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The Potential of Intrinsically Disordered Proteins as Drug Targets

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by

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ABSTRACT

Tumor necrosis factor α-induced protein 3-interacting protein 1 (TNIP1) is a negative regulator of inflammatory signaling in several diseases. TNIP1 is also an intrinsically disordered protein (IDP), which makes it difficult for current drugs to affect it. More research on IDPs could lead to novel drugs targeting TNIP1, leading to improved therapies for patients with acute and chronic inflammatory diseases. The main difference between IDPs and the more common ordered proteins is that IDPs are flexible, a characteristic of TNIP1 which was demonstrated in this study via protease sensitivity. Ordered proteins are rigid, which means that they only have one well-defined three-dimensional structure. The flexibility of IDPs allows them to have multiple conformations that they can switch between quite easily. However, switching between conformations makes it much harder to solve for the structure of an IDP. Since developing drugs relies heavily on knowing a protein's structure, IDPs have not yet been common therapeutic targets. Several screening approaches for new IDP-targeting drugs are considered here, including those driven by artificial intelligence. There have been some reports of successful small molecule screens, but finding a universal technique is still in high demand. Currently, it is thought that drugs binding to multiple conformations of IDPs may be beneficial over a drug only binding a single conformation. Since 20-30% of the proteins in our body are IDPs, continued characterization of IDPs could lead to better drug designing methods, more structural information about TNIP1, and a better multifaceted approach for treating psoriasis, cancer, Parkinson's disease, ischemic vascular diseases, and beyond.

INTRODUCTION

All of the proteins in our body can be characterized by their structure. The structures of these proteins are largely due to their unique amino acid sequences. Typically, the amino acid sequences help the protein fold into a stable, ordered, and specific three-dimensional conformation. In many cases, these well-defined ordered proteins have biological activity in our body. However, there are proteins that can have biological activity without a stable three-dimensional structure. These proteins are called intrinsically disordered proteins (IDPs), which are unfolded, flexible, and capable of rapidly changing conformations. IDPs play a major role in many biological processes and disease states in humans [1]. Reduced function of tumor necrosis factor α–induced protein 3-interacting protein 1 (TNIP1), an IDP, has been linked to inflammatory skin diseases [2,3]. Gaining a better understanding of the varying conformations of TNIP1 could lead to a promising drug target in inflammatory skin disease states. To support this connection between IDP conformation and drug targets, the distinction between IDPs and ordered proteins must be made.

While ordered and disordered proteins may seem like two separate binary states, the amount of disorder in proteins is described as a continuum [4]. For example, there are hybrid proteins that are described as a mix between ordered and disordered, which is due to intrinsically disordered protein regions (IDPRs) within their structure. Also, it is important to note that neither of the extremes on this continuum exist. All proteins have some degree of movement, but they cannot be completely chaotic either. The structure disorder-continuum is complex, so distinguishing between ordered proteins and IDPs can be challenging. However, some [5] theorize that a protein can be considered an IDP when it cannot be described by having a single three-dimensional conformation, or at least within a series of snapshots. In some instances,

determining whether a protein fits this IDP definition of "conformational uncertainty" involves an environmental context, such as an interaction with a partner protein [5].

Human Proteome:

To put IDPs in the context of the human proteome, IDPs were described as proteins that have more than 30% of disordered residues in their polypeptide chain. Using this quantitative threshold, over twenty thousand proteins in the human proteome were analyzed and 32% were characterized as IDPs, 19% characterized as containing IDPRs, and 49% characterized as ordered proteins [6]. However, there is a misconception that IDPs are uncommon, which may be due to the fact that only 0.9% of eukaryote proteomes are fully (highly) disordered [7]. Given the large presence of IDPs in the body, it is important to understand the various functions that they carry out.

IDP functional diversity:

Unlike typical ordered proteins, IDPs are flexible which allow them to have a multitude of conformations and therefore a variety of functions. This structure-function relationship is evident when looking at various disease states in humans [1]. In Parkinson's disease there is an IDP called α-Synuclein (α-Syn). The mechanism of α-Syn-related pathology is not well-defined, but it is theorized that it is involved with neuronal transmission as well as membrane thinning, curvature, and remodeling. There has also been mounting evidence that associates α-Syn dysfunction with damage of multiple organelles and α-Syn genetic abnormalities with familial, early onset Parkinson's disease [8]. There is an IDP called c-Myc, which acts as a transcription factor of various genes that regulate many functions involved with cancer. The overexpression of

c-Myc leads to the proliferation of many human cancers such as, breast cancer, colon cancer, and cervical cancer [9]. In psoriasis, TNIP1 was found to directly control IL-17A-mediated gene regulation in keratinocytes [10]. Due to its flexible nature, TNIP1 can also repress intracellular signaling and transcription by binding to multiple factors such as, RAR, PPAR, and NFκB [11,12,13]. Loss of TNIP1 leads to increased chemokine recruitment and ultimately inflammation that is consistent with psoriasis [2]. Gaining a better understanding of the varying conformations of TNIP1, and other IDPs, could lead to more information about how IDPs can be targeted in our body. Investigating how IDPs can change their conformation when bound to partner proteins and in their free state provides a foundation that is necessary for drug design.

IDPs IN A BOUND STATE

Intrinsically disordered regions (IDRs) within IDPs give IDPs their flexibility. This allows for IDPs to bind to many different proteins and have one protein bind to multiple places on the same IDP. These mechanisms are referred to as one-to-many binding and many-to-one binding respectively. One-to-many binding allows for p53, a tumor suppressor IDP, to bind to over 100 different partners. p53 utilizes its many IDRs that each bind multiple partners, which enables p53 to bind to all of its different targets. Many-to-one binding allows for 14-3-3, an IDP [14], to have one protein bind to many of its 200+ IDRs. These interactions are called many-to-one binding because many of the IDRs on 14-3-3 are able to bind one identical amino acid sequence [14]. One-to-many binding and many-to-one binding show how one dysfunctional IDP can have a large effect on multiple signaling pathways within the body.

Most IDPs become more ordered after they are bound to their target. Unfortunately, there are some IDPs that maintain their disorder even after binding to their target, which makes

characterizing their changing conformations difficult [15]. However, this unusual phenomenon can be predicted using a FuzPred algorithm. The algorithm uses the sequences of over two thousand complexes to predict whether an IDP will transition from disordered-to-ordered, disordered-to-disordered, or somewhere between the two ends of the spectrum [16]. Given that understanding the various conformations of IDPs is imperative for drug design, it is important to discuss how the IDPs that transition from disordered-to-ordered when binding are able to do so.

Conformation when binding:

The transition of IDPs from unbound to bound (disordered-to-ordered) and bound to unbound (ordered-to-disordered) can be guided by the electrostatic interactions between the IDP and the binding site. As shown in p53 fragments, the electrostatic interactions predominate at a long-range (10-20 Å), which facilitates the IDP folding into a more stable conformation. Once the IDP is close to the binding site, van der Waals forces predominant, which stabilizes the ordered conformation of the IDP. When the IDP is becoming unbound and transitioning from ordered-to-disordered, some order may be retained in the short-term depending on the distance between the IDP and its receptor. If the rate of unfolding is faster than the rate of unbinding then the IDP will regain its disorder before the IDP has lost all of its contact points with its receptor. However, the IDP can briefly maintain its stable bound conformation if the rate of unbinding is faster than the rate of unfolding [15]. The mechanism by which the IDP actually binds to its target is affected by a number of factors.

IDP binding mechanisms:

IDPs can bind to their targets in a few ways, "Several possible binding mechanisms for coupled folding and binding have been identified: folding of the IDP after association with the target ("induced fit"), or binding of a prefolded state in the conformational ensemble of the IDP to the target protein ("conformational selection"), or some combination of these two extremes" [17]. The actual reactions behind these mechanisms are independent from one another, and depend on the conformational propensities of both the IDP and the receptor it is targeting [17]. It is also important to note that becoming ordered when bound does not correlate with an increased affinity for the receptor. The various intermediate conformations that may be involved with binding are independent from one IDP to another. In addition, the intermediates that are involved when an IDP binds to its receptor can depend greatly on the cellular environment that the IDP is in. IDPs that undergo different conformational changes as a result of the environment or propensities of its target protein use a binding mode known as a context-dependent. The environment can also dictate how the IDP changes between its various conformations when it is unbound [16].

IDPs IN AN UNBOUND STATE

When IDPs are unbound and in their free form, their conformation depends on two major variables: the IDP's conformational propensities and the environment. Even in stable physiological conditions, IDPs can undergo many conformational changes on their own. The rapid changing between sub-states is likely due to small energy barriers [15]. The small energy barriers allow for the IDP to change its conformation rapidly on its own, but this also allows for

the environment to have a notable impact on IDPs. Before analyzing the effects of environmental factors, the structural properties of IDPs in physiological conditions needs to be discussed first.

Two important properties of IDPs when they are in their free form are their low hydrophobicity and a relatively high net charge [18]. IDPs possess these properties due to the specific amino acids that are used to construct their primary structure. IDPs have a biased amino acid sequence that is skewed towards more disorder-promoting amino acids (Pro, Arg, Gly, Gln, Ser, Glu, Lys, and Ala) than order-promoting amino acids (Cys, Trp, Tyr, Phe Ile, Leu, Val, and Asn) [19]. The disorder-promoting amino acids group has more amino acids with net charges at physiological pH, while the order-promoting amino acids group has more amino acids with aromatic hydrophobic rings. The balance between these two groups can determine whether the protein as a whole is ordered or disordered. It is likely that the charges on the protein promote repulsion and unfolding, while the hydrophobic interactions promote attraction and folding [18]. Having the right combination of these interactions can not only determine whether a protein is disordered, but also if it remains functional. Although these two interactions are predictable at physiological conditions, they can be greatly influenced by environmental factors, such as pH, proteases, and temperature [20].

Environmental factors:

The first environmental factor to be considered is pH, which can both induce further disorder and help the IDP maintain its physiological structure. Decreasing the pH has been shown to induce disorder in a human IDP called LL-37, which requires an α-helical conformation for optimal antibacterial activity. At a pH of 5 the α-helices on LL-37 begin to degrade, and at a pH of 2 LL-37 becomes entirely disordered. On the contrary, increasing the pH

to 13 resulted in retention of the α-helical structures of LL-37. It is thought that the unfolding of α-helices is brought on by protonation of side chains, which increases net charge and therefore the repulsive force. While increasing folding is driven by reducing repulsive forces due to neutralization of charged side chains [21].

The next environment factor to consider is proteases. The flexible nature of IDPs makes them susceptible to degradation from many proteolytic enzymes [19]. Proteases are able to bind to degradation sites on IDPs because they are unfolded and easily accessible. A common example of a degradation site on an IDP is a PEST sequence (P = Proline, E = glutamic acid, S = serine, T = threonine). PEST sequences are characterized as being flexible, unstructured, and filled with disorder-promoting amino acids. Due to IDPs high sensitivity to proteases, IDPs tend to have much shorter half-lives than ordered proteins [22].

Finally, it is important to consider temperature, which has been shown to induce structural changes in IDPs. For example, a change a temperature can alter the stabilization of an α-helix conformation [23]. It has also been shown that increasing temperature can induce partial folding in α-Syn. Temperature-induced partial folding is supported by the fact that "the hydrophobic interaction has the unusual property of increasing in magnitude at higher temperatures due to the large change in heat capacity with temperature…" [24]. The partial folding of IDPs from elevated temperature can be reversible by lowering temperatures, which promotes disorder [20]. In fact, IDPs have been shown to be resilient to many of these environmental changes.

Resilience to harsh conditions:

IDPs are highly resilient to harsh conditions because they avoid aggregation, remain soluble, and can either maintain their function or quickly regain it when conditions normalize. For example, many IDPs were found to be unaltered after being heated to 90℃. Also, IDPs have been shown to avoid precipitation after acid treatment, which is attributed to their low hydrophobicity content. In ordered proteins, hydrophobic residues becoming exposed to solvent are thought to be a main component to their subsequent aggregation and precipitation. IDP's ability to avoid aggregation and precipitation allows them to stay in solution, while typical ordered proteins do not. If the solution is centrifuged, most of the ordered proteins are found in the pellet, while the IDPs are isolated in the supernatant. Although the solution is not completely pure, it still has been useful for researchers to analyze the IDPs that are present [25]. Overall, environmental factors can induce several structural changes in IDPs, but compared to ordered proteins, IDPs are highly resilient. It has been suggested that IDPs fit the concept of "one cannot break what is already broken" [19].

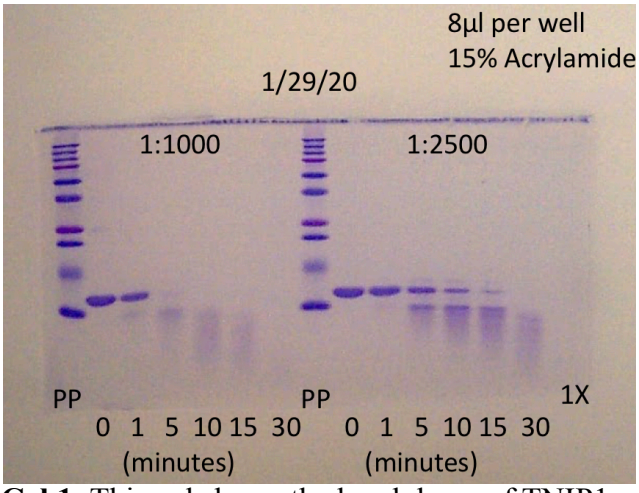
Having a strong understanding of the various environmental factors within the body is very important when considering the conformation of IDPs, but also for drug design. When researchers were attempting to stabilize an IDP called DKK2, it was discovered that DKK2's conformation was more sensitive to the experimental conditions than the stabilizing molecule developed [26]. The pH and trehalose concentration were two experimental conditions with effects on the solubility of DKK2. Reducing the pH had the largest effect on DKK2's solubility, with a significant reduction occurring when the pH fell below 9 [26].

LABORATORY EXPERIMENTS

LEGEND

Ratio → Trypsin:TNIP1
PP → Marker proteins
1X → Laemmli buffer control
µg per well → amount of protein in each lane
% acrylamide → determines magnitude of protein shift
Try → Trypsin
Chy → Chymotrypsin

IDPs susceptibility to proteolytic degradation by many different proteases causes them to have much shorter half-lives than ordered proteins. Since a decrease in TNIP1 is associated with disease, identifying molecules that stabilize its active form and increase its half-life could be an important strategy for drug design. Trypsin and chymotrypsin are two examples of proteases that can decrease IDPs' half-life. Proteases are able to bind to known degradation sites on IDPs, which could help identify regions on TNIP1 to target for drug design. Due to TNIP1's susceptibility to proteases, I was able to learn more about its structure through a series of experiments. The experiments began by exposing TNIP1 to trypsin and chymotrypsin at two different temperatures (37°C and 25°C).

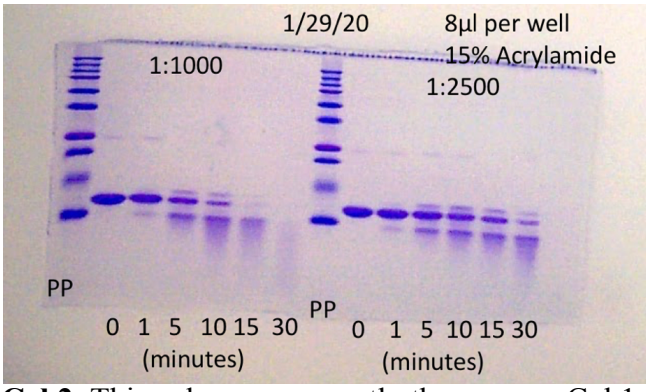


Gel 1: This gel shows the breakdown of TNIP1 over time at two different trypsin to TNIP1 ratios at 37°C. Samples were taken at different time points once the reaction began. When there is less TNIP1 present, it was gone before the 5-minute sample. When TNIP1 was more abundant, it was still slightly visible at the 15-minutes mark.

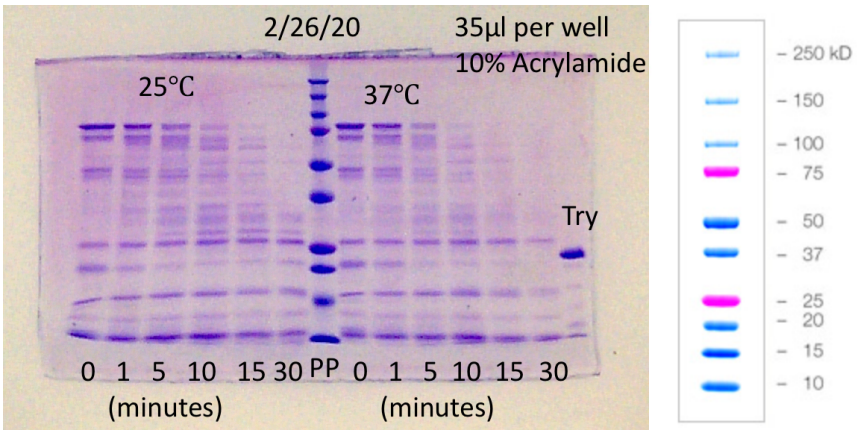
The first step taken was to confirm that trypsin has increased activity at 37°C compared to 25°C, and that TNIP1 is broken down faster when the ratio of trypsin to TNIP1 is increased. These experiments used a purified UBAN fragment of TNIP1 because the fragment has been

associated with the anti-inflammatory function of TNIP1 [27]. Additionally, using only a fragment of TNIP1 results in increased clarity. The gels from these experiments are shown as Gel 1 and 2.

The next step was to purify full length TNIP1 from a nickel column. Although TNIP1 is difficult to use in size exclusion chromatography, the resulting fractions were pure enough to proceed with the experiment. These samples were used to compare the effects of trypsin and chymotrypsin. This was done by running both enzymes on one gel at optimal conditions. The resulting gel showed that the breakdown of TNIP1 was similar between the two enzymes, but that trypsin worked faster than chymotrypsin. To further analyze the differences between trypsin and chymotrypsin, comparisons of their functions were evaluated at 25°C and 37°C. Full length TNIP1 is the uppermost band and is clearly broken down over time. While the



Gel 2: This gel was run exactly the same as Gel 1, but the reactions were carried out at 25°C instead of 37°C. Comparing Gel 1 and Gel 2 definitively shows that trypsin functions more optimally at 37°C than 25°C. On Gel 1 (37°C), the TNIP1 band (1:1000) dissipates between the 1-minute and 5-minute samples. On Gel 2 (25°C), the TNIP1 band (1:1000) dissipates between the 10-minute and 15-minute samples. The change in activity is apparent when comparing the 1:2500 ratios as well.

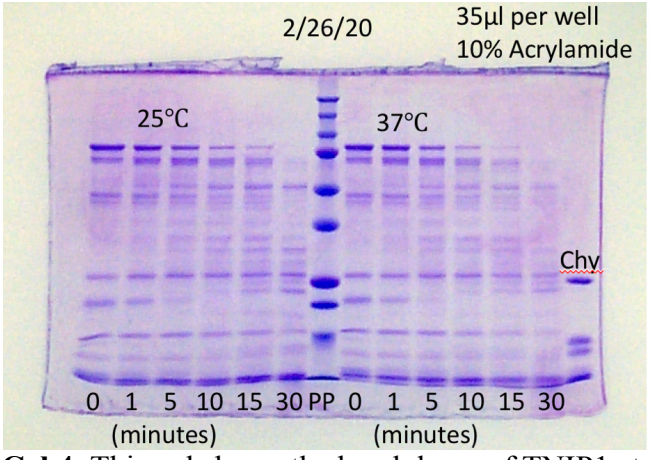


Gel 3: This gel shows the breakdown of TNIP1 at two different temperatures by trypsin.

sample is not entirely pure, the approach is still valid because I was simply assessing the changes to the different bands over a set period of time in the presence of proteases. These gels are labeled as Gel 3 and Gel 4.

In Gel 3, the band at the bottom of the gel seems to remain unchanged throughout the duration of the reaction. This could be an ordered region of TNIP1 that does not get recognized by trypsin. In addition, the band above the 25kD mark also seems relatively unchanged, or even slightly denser as the reaction occurs. This band could be another ordered region, or potentially a fragment that accumulates over time. Many results seen in the trypsin gel are similar to the chymotrypsin gel.

In Gel 4, the band right below the full length TNIP1 becomes the same density as TNIP1 over time. This fragment could potentially be an ordered region, or is simply less likely to be recognized by chymotrypsin than trypsin. Additionally, a band appears in the 30-minute wells right above the 50kD mark. This band is not present in the trypsin gel, which suggests that the band may be due to fragments accumulating over time. The difference in fragments between the two gels is likely due to the differences between trypsin and chymotrypsin's ability to recognize cleavage sites within TNIP1.



Gel 4: This gel shows the breakdown of TNIP1 at two different temperatures by chymotrypsin.

Since the recognition sites of trypsin and chymotrypsin are well documented [28], analyzing the fragments from the gels could provide valuable information about where TNIP1 is cut and thus disordered. A suspected disordered fragment from one of the gels was cut out and analyzed by mass spectrometry (MS). The initial assessment of intrinsic disorder in TNIP1 was done by using a primer for in silico experiments, which were also coupled with in vitro techniques [29]. This has now been expanded on because the sample from the MS work in conjunction with further analyses have led to more evidence of TNIP1 being an IDP and an overall greater depiction of TNIP1's structure. Specifically, the UBAN domain demonstrated significant intrinsic disorder when analyzed by nuclear magnetic resonance (NMR). This provides more evidence that the UBAN domain plays a major role in carrying out the various functions of TNIP1 [27]. Improving our understanding of TNIP1's structure is important for learning more about how it functions as well as for developing small molecule stabilizers that increase TNIP1's half-life.

A stabilizer would likely need to protect TNIP1 from degradation by a variety of proteases. This could lead to TNIP1 performing more of its functions, which could potentially have benefits in disease states that are associated with decreased TNIP1. However, this is currently theoretical, so further steps need to be taken to investigate if TNIP1 is even a good candidate for small molecule binding.

DRUG DESIGN

There are an abundant amount of IDPs in the human body and they play a central role in psoriasis, cancer, Parkinson's disease, etc.; thus, learning more about their potential as a drug target is critical [1]. The cavities of IDPs were examined for druggability and it was found that

they have a druggable probability of 9%, while ordered proteins only have a druggable probability of 5%. Druggability was calculated using a proven algorithm from a program called CAVITY [30]. The algorithm is able to detect whether the geometric shape in a protein's cavity is suitable for ligand binding. Since determining druggability in protein cavities requires a known conformation, it is fair to wonder how IDPs were analyzed using this algorithm. The IDPs were bound to partner proteins leading to a transition from disordered-to-ordered, which allowed researchers to identify their conformation when bound. All of the cavities for IDPs were based purely off of their conformation when they were bound. Although the data shows that IDPs are promising drug targets, actually discovering effective drugs is difficult because IDPs do not exist solely in a bound conformation as used in this experiment [30].

In the case of TNIP1, the UBAN fragment has evidence that suggests the presence of two molecular recognition features called MoRFs. These MoRFs are important for carrying out the various functions of an IDP because they have the ability to undergo disorder-to-order transitions. The discovery of potentially two MoRFs in the UBAN fragment of TNIP1 is also supported by the protease experiments. The results clearly demonstrated that the UBAN fragment is highly susceptible to degradation by proteases in multiple environments, which indicates that disorder is present [27]. Identifying MoRFS is a critical step for understanding function and potential for drug design. Stabilizing MoRFs has been found to acceleration the association rate constants for transitioning the IDP into the bound state [31]. Therefore, they could serve as a potential target within an IDP to increase its functionality.

IDP drug design methods:

Although traditional drug design methods rely on a well-defined structure, there has been some progress in IDP drug design due to a structure-based virtual screen approach. To carry out this approach, binding sites of c-Myc had to be identified. Using CAVITY, circular dichroism (CD), and NMR, c-Myc was inspected for possible binding sites [9]. CD is a quick and effective method to characterize protein conformational changes and secondary structures. The spectra of proteins are greatly influenced by its conformation, so CD can be used to monitor conformational changes due to temperature, denaturants, binding interactions, etc. This makes CD a valuable tool for characterizing the conformations of IDPs. NMR is also an important asset to this experiment because it can give residue-specific information, which CD cannot [32]. Together, these tools were able to identify three binding sites on c-Myc. The next step taken was virtually screening libraries for ligands that have activity in any of the identified binding sites [9]. A molecular docking methodology called GLIDE uses conformational, orientational, and positional space to identify ligands that could bind [33]. This method has led to the discovery of 7 molecules that not only bind to the disordered region on c-Myc, but also inhibit its dimerization. Since overexpression of c-Myc has been associated with a variety of cancers, preventing its dimerization could pose as a potential therapy for cancer [9]. Developing drugs that inhibit IDPs is important for proteins that are overexpressed in diseases states like c-Myc. However, it is also important to research drugs that can increase the activity of IDPs that are under expressed in disease states, such as TNIP1 for treating psoriasis.

IDP-stabilizing drug design:

Ligands that can stabilize the active form of an IDP are already being researched. An example of this is seen when looking into ligands that bind DKK2, an IDP that modulates cancer and ischemic vascular diseases [26]. It was hypothesized that since DKK2, like many IDPs, are able to bind RNA-binding proteins (RBPs), then additionally adding an RNA ligand may contribute to the stabilization of DKK2. This was put to the test and an RBP, LysRS, and an RNA ligand were able to promote the soluble, stable, and functionally active form of DKK2. Although the buffer conditions were shown to have a significant effect on the solubility of the complex, the activity shown in optimal conditions was similar to a known antagonist of the same pathway. Producing a stabilized and functional form of an IDP by binding the IDP to an RNA ligand could be a promising method for other IDPs needing stability [26], such as TNIP1. Another important factor to consider is the effect of RBPs on the regulation of microRNAs (miR). In fact, 92% of RBP interact directly with at least one miR locus [34]. This is an important interaction to consider because miR have been shown to alter IDP expression. For example, miR-27a is a known negative regulator of DKK2 at the translational level [35]. Therefore, RBPs altering miR expression could lead to a downstream effect on an IDP. The potential clinical effects of the interactions between RBPs, miR, and IDPs should be considered when using RBPs for IDP stabilization.

Additional IDP drug design factors to consider:

Although IDP drug design seems to be focused on effecting a specific conformation of an IDP, there have been some thoughts about drugs that bind multiple conformations rather than just one. The 7 active compounds that inhibited c-Myc dimerization from the virtual screen

approach all showed multi-conformational-affinity rather than high-conformational-specificity. Given that all the active compounds demonstrated multi-conformational-affinity, it was hypothesized that IDPs may prefer to bind multi-conformational-affinity ligands in preference to high-conformational-specificity ligands. Given the rapid dynamic nature of IDPs, multi-conformational-affinity may be described as a ligand cloud binding to a protein cloud [9]. The ligand and protein cloud concept may be insightful for drug design, but it could spell trouble from a clinical standpoint because it could lead to unintended effects in other pathways associated with the IDP.

As previously discussed, the flexibility of IDPs allows for them to have a variety of functions even within just one pathway, such as TNIP1 and inflammation [1]. One-to-many and many-to-one binding allows for a range of effects for just one IDP, so drugs that bind IDPs have the possibility of also exhibiting a range of effects. Inhibiting or stabilizing multiple conformations could prevent IDPs from fulfilling their functions in pathways not related to the disease state. In addition, if IDP expression is altered by RBPs or miRs then unintended consequences could still arise. Unknowingly inhibiting or stimulating pathways could lead to the IDP-targeting drug being toxic or ineffective.

Drug design methods for IDP stabilizers:

Currently, the drug design methods for stabilizing IDPs have yet to be widely successful. However, a few methods have led to the identification of small molecule stabilizers for IDPs (see Table 1). Unfortunately, these current methods are vastly inefficient and the newer strategies have little evidence to support that they can be easily replicated for other IDPs. Since TNIP1's role in disease has become clearer, it is important that we can take the next step by developing

drugs that can stabilize it. In order to successfully discover lead compounds, the current methods need to be modified to better suit IDPs. I was able to get a thorough idea of what strategies are being employed for other IDPs by conducting a comprehensive literature search.

I began the search by compiling a list of terms about the topic. The list was used to find literature regarding small molecule stabilizers for IDPs and the associated techniques that were successful. An important part of the process was tracking the combinations of terms I used, so I could avoid repetitive searches and to evaluate what terms were working the best. After optimizing the combinations of the search terms, I was able to gather an extensive list of resources about drug design for IDPs. I included the sources that identified a small molecule that could stabilize an IDP in Table 1 (next page).

Evidently, there has not been a lot of success in this area. An improved protocol for high-throughput screening (HTS), fragment-based drug design (FBDD), and thermal-shift assays (TSA) is still highly desired to increase chances of identifying hits. Many computational methods have been proposed as well to help supplement these current strategies. Finding one universally applied technique to optimize the benefits from each of these protocols could at least be a step forward in improving the drug design process. Also, the protocols for identifying small molecule stabilizers have not been proven to be successful in other IDPs. Therefore, I cannot confidently say that the current protocols can efficiently identify stabilizers for TNIP1. Since IDPs are unstructured, may require multi-conformational affinity, and have a low success rate for small molecule identification, I propose that the solution for IDP and TNIP1 drug design will come in the form of artificial intelligence (AI).

Table 1: Summary of Small Molecule Drug Design for IDP

Protein Stabilized	**Reference**	**Technique Used**	**Advantages and Disadvantages**
Foxm	Bioorg Chem. 2017 Feb;70:12-16. [36]	High-throughput screening (HTS)	Can rapidly screen for activity of 100,000+ compounds daily. Not small molecule stabilizer specific, so many false positives. A ligand binding to an IDP and the functional outcomes are especially difficult to detect, so success rates are as low as 0.04% [37].
PGC-1a	Diabetes. 2013 Apr;62(4):1297-307. [38]		
p53	Proc Natl Acad Sci U S A. 2012 Jul 17;109(29):11788-93. [39]		
PGC-1a	Mol Metab. 2018 Mar;9:28-42. [40]	Cell-based HTS	Similar to previous HTS, but is designed to specifically target PGC-1a activity from increased protein stability.
αSyn	Sci Rep. 2019 Nov 18;9(1):16947. [41]	High-throughput chemical microarray surface plasmon resonance imaging	The addition of SPR allows for improved detection of binding events
14-3-3	J Med Chem. 2020 Jul 9; 63(13): 6694–6707. [42]	Fragment-based drug design (FBDD)	Uses smaller starting structures to identify druggable hotspots, which can be developed into small molecules [43]. However, a major drawback is that most identified fragments are silent in assays used for HTS. Therefore, x-ray crystallography or NMR studies may need to be used as a supplement.
NUPR1	Sci Rep. 2017 Jan 5;7:39732. [44]	Thermal-shift assay (TSA)	Hits were classified as compounds that increased the temperature needed for disorder. The assay is specific to a stabilizing effect and is a good fit for small-scale screens. However, the assay is much less reliable for proteins with intrinsic disorder due to non-ideal denaturation profiles [45].

Artificial intelligence in drug design:

AI could help piece together our current strategies to create a synergistic effect. Researchers have attempted to screen IDPs for small molecule modulators by incorporating AI, machine learning (ML), and deep learning (DL) into the scientific process. AI can be defined as "devices that 'perceive their environment and take actions to maximize their chance of success at some goal'" [46]. ML and DL are considered subsets of AI. ML is able to learn and improve its algorithm and therefore its performance over time, while DL "is capable of handling extensive raw and complex data where it operates by mimicking deep neural networks (DNNs) and learning processes of the human brain" [47].

AI, ML, and DL are already in use to better understand the complex protein-protein interaction systems in different disease states. This is illustrated in Figure 1. For instance, ML

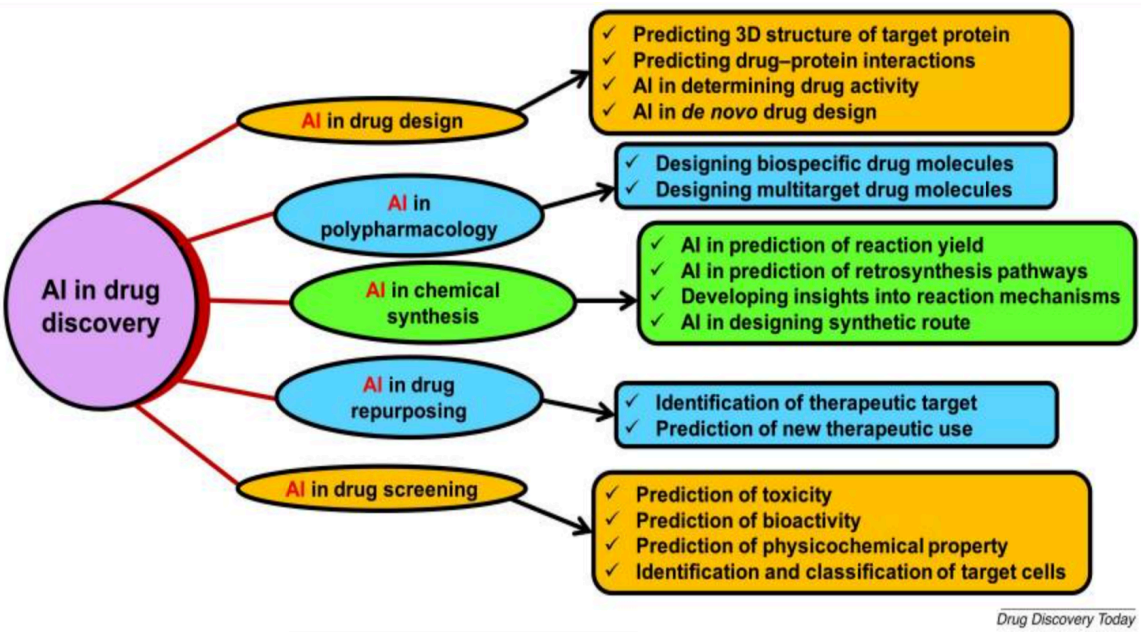


Figure 1: This image summarizes the uses of AI in drug discovery [48].

can be used for preparing protein-protein interaction (PPI) libraries and for evaluating drug-likeness and ADMET properties of initial PPI hits [47]. For IDPs, DL is already being used to

identify MoRFs. The DL method only requires the protein sequence for its predictions and is already outperforming previous models [49]. After MoRF identification, the next step is to characterize these structural features to help clear up TNIP1's role in disease. This process is already underway because ML can provide a convenient framework for analyzing disorder-to-order transitions in IDPs. A ML technique used for carrying out this analysis is called anharmonic conformational analysis (ANCA), which is able to accomplish this by using fourth-order statistics. This approach has led to the identification of intermediate conformational states of an IDP called BCL2. These intermediates enable BCL2 to undergo partial unfolding upon binding to its partner protein BECN1. Also, AI techniques in conjunction with Bayesian approaches can be used to integrate the experimental and computational simulations to better our understanding of energetics between IDPs and their respective binding partners. This could ideally lead to an improved insight into the mechanisms of IDPs [49].

A protocol [37] for efficiently identifying small molecule ligands of disordered proteins was created and two novel ligands with promising activity for degenerative amyloid disorders were identified. A key component of this protocol was using ML for identifying and utilizing chemical characteristics to help focus the screening attempts. One of the ligands is even able to delay the tau aggregation reaction *in vitro*. The ligands are chemically different than any other compound known to affect this process, which indicates that they are interacting with a different chemical space. Also, these ligands were identified out of just a total of ten compounds that were characterized *in vitro*. Given that typical success rates with high-throughput screening are quite low, this new protocol offers a drastically improved approach. This same approach can easily be applied to TNIP1 and other IDPs, which makes the protocol enticing for IDP drug design in any disease state. Although a substantial amount of additional drug development is still required, this

new method offers a starting point at the minimum [37]. AI evidently can help us learn about IDP conformations, functions, druggable potential, and even help us find potential active ligands.

Current IDP therapies:

There have been two instances where drugs that target IDPs or IDRs have made clinical trials. Both were discovered by a phenotypic screen of a natural compound library, which is a comparable method to cell-based HTS [50,51,52]. The first was Trodusquemine [53], but the most noteworthy is a drug indicated for prostate cancer called ralaniten, which binds to an IDR on the androgen receptor [52]. Although the clinical trial only included 28 participants, ralaniten was well-tolerated and kept several patients stable for over one year. Unfortunately, ralaniten displayed poor pharmacokinetics (PK), which led to a high pill burden. The next-generation of ralaniten has improved PK and stability, and is currently recruiting for a phase 1 trial under the name of EPI-7386 [54]. The lack of IDP-specific drugs currently available should not reflect IDPs as a limitation for drug development, but instead should represent how IDP drug development is still very much in the early stages [55]. While ralaniten and its analogs promote optimism for IDP-targeting drugs in the future, there is still much to be learned about IDPs and their functions in many disease states. IDPs are highly abundant in the human proteome, so the potential for IDPs in medicine is untapped. More research in this area could lead to a multitude of novel mechanisms and FDA-approved drugs.

CONCLUSIONS

- Discovering drugs that effectively target IDPs could lead to major breakthroughs in psoriasis, cancer, Parkinson's disease, ischemic vascular diseases, and beyond.

- IDPs use both one-to-many and many-to-one binding mechanisms, which allows them to have several biological functions in our body. This makes them valuable drug targets in many different disease states, but this could also increase the chance of side effects if IDP-targeting drugs make it to the market.
- Thirty-two percent of the human proteome is IDPs. This makes the use of IDPs in medicine incredibly underutilized. If researchers are able to refine drug design methods for IDPs, then there could be a subsequent spike in IDP-targeting drugs moving into clinical trials.
- It can be determined if IDPs become ordered after binding to partner proteins using the Fuzpred algorithm. It could be beneficial to apply this algorithm to IDPs associated with disease because using an ordered bound structure is a crucial part of drug discovery. Focusing efforts strictly towards IDPs with ordered conformations after binding could lead to more clinical trial success.
- Multi-conformational-affinity may be the approach for current IDP-targeting ligands, but it is fair to question whether high-conformational-specificity may be used more frequently in the future. Although IDPs may tend to bind multi-conformational-affinity over high-conformational affinity, it is justified to believe that multi-conformational-affinity may lead to more pathways being affected. Whether this leads to a higher efficacy or more side effects is yet to be determined, so a ligand binding by high-conformational-specificity cannot be ruled out.
- Although IDPs are highly resilient and can maintain their function in harsh conditions, the environment can still change their conformation which can affect how tightly ligands bind. Also, IDPs have a short half-life due to their relatively open conformation making

them highly accessible and thus susceptible to the presence of proteases in the human body. If a ligand cannot keep up with the production of IDPs then the intended effect will not be apparent or have a short duration of action. This may be why ralaniten had a high pill burden.

- Designing small molecule stabilizers for IDPs has been proven to be challenging. The current methods each have their own advantages and disadvantages, but a universal protocol is still in high demand. A potential solution could be AI, which can be used to piece together the puzzle of IDP drug design.

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