the lack of efficient design strategies in the past have limited their applicability as a new therapeutic modality. However, rational discovery approaches are beginning to be employed to identify new molecular glues. These include HTS, systemic data mining, scalable chemical profiling and chemical genetics [82,99,123]. Moreover, the development of new computational tools to predict the protein-protein interfaces and interactions induced by molecular glues in the complex, could aid virtual screening and SBDD of new small molecule degradative PPI enhancers [144,145]. Most molecular glues identified so far have a clear bias to preferentially bind to either the protein-of-interest or the adaptor protein [103]. An emerging strategy to enhance the chances of discovering molecular glues in the literature involves fragment screening using focussed libraries, based on binding motifs for either protein in the pair, thereby increasing the likelihood of molecular recognition [82,83].

Despite the encouraging progress made on molecular glues in the recent past, research on this modality is still in its infancy and there is a long way before they can be rationally developed into clinical candidates [98,126,146]. Yet, pioneering work in the literature, especially by Simonetta et al. has highlighted that structure-based rational optimisation approaches are highly desirable and can be successfully implemented in molecular glue-degrader discovery programmes targeting oncogenic transcription factors similar to NRF2 [84]. Most current research pays close attention to identifying extensive screening strategies, coupled with developing mechanistic validation methods [122]. Advances in computational chemistry, crystallography and biochemical technology can deepen understanding of the structural biology, molecular mechanisms and medicinal chemistry of molecular glue degraders [82]. Taken together, these can play an increasingly important role in discovering new molecular glues and potentially translating these strategies into practical clinical applications, thereby providing hope for breakthroughs in undruggable target classes.

Data availability

No data was used for the research described in the article.

Acknowledgements

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