Biomolecular Targeted Covalent Inhibitors

Subjects: Medicine, Research & Experimental

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Introduction of artificial structure(s) into a middle-sized therapeutic biomolecule, which would often add a superior function to the drug molecule, is easier than that into an antibody. In this direction, introducing a reactive warhead structure into such therapeutic biomolecules to create BIOmolecular Targeted Covalent Inhibitors (abbreviated as bioTCIs). It would create precise and safe covalent drugs that semi-permanently inhibit the target protein activity upon binding and the duration of the drug effect is only limited by the target protein turnover. Regardless of the modalities, all bioTCIs reduce the skepticism of small-molecule TCIs' safety concerns because bioTCIs can stringently recognize and conjugate only to the target proteins. Among them, oligonucleotide-type bioTCIs possess unique features, such as nuclease resistance and on-demand-reversal of the drug action with the complementary-strand antidote, which circumvents another major limitation to clinical translation of the aptamer drugs.

covalent aptamer | protease/nuclease resistance | warhead | middle-molecule covalent drug

1. History and General Principle of bioTCI

1.1. From Small Molecular TCI to bioTCI

Figure 1 summarizes the features of the four representative TCI modalities, including the middle-molecules, and **Figure 2** shows a simplified history of their development. Among them, small-molecule covalent drugs are not new [1][2][3]. A prototypical example is aspirin, which has been in distribution since 1899, but the covalent mechanism of inhibition was revealed only in the 1970s [4][5]. Indeed, one-third of FDA-approved enzyme inhibitors, including blockbuster drugs such as warfarin, proton-pump inhibitors, and β-lactam antibiotics, of major clinical utility targeting various diseases are covalent drugs [6][7]. Most currently active covalent drugs were discovered by coincidence and the mechanism of covalent inhibition revealed only after the drugs' usefulness had been established [7]. Rational design of the small-molecule TCI started in the late 20th century [8][9], and the first approved rationally designed TCI appeared in 2013 [2][6]. Thereafter, the numbers of patents, publications, and the FDA approval of TCIs increased dramatically [2][3]. As with conventional drugs, the major development in covalent drugs focused on small molecules because of the desirable features such as easy production, lack of immunogenic response, and the possibility of oral administration. However, it is also well-known that such small molecule covalent drugs, including those that are FDA-approved, show a concentration-dependent off-target binding where the interaction between the small-molecule and a protein target is strongly influenced by the hydrophobic interaction [10][11]. Such hydrophobic interaction domains are ubiquitously present in many proteins, resulting in off-target binding of the small molecules [12]. To circumvent this limitation of small molecule non-specificity, more recent rational TCI design has gradually shifted to TCI bio-oligomers/polymers (i.e., bioTCIs including TCI peptides,

proteins, and nucleic acids), which have higher target specificity and selectivity [13][14][15][16][17]. Among the bio-oligomers/polymers, middle molecule-type target binders (i.e., peptides [18] and oligonucleotides [19]) are traditionally recognized as less suitable drug modalities because of the short in vivo half-life from protease and nuclease digestion, and rapid renal clearance. However, enabling these middle molecules to bind covalently to the target protein could prolong the drug action, regardless of the macroscopically observable pharmacokinetic (PK) half-life of the unbound free drug, because of the irreversible nature of covalent binding. Covalent binding to the target protein appears to endow the binders with relative protease [20] and nuclease resistance [21][22], further prolonging the drugs' in vivo half-life. This shift in the effector molecules from small to middle has resulted in the resurgence of TCI development.

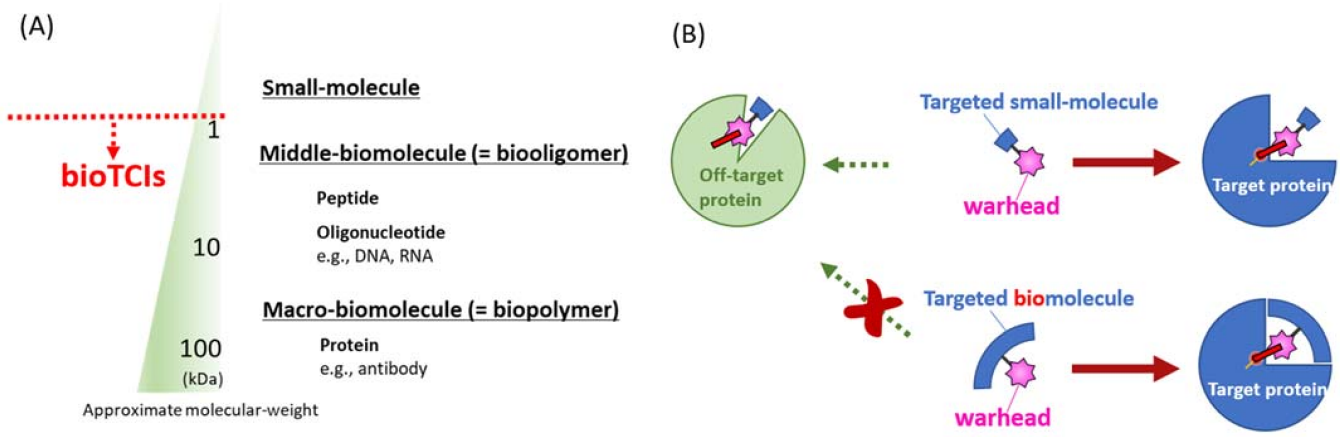

Figure 1. Reported modalities of targeted covalent inhibitors (TCIs). (**A**) Different TCI modalities are categorized as small, middle, or macro-molecules according to their molecular mass. This classification by the molecular mass is not absolute, with many overlaps. Peptidic and nucleotidic aptamers discussed in this entry are defined as middle-molecules by such a molecular mass classification. (**B**) The most distinguishing difference between conventional small-molecular TCIs (upper) and bioTCIs (lower). Because of the multi-point recognition of the target protein by the bioTCI, off-target covalent conjugation toward target-unrelated protein will be suppressed. The cost for the stringent target recognition via the molecular-weight increase of bioTCIs is the difficulty in inhibiting intracellular proteins because of the limited membrane permeability.

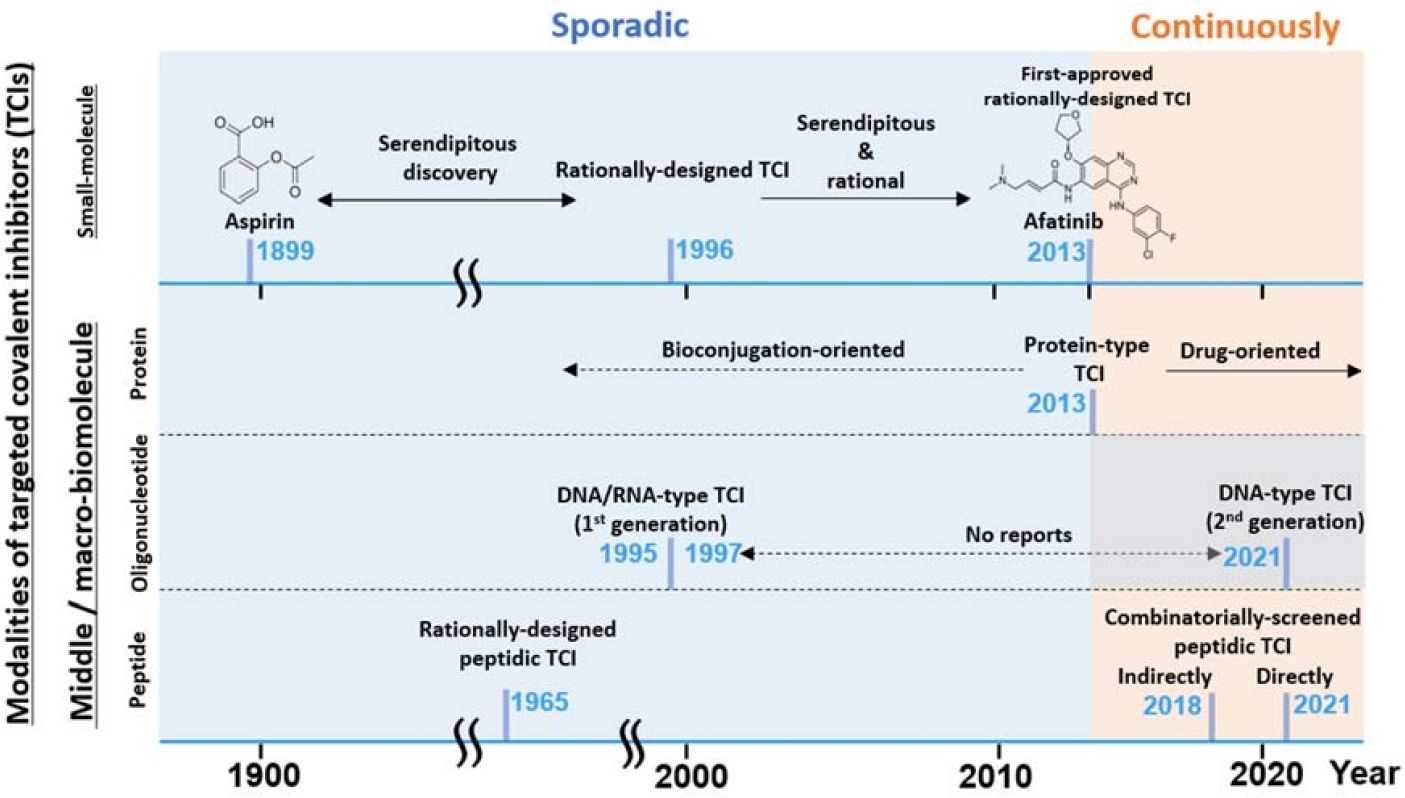

Figure 2. A graphical summary of the brief TCI history and recent hot topics described in this entry. TCI modalities listed (left axis) vs. timeline (horizontal axis) and the notable development are listed. TCI development has been mostly sporadic until recently with the renewed interest [7] in TCI drugs.

1.2. Warhead Design and Introduction into Middle/Macro-Biomolecules

bioTCI combines the inherent enhanced specificity of the middle/macro-biomolecules and covalent-binding to the target protein enabled by incorporating a weakly reactive electrophile (i.e., a warhead) to further enhance the affinity and selectivity for the intended target (**Figure 3**) [13][14][15]. Many warheads, classified by reactive groups, targeted nucleophilic amino acids, and the mechanism of covalent inhibition, have been reported in the literature and summarized in recent reviews [6][23][24][25]. Choosing the warhead to enable the covalent interaction between the middle/macro-biomolecules and the target is critical in creating a bioTCI. Adjusting warhead electrophilicity is the key for developing covalent drugs [26], regardless of the drug modality; too much electrophilicity would induce non-specific reactions to any nucleophilic functional groups of any unrelated proteins. Thus, weak electrophilic warheads which do not react with the nucleophilic functional group at a dilute concentration are needed [27][28]. For example, Michael-addition, conventional nucleophilic substitution-(S_N2, S_NAr), or sulfur fluoride exchange-(SuFEx) reaction-type warheads have been often used in the development of bioTCIs (**Figure 3**) [15].

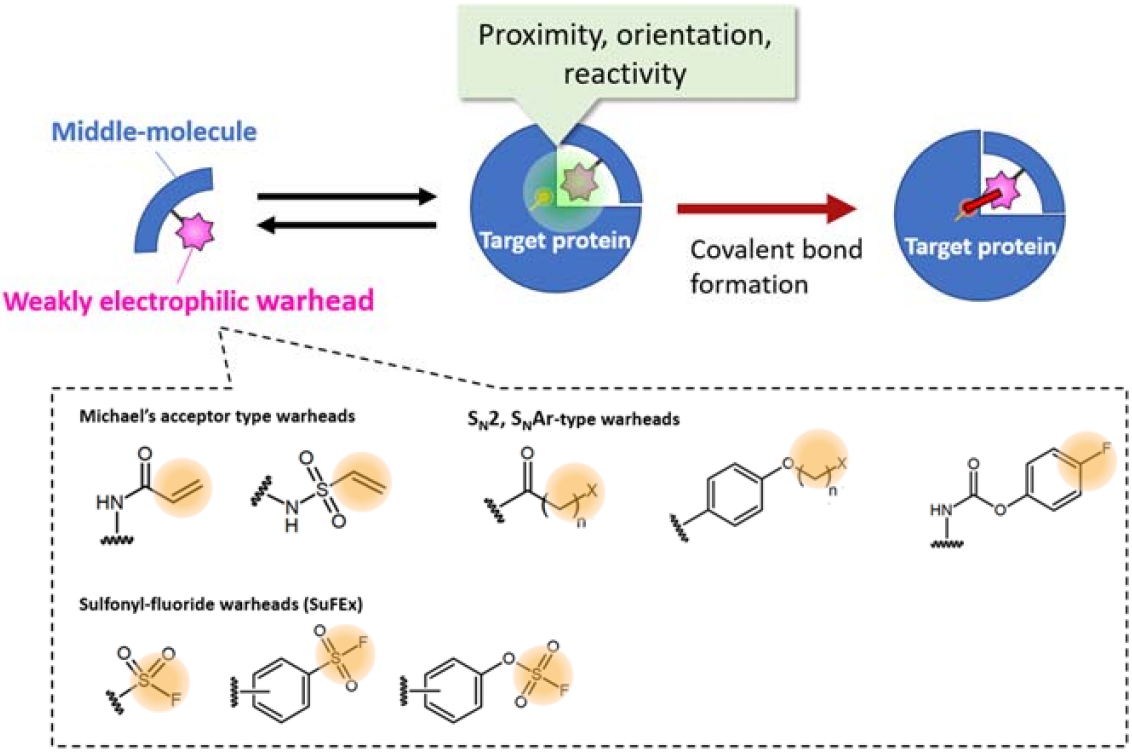

Figure 3. Selected examples of warheads for creating a bioTCI. For the appropriate covalent bond formation, both proximity, orientation, and optimized reactivity between the warhead and a nucleophilic amino acid of the target protein are needed. For a comprehensive classification of the warheads, see [23][29]. Synthetic strategies of SuFEx-type warheads, summarized in the latest review by am Ende and Ball's group [30] is very informative.

In most cases, bioTCIs are created by a rational design through introduction of the warhead into the targeted middle/macro-biomolecules at a specific position (i.e., placement) [15] via chemical modification [17] including bio-conjugation [14][31] or unnatural amino acid (Uaa) incorporation via genetic code expansion [16]. Usually, the position is determined based on the three-dimensional structure of an inhibitor/target complex. The rationale is that by introducing a warhead to an appropriate position of the binder, the location of the warhead becomes physically closer to a destination nucleophilic amino acid of the target protein. This proximity effect increases the local effective concentration of the warhead/nucleophilic amino acid and promotes the appropriate covalent binding reaction [2][7][15][27][28]. The positional determination is often time-consuming, and the covalent bond sometimes cannot form despite the seemingly appropriate introduction a warhead to the inhibitor. In fact, when a SuFEx-type latent-electrophilic warhead [23][30][32][33][34], which can theoretically react with any nucleophilic amino acids [35][36][37], was introduced into the 22nd leucine (L22) position of a Mdm2/4-binding staple peptide guided by the structural information, a covalent bond did not form despite a histidine and lysine in the warhead's vicinity on the target protein. Unpredictably, the expected covalent bond formed when a regioisomer of the warhead was used (**Figure 4**A) [38]. When a SuFEx-type Uaa-warhead was rationally introduced into the 75th glutamine, 77th asparagine, and 129th alanine (Q75, D77, A129) of the PD-1 protein, respectively, only A129-mutated PD-1 reacted with PD-L1 (**Figure 4**B) [39]. These results suggested that a simple proximity between the warhead and the receptive target amino acid may not be sufficient to facilitate the covalent reaction; stringent proximity and proper orientation, as is typical for a S_N2-type reaction, between the warhead and the nucleophilic amino acid may be

needed. A deeper understanding of the “matchmaking environment” [27][28][35][40] surrounding the warhead enabling the covalent reaction with the target is desirable. Rationale approaches based on structural information have not consistently worked and a more robust alternative method, not simply relying on trial-and-error for optimal warhead placement, is essential for this important area of drug development to progress.

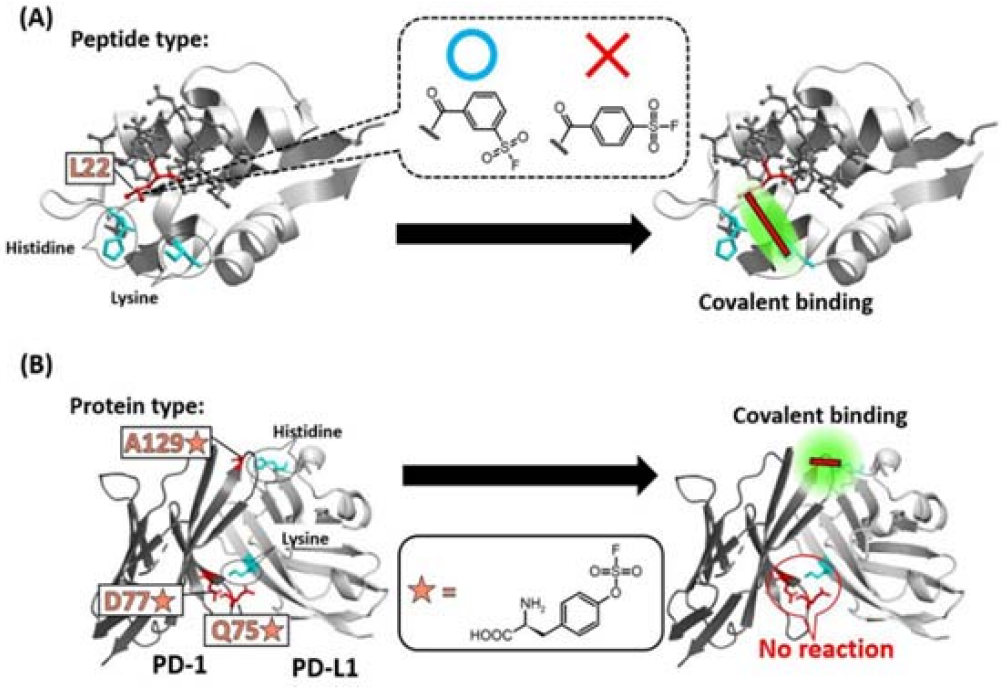

Figure 4. Specificity of the position of warhead incorporation for a successful covalent-bond formation. (**A**) SuFEx-type warhead isomers were introduced into the 22nd leucine (L22) position of a Mdm2/4 (light gray) binding staple peptide (dark gray). Only meta-substituted regioisomer reacted with a lysine of Mdm2/4. (**B**) Q75, D77, A129 of PD-1 (dark gray) was mutated into SuFEx-type Uaa (orange star). Only A129-mutated PD-1 reacted with a histidine on PD-L1 (light gray).

Among the bioTCIs, the development of peptidic inhibitors started early (i.e., in the middle of 1960s) [41] because position-specific chemical modification of the warhead can be accomplished through the historically established solid-phase peptide synthesis [42] followed by the post-synthesis chemical modification [17]. Besides the rational introduction of a warhead into the targeted peptide, both irreversible- and reversible-peptidic TCIs have been discovered, starting from screening of natural products [43]. The former and the latter examples are peptide-epoxides (e.g., epoxomicin) [44], and peptide-aldehydes (e.g., flavopeptin) [45], respectively. Structurally optimized variants of the natural peptides (i.e., peptidomimetics [46][47] TCIs) have been an active area of investigation. Natural peptides and peptidomimetics are both included as peptidic TCIs in this entry. Indeed, the peptidic TCIs are the most developed and promising modality among the bioTCIs, and currently several (e.g., Carfilzomib) have been approved by the FDA [48]. Current progress and modern history of the peptidic TCIs are summarized in informative recent reviews [13][15].

In contrast, the development of the proteinic TCIs has been slow because the specific chemical modification of a protein is a huge challenge and, traditionally, only the Uaa incorporation methodology [49] has been performed. However, the warhead-endowed Uaa promiscuously reacts to off-target biomolecules resulting in interruption of translation or cytotoxicity [15]. This promiscuous reaction has been elegantly overcome by Wang in 2013 through fine-tuning of the Uaa-warhead electrophilicity by proximity-enabled reactivity such that the Uaa does not react with off-target natural amino acids and other biomolecules under physiological conditions [39]. With this breakthrough, the proteinic TCI development has sped up [50][51] as well-summarized in recent reviews [15][52].

1.3. Pros and Cons of bioTCI over Non-Covalent Biomolecular Targeted Inhibitors

A major advantage of the bioTCI not shared by non-covalent biomolecular drugs is the prolonged duration of the drug effect and a less frequently required dosing, lessening the burden to patients. As shown in **Figure 5**, kinetic studies of the covalent bond formation between the TCI and the target protein follow a two-step process where the reversible initial docking of the compound to the target is followed by an irreversible covalent bond formation, resulting in a drug–protein conjugate that is not affected by the classical equilibrium kinetics of binding. Instead, the overall inhibition efficiency of TCI is better described by a derived PK parameter k_{inact}/K_I accounting for the irreversible second step binding [2][6][27][28][53][54][55]. A corollary to this two-step target recognition is that a non-specific covalent bonding of the warhead to the target seldom occurs, which is desirable and a consequence of the choice of warhead with reduced reactivity but increased specificity. The compromise between reactivity and specificity also results in a rather slow covalent bond formation requiring 10s of minutes to hours for completion. However, this alone is not a major drawback for a practical application since drugs are often continuously infused to attain a specific clinical endpoint and the bioTCI could be continuously infused to maintain the required serum concentration of the drug for the duration necessary for covalent bond formation with the target. The irreversible nature of the covalent bond formation assures us that, even if the bioTCI shows a relatively low affinity, the gradual shift in the equilibrium between the free and drug-bound target should cause a complete inhibition of the target. The non-equilibrium covalent binding of the bioTCI would also overcome any competing endogenous substrate(s) which binds to the same docking site of the target protein [2]. A prolonged inhibition of the target protein is expected from the extension of the pharmacological half-life regardless of the half-life of the free drug. The drug effect of the covalently bound bioTCI should far outlast even after the clearance of the unbound drug in the serum. An excellent theoretical treatment of TCI kinetics can be found in [53], and experimental data for small molecule TCI [56] and aptamer TCI [21][22][57] have been reported.

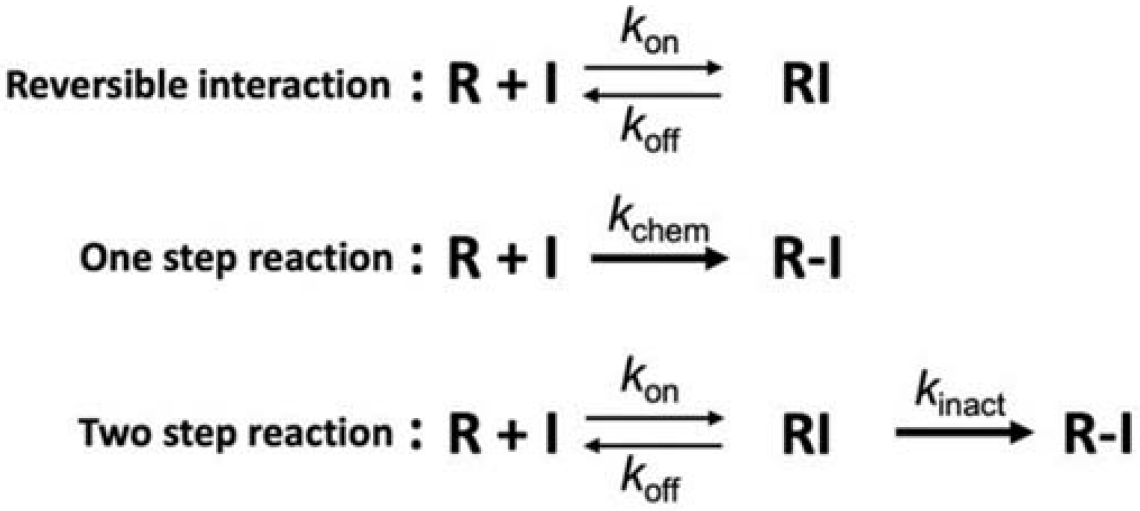

Figure 5. Mechanisms of covalent interaction [54][55]. Interaction mechanism between the protein (R) and the inhibitor (I) of non-covalent-type (**top**), conventional covalent-type including non-specific conjugation (middle) or targeted covalent-type (**bottom**). k_{inact} and k_{chem} are defined as the inactivation rate constant for the 2-step irreversible inhibition and the reaction rate constant for the 1-step irreversible conjugation, respectively. For the TCI (bottom), first step equilibrium (k_{off}/k_{on}) is a part of the inactivation constant ($K_I = [k_{off} + k_{inact}]/k_{on}$), which includes the TCI's *affinity* and resembles the concept of the dissociation constant of a non-covalent binder (i.e., $K_D = k_{off}/k_{on}$; top). However, overall performance of the TCI is represented as a *non*-equilibrated parameter of k_{inact}/K_I (i.e., the inactivation efficiency) which resembles the catalytic efficiency of enzymes (i.e., k_{cat}/K_m) .

Given the long drug effect, the potential risk for irreversible adverse drug effects (ADE) by TCI binding to off-targets has been a major concern, and perhaps the main reason for the hesitancy for a wide acceptance of TCI as a drug platform. To minimize ADEs, TCIs require exquisitely high target specificity [7]. Although many small molecule-, peptide-, and protein-type covalent drugs have been developed, none have overcome the potential risks of irreversible ADEs. In the following section, the researchers discuss a very recent development in reversible peptidic and nucleotidic TCI where, in the latter, the pharmacological effect is reversible even while still covalently bound to the target.

2. Recent Hot Topics of bioTCI

2.1. Combinatorial Screening of Peptidic TCI: A Well-Developed Modality

Despite the long track record of success, rational design and/or natural screening for peptidic TCIs cannot meet the demands of the ever-increasing broad range of different target proteins. Alternatively, combinatorial screening methods are widely used to discover peptidic binders as they allow for the rapid generation of a candidate library with a large diversity [58]. Theoretically, peptidic TCIs [13][15] can also be obtained via the combinatorial screening by introducing a warhead into a designated position of the library peptides. Practically, control of the warhead reactivity [29] during the library construction and selection is difficult, and the warhead in the library often forms promiscuous covalent bonds between biomolecules [59]. To get around this problem, an *indirect* combinatorial

method was implemented to first select for a targeting peptide using a mock-warhead-introduced peptide library on the T7 phage. After the selection and peptide-sequence identification, the desired target-selective covalent binding was observed when the unreactive mock warhead was replaced by a reactive warhead (**Figure 6**A) [59].

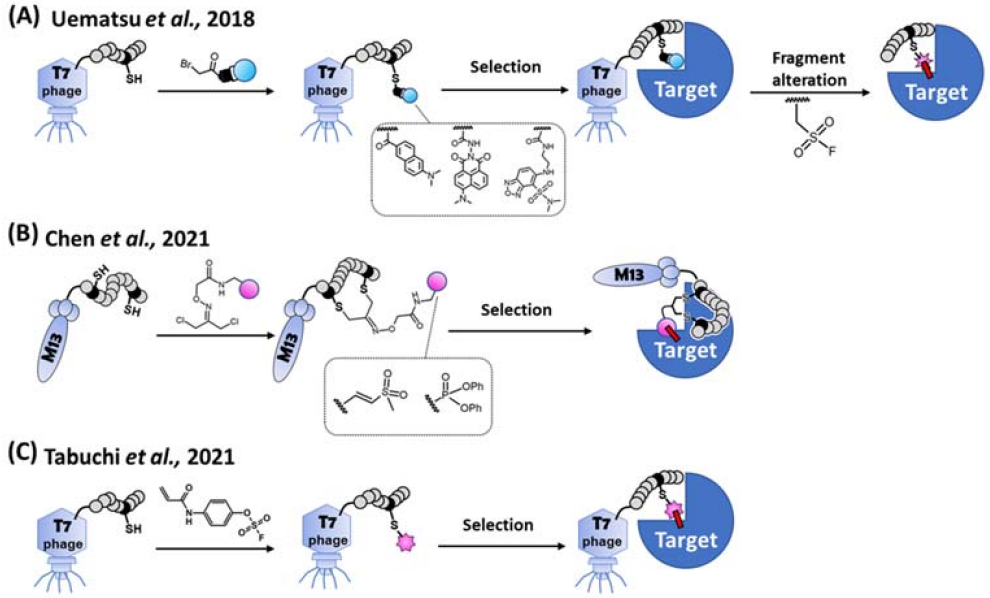

Figure 6. Methods for the combinatorial screening of peptidic covalent binders. (**A**) Indirect screening. Library peptide on the T7 phage is modified by each bait fragment (i.e., unreactive mock warhead), respectively. After the selection of a target protein, a consensus sequence of a peptide is obtained. The bait fragment of the peptide is alternated to a SuFEx-type warhead for obtaining a covalent binder [59]. (**B**) Bogyo's direct screening method. A cysteine-reactive vinyl sulfone or a serine-reactive diphenylphosphonate is introduced to the library peptide on the M13 phage. A covalent binder is directly selected from the warhead modified cyclic peptide library [60]. (**C**) The direct screening method. Aryl-fluorosulfate (AFS) warhead is introduced to the library peptide on the T7 phage. A covalent binder is directly selected from the AFS-modified peptide library via reactivity and affinity-based co-selection [40].

In 2021, *direct* combinatorial screening via the phage display was independently reported by Bogyo's group and us (**Figure 6**B,C). Bogyo's group designed two least-reactive warheads to minimize the promiscuous reactions between the biomolecules, and successfully selected peptidic TCIs using the M13 phage display [60]. They stringently regulated the reactivity of each warhead against cysteine or serine independently, and a bifunctional linker attached to each warhead. The free ends of the bifunctional linker were conjugated with two designated cysteines on a randomized peptide via the thioether linkage. After bio-panning using the warhead-introduced peptide library, cyclic peptidic TCIs against cysteine- and serine-proteases were obtained. Using another approach, Taki's group introduced a latent-electrophilic aryl fluorosulfate (i.e., fosylate [61]; Ar-OSO_2F) warhead [30][62] which is completely inert and activated only in a matchmaking (i.e., enzyme-like) microenvironment [27][28][35] created between the target protein and an appropriate peptide during the reactivity and affinity-based [63] co-selection process of the T7 phage display [40]. The fosylate warhead minimized the promiscuous reaction during the library's construction/selection, and a TCI was obtained with only 2 rounds of bio-panning. Non-specific and non-covalent

interactions between target-unrelated biomolecules were eliminated during a harsh washing step with a urea and SDS containing buffer, while the robust T7 phage still kept its infectivity [64].

Another direct screening system using M13 phage display extended the possibility of finding reversible peptidic TCIs, as demonstrated by Zheng and Gao [65]. A lysine-targeted warhead (i.e., 2-acetylphenylboronic acid) was attached to a library peptide displayed on the phage, and cyclic TCIs reversibly conjugating to SrtA and SARS-CoV-2 spike RBD proteins were selected. This new modality endowed a long target-residence time of the drug without permanent conjugation, as in bioTCIs, and reduced the drug clearance and risk of immunogenicity [2][13]. A reversible covalent binding of cyclic TCIs to off-targets should reduce the chances of a prolonged ADE [66].

Antidote-reversible small-molecule TCIs targeting thiols, alcohol, and amines have been described [67], but none have been reported for a peptidic TCI. These approaches may be better described as a reversal of the covalent bond formation rather than an antidote reversal of the drug effect, even while the TCI is still covalently bound to the target protein. A truly neutralizable warhead/antidote pair (e.g., benzoxaborole/reduced-glutathione) [68][69], where the covalent bond is maintained but the non-toxic antidote rendering the drug inactive, has been proposed for peptidic TCI, but this technology is yet to be implemented. Discovery of a technology that will allow on-demand and specific reversal of peptidic TCIs by addition of non-toxic antidote molecules, as discussed in the next section for nucleotide TCIs, is one key future direction.

2.2. Nucleotidic TCI: A Developing Modality

Single-stranded DNA or RNA oligonucleotides form complex folded structures and a specific aptamer that binds to the desired target [70] can be identified by a repetitive screening of an aptamer library against the target by a process termed systematic evolution of ligands by exponential enrichment (SELEX) [71][72][73]. The starting library of N random sequence possesses 4^N complexity (i.e., ~10^{12} for N = 20, and 10^{24} for N = 40), favoring the discovery of an aptamer with a specific sequence necessary for binding to the desired target. Many high affinity aptamers with an affinity in the pM range or better have been selected, curated (Apta-Index, http://www.aptagen.com/; RNAapt3D, https://rnaapt3d.medals.jp/, both accessed on 6 February 2023) and readily available from many commercial vendors. Aptamers have been touted as potential antibody replacements given their high specificity and affinity [72][74] but the limitation of a very short in vivo half-life has prevented their practical application [70]. In theory, these already identified aptamers can be rendered potential TCIs with a long drug half-life just by incorporating a warhead, as recently demonstrated for the thrombin binding aptamer [14][22] and SARS-CoV-2 spike protein binding aptamer [31][57]. Whether other warhead-introduced aptamers will conjugate with the target protein depends on the orientation of the aptamer to the target dictating the proper positioning of the warhead on the aptamer, and the availability of the interaction-capable amino acids on the target protein. Such a trial-and-error approach to creating a TCI from a pre-selected aptamer can be circumvented by directly selecting for a covalently binding aptamer during the SELEX process akin to the phage-library approach established for the peptidic TCIs.

Smith and colleagues were the first to report covalently binding RNA and DNA aptamers targeting the neutrophil elastase [75][76] obtained by a direct combinatorial screening method denoted the blended SELEX. This first-

generation nucleotidic bioTCI was selected by utilizing a splint-DNA comprising a small-molecule-TCI as the elastase-specific warhead, a spacer, and a 3′ fifteen base pair overlap complimentary to the forward primer region of the aptamer library. The mobility-shifted aptamer-bound protein band was gel-isolated, and the eluted aptamer was amplified by PCR to generate the aptamer pool for the second-round selection. The optimized bioTCI from the library enhanced the target selectivity and specificity of the original small-molecule-TCI. This method has a major advantage in that an unmodified conventional aptamer library is used as the input. The selected aptamer can be readily separated from the covalently bound splint DNA by heating, since only the partial double strand connects the splint DNA to the unmodified library aptamer pool. An expansion of this attractive method of combinatorial screening for nucleotidic bioTCIs using a *generalized* warhead capable of interacting with any desired target protein, instead of the neutrophil elastase-specific already-known TCI as the warhead, has not been reported.

The second-generation nucleotidic bioTCI is based on a pre-identified DNA aptamer showing high affinity for the target, and a warhead directly conjugated at a specific position of the DNA [14][22][57]. It should be noted that TCI aptamers created in such a fashion are better described as a tethered-TCI (TeTCI) (**Figure 7**) since the protein domain forming the covalent bond is outside [6][77][78] the actual aptamer docking domain. The long linker between the warhead and the aptamer serves as a chain that tethers the aptamer to the target protein. The TeTCI is conceptually different from most of the small molecule or even the peptidic TCI where the covalently binding residue is within or near the docking domain [7]. Three methods for introducing a warhead into an aptamer to create a TeTCI have been reported over the last two years. Tabuchi et al. [14] replaced a thymine (T) residue of the thrombin binding aptamer (TBA) with an octadiynyl-dU (OctdU) containing an alkyne group based on the structural information of TBA-bound thrombin [79], and a benzenesulfonyl-fluoride warhead introduced by the copper-catalyzed azide–alkyne cycloaddition (CuAAC; also known as a click chemistry). The starting 15-mer TBA aptamer selected by the conventional SELEX is a well-studied thrombin binding G-quadruplex aptamer [80]. Warhead introduction at the 3rd T residue of the TBA (i.e., TBA3) resulted in an efficient TCI reacting with thrombin, while the same warhead placed at T9 facing away from the target protein resulted in only a weak covalent-binding ability. The resulting TBA3 covalently bound to thrombin and inhibited the enzymatic activity of the target. TBA3 demonstrated nuclease resistance, and the TeTCI remained bound to thrombin, and intact, even after 24 h of digestion with DNase I [21]. As expected, the addition of the complimentary-strand (CS) oligonucleotide against the aptamer sequence as an antidote [81] reversed the thrombin inhibition, and the CS antidote rendered the thrombin-conjugated TBA3 nuclease-sensitive [14]. The reversal by the CS antidote was swift, probably because the relatively long tether placed between the warhead and the aptamer did not interfere with either the double-strand (DS) formation between the aptamer and the CS, or the exposure of the DS towards the outside of the binding pocket on the target protein accessible to nuclease digestion (**Figure 8**). The researchers have applied the same technology and confirmed the creation of a TeTCI targeting SARS-CoV-2 S-protein RBD domain from a previously reported DNA aptamer. Multiple warhead introduction into a single aptamer showed greater inhibition than the corresponding monoadduct.

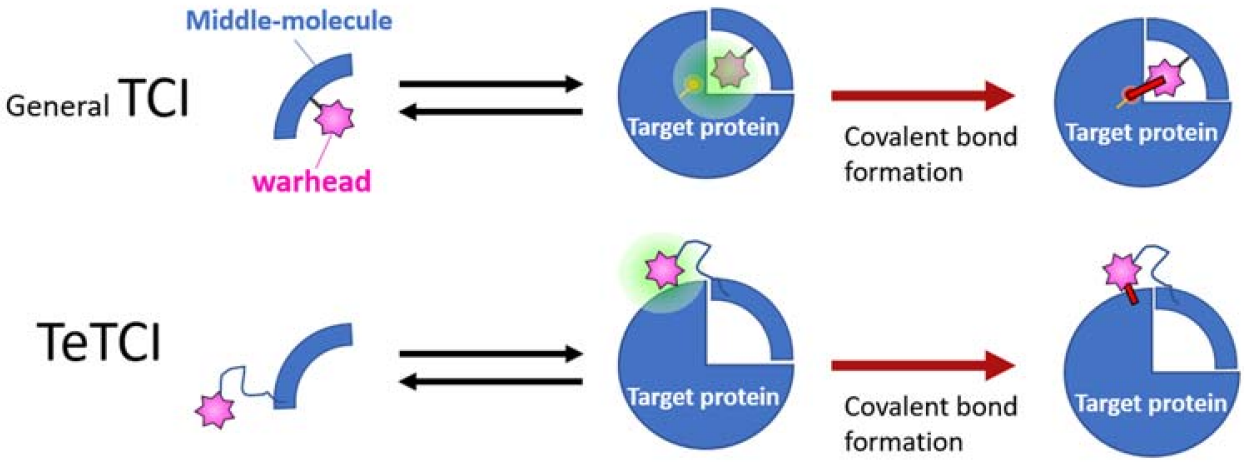

Figure 7. A graphical summary of the conventional vs. tethered TCI (abbreviated as TeTCI). The general TCI (top row, blue quarter) endowed with a warhead (pink star) follows a two-step binding to form a covalent bond with the amino acid (s) usually within the TCI docking domain of the target protein (right). A TeTCI (second row) where the warhead attaches to the drug modality (e.g., a nucleotidic aptamer) through a long linker similarly binds to the target, but the site of covalent attachment is outside the docking domain. This distinction between the drug docking site and the site of covalent bond formation (i.e., general vs. TeTCI) is not modality dependent since some small molecule TCI endowed with a warhead with a long linker also reacts with residues outside of the presumptive docking domain.

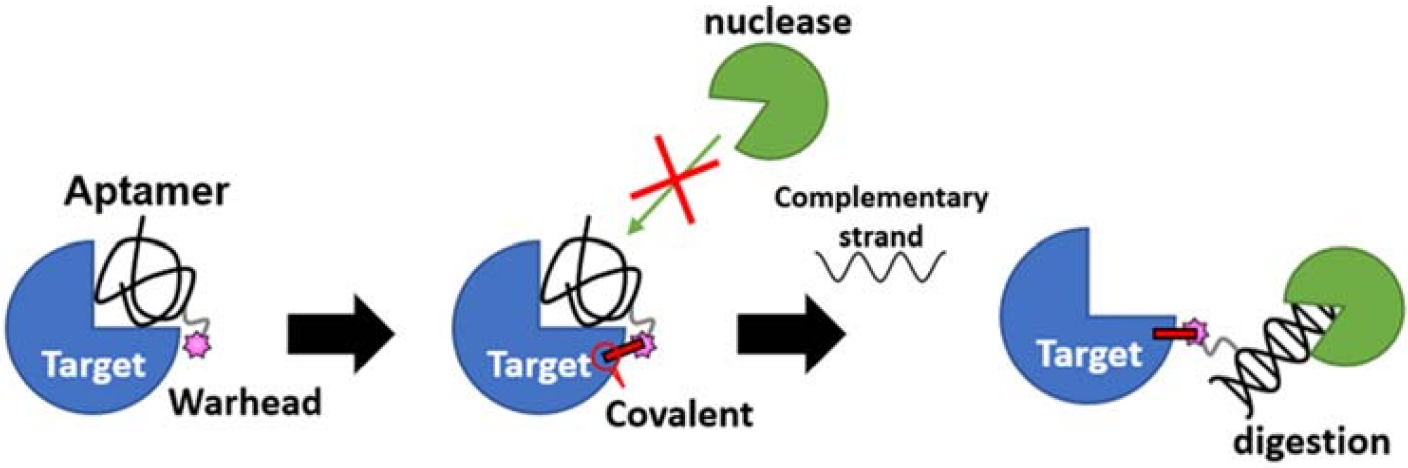

Figure 8. A reversible antidote mechanism of nucleotidic TCI. Once the irreversible covalent bond is formed on the target, the aptamer becomes nuclease (green packman) resistance. When the aptamer is dislodged from the binding site by the on-demand addition of the complimentary strand antidote, it becomes sensitive to nuclease digestion even though still covalently bound to the target.

TBA possessing an alternative inverse electrophile, as reported by Tivon et al., showed the same results. The conjugation efficiency of their aptamer TCI with thrombin depended on where in the aptamer the warhead was introduced, and the enzymatic inhibition and relative nuclease resistance of the aptamer-conjugated thrombin were reversed by the CS antidote [22]. The availability of precise structural information of TBA bound to thrombin enabled

the rational determination of where to introduce the warhead for this aptamer. Structural information is usually unavailable for most aptamers bound to its target, and the determination of the position of warhead introduction becomes a labor-intensive trial-and-error process where every T residue is replaced with an OctdU and screened for efficient covalent binding to the target. Qin et al. [57] reported a potential docking-structure-independent method of warhead introduction by simply extending the 3′ end of an aptamer with a phosphorothioate (PS)-linked nucleic acid tail, and subsequent introduction of a warhead through a simple nucleophilic reaction between a Br-warhead and the S-atom of the PS linker. The authors chose two SARS-CoV-2 S-protein binding aptamers previously selected by the conventional SELEX [74][82] and tailed the 3′ end with 7 T residues possessing 1, 3, 5, or 7 PS bonds. Subsequent methyl-benzenesulfonyl fluoride introduction by the nucleophilic substitution reaction rendered the aptamer a TCI with the 7 PS-tail showing the best covalent bond formation with the target protein. Whether this structural information-independent approach to create a TCI by the PS tailing can be extended to other aptamers or whether the PS-tailing bound aptamer is reversible by the CS antidote is unknown.

TeTCI, created by introducing a warhead with a relatively long linker, might increase the non-specific binding to unintended off-targets. TeTCI examined to date show specificity for the intended target in the presence of serum, suggesting that even a tethered warhead requires a proper matchmaking environment guided by the aptamer docking to the intended target for the covalent bond formation. However, further studies are needed to determine whether the TeTCI's matchmaking environment is as selective and rigorous as the conventional TCI where the docking itself appears to create the environment conducive to the covalent bond formation. Alternatively, incorporation of sulfamoylfluoride-functionalized nucleosides [83] where the modified nucleosides can directly undergo SuFEx with the target with no long linker could result in a traditional nucleotidic TCI where the site of covalent bond formation is within the docking pocket.

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