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Chemically programmed antibodies

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Abstract

Due to their unlimited chemical diversity, small molecules can rival monoclonal antibodies (mAbs) with respect to specificity and affinity for target molecules. However, key pharmacological properties of mAbs remain unmatched by small molecules. Chemical programming strategies have been developed for site-specific and covalent conjugation of small molecules to mAbs with unique reactivity centers. In addition to blending favorable features of small molecules and mAbs, chemically programmed antibodies (cpAbs) are economically attractive because they utilize the same mAb for a virtually unlimited number of target molecule specificities, reducing manufacturing costs and shortening drug discovery and development time. Preclinical studies and clinical trials have begun to demonstrate the broad utility of cpAbs for the treatment and prevention of human diseases.

Keywords

Pharmaceutical; monoclonal antibody; small molecule; site-specific conjugation; covalent conjugation

Monoclonal antibodies versus small molecules

Monoclonal antibodies (mAbs) have become clinically and commercially highly successful pharmaceuticals. Over the past two decades >30 mAbs were approved by regulatory agencies in the USA and EU [1]. A similar number of mAbs is currently in phase III clinical trials [2]. mAbs have already benefitted millions of patients worldwide. Whereas cancer and autoimmune diseases have remained the dominant indications, mAbs for the treatment or prevention of infectious, cardiovascular, neurodegenerative, and other human diseases have also been marketed or have advanced to late stages of the drug pipeline. In some indications, such as CD20+ B-cell non-Hodgkin lymphoma (*rituximab*) and HER2+ breast cancer (*trastuzumab*), mAbs are now an integral part of standard first-line therapy. For certain rare human diseases, such as paroxysmal nocturnal hemoglobinuria (*eculizumab*), mAbs are or are projected to become the only approved drugs. In accord with their clinical success, global sales of mAbs totaled >\$50 billion in 2012 and are expected to rise at an annual growth rate of >5% over the next decade (www.bccresearch.com).

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What are the reasons behind the boom of mAbs? Key features at the center of both their clinical and commercial success are precision and pharmacology. The precision of mAbs is in one part founded on their high specificity and affinity for antigens and in another part due to the local confinement of the large antibody molecule (~150 kDa) to the circulatory system and interstitial space. Small molecules (<1 kDa), by contrast, have unlimited access to nearly all extracellular and intracellular niches, making activity and toxicity profiles less predictable. The larger size of mAbs, along with its recycling through the neonatal Fc receptor (FcRn) of endothelial cells, monocytes, and macrophages also results in a prolonged circulatory half-life when compared to small molecules. In addition to interacting with FcRn, the Fc fragment of mAbs in Immunoglobulin G (IgG) format, mediates their effector functions, including complement-dependent cytotoxicity (CDC) by recruiting complement protein C1q and antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) by interacting with Fc γ receptors expressed on NK cells, macrophages, and other leukocytes. CDC, ADCC, and ADCP are collectively known as the effector functions of mAbs. Fc optimization through rational design and directed evolution has allowed the tuning of the circulatory half-life as well as effector functions [3]. Thus, the Fc fragment is crucial for the favorable and tunable pharmacokinetic (PK) and pharmacodynamic (PD) properties of mAbs when compared to small molecules. Being small and synthetic, however, also has advantages. In contrast to mAbs, small molecules can readily diffuse through tissues and cells and can be made orally available. Small molecules are also cheaper to manufacture. The production of mAbs, which are heterogeneous glycoproteins expressed in mammalian cells and purified by a multistep chromatography process [4], is considerably more complex than the production of small molecules. Although the higher manufacturing costs contribute only a fraction to the current market price of brand name mAbs, they will limit the savings one can expect from generic and biosimilar mAbs in the future.

Chemically programmed antibodies (cpAbs) address some of these challenges by blending favorable features of mAbs and small molecules (Table 1, Figure 1). Pharmacologically, cpAbs equip small molecules with the PK and PD properties of the antibody molecule. Economically, cpAbs are expected to reduce manufacturing costs and shorten drug discovery and development time (Figure 2). This review delineates the molecular concept and architecture of cpAbs, discusses their preclinical and clinical performance, and delivers an outlook on this new class of pharmaceuticals.

Molecular concept and architecture of cpAbs

Conventional mAbs (Figure 1A) bind antigens by virtue of their complementarity-determining regions (CDRs), which are on certain β -turns of the antiparallel β -sandwich that makes up the immunoglobulin (Ig) fold. Up to six CDRs, three from each light and heavy chain variable domain, can contribute to the antigen binding site, potentially providing large surface areas mediating the interaction of paratope (antibody) and epitope (antigen) [5]. The proportion and complementarity of this interface explains the high specificity and affinity with which antibodies can bind antigens. Antigen recognition by cpAbs is fundamentally different in that it is mediated by a synthetic component, which is site-specifically and covalently attached to an antibody component (Figure 1B).

The antibody component equips the synthetic component with the circulatory half-life, bivalence, and effector functions of conventional mAbs. Although not directly contributing to antigen recognition, the antibody component also endows the synthetic component with bulkiness, which can augment its ability to interfere with ligand-receptor interactions. The only formal requirement for the antibody component of cpAbs is a unique reactivity center. At a minimum, the antibody component is an Fc fragment with a unique reactivity center at

the *N*- or *C*-terminus. The synthetic component of cpAbs consists of: (i) a pharmacophore (a peptide, a peptidomimetic, or other small molecule) that binds with high specificity and affinity to an extracellular (membrane-bound or secreted) antigen; (ii) a reactive (usually electrophilic) group that permits site-specific and covalent conjugation to a unique reactive (usually nucleophilic) residue of the antibody component; and (iii) a linker spacing pharmacophore and reactive group.

In the most basic molecular assembly, termed *Fc-based cpAb*, the synthetic component replaces the Fab fragment of a conventional mAb (Figure 1B **center**). Alternatively, the antibody component is a conventional mAb with a unique reactivity center in its paratope suitable for chemical programming. The resulting cpAb (Figure 1B **left**), termed *IgG-based cpAb*, closely resembles a conventional mAb with respect to shape and size. This molecular assembly is found in the first cpAbs that were described a decade ago and entered clinical trials shortly after [6]. Overall, irrespective of these various molecular assemblies, the antibody component of cpAbs serves as a carrying moiety and the synthetic component serves as a targeting moiety. Recently, *Fab-based cpAbs* have been introduced, where the antibody component serves simultaneously as carrying and targeting moiety [7] (Figure 1B **right**). Thus, Fab-based cpAbs have two targeting moieties and constitute a new class of bispecific antibodies.

A key characteristic of cpAbs is the *site-specific* conjugation of the synthetic component to the antibody component. A defined molecular assembly mandates site-specific conjugation. Corresponding to conventional mAbs in IgG format, which comprise two Fab and one Fc fragment (Figure 1A), cpAbs combine a fixed number of targeting and carrying moieties in a demarcated arrangement (Figure 1B). By contrast, random conjugation of synthetic component and antibody component results in a mixture of conjugates, exhibiting a range of stoichiometries and batch-to-batch variability. In addition to causing product heterogeneity, random conjugation can impair the pharmacological properties of the antibody component [8]. Another key characteristic of cpAbs is the *covalent* conjugation of the synthetic component to the antibody component. Covalent conjugation can be reversible or irreversible.

Features of the antibody component of cpAbs

The site-specific and covalent conjugation of a synthetic component requires the presence of unique reactivity centers in the antibody component. Three unique reactivity centers have been predominantly used to generate cpAbs: a reactive lysine (K) residue in the paratope for the assembly of IgG-based cpAbs, engineered *N*-terminal cysteine (C) or *C*-terminal selenocysteine (U) residues for Fc-based cpAbs, and an engineered *C*-terminal selenocysteine residue for Fab-based cpAbs (Figure 1C). The unique reactivity centers that are defined by these three different natural amino acids are discussed separately in the following. In addition, an *N*-terminal alanine-lysine-threonine (AKT) tripeptide [9], which constitutes a substrate for pyridoxal 5'-phosphate-mediated transamination, and the unnatural amino acid para-acetylphenylalanine (pAcPhe) [10,11] were recently deployed as unique reactivity centers for Fc-based and Fab-based cpAbs, respectively (Table 2).

Reactive lysine—The original concept of cpAbs [6] was based on a group of mAbs that were induced by reactive immunization of mice and then cloned, expressed, and purified by hybridoma technology [12,13]. Reactive immunization can be defined as the induction of antibodies that covalently bind a reactive immunogen [14]. A 1,3-diketone hapten was used as a reactive immunogen for the induction of antibodies with a reactive lysine residue in the paratope (Figure 3A). The crystal structure of one of the anti-1,3-diketone hapten mAbs, 33F12, revealed that the paratope is a hydrophobic cleft >11 Å deep with the reactive lysine

residue, K99, at its base [15]. K99 of the heavy chain variable domain of mAb 33F12 and other anti-1,3-diketone hapten mAbs lies in close proximity to the third CDR and arose by somatic mutation [13,16]. The hydrophobic microenvironment disfavors the protonation of the ϵ -amino group of K99, thereby substantially reducing its pKa and allowing a reversible covalent interaction with the 1,3-diketone hapten to form an enaminone stabilized by imine-enamine tautomerism (Figure 3A). The covalent conjugation was confirmed in a crystal structure of mAb 33F12 in complex with the 1,3-diketone hapten [17]. Collectively, K99 of anti-1,3-diketone hapten mAbs has become an extensively proven unique reactivity center in antibody components of cpAbs. Nonetheless, unique reactivity centers in the paratope that mediate site-specific and covalent conjugation are not limited to reactive lysine residues, providing additional opportunities for the generation of cpAbs [18–22]. For example, alternative unique reactivity centers include cysteines engineered by rational design [19] and tyrosines selected from antibody libraries by phage display [21].

N-terminal cysteine—As discussed above, natural amino acids in the microenvironment of the paratope can have unique reactivity. Outside of a paratope or in its absence, the availability of unique reactivity centers in the antibody component is limited. An engineered *N*-terminal cysteine residue, C1, in Fc fragments provides an example of a unique reactivity center used for the generation of cpAbs in the absence of a paratope [23]. Exploiting the concept of native chemical ligation [24], the naturally occurring 1,2-aminothiol group of C1 is reacted with a thioester derivative resulting in the formation of an amide bond (Figure 3B).

C-terminal selenocysteine—Another example of a unique reactivity center used for the generation of cpAbs in the absence of a paratope is an engineered *C*-terminal selenocysteine residue, U234, in Fc fragments [25]. Selenocysteine, also known as the 21st natural amino acid, is inserted at the *C*-terminus by equipping an antibody expression cassette with a TGA codon followed by a hexa-histidine encoding sequence, a TAA stop codon, and a 3' untranslated region (UTR) with a selenocysteine incorporation sequence (SECIS) element [25]. In mammalian cells (in the presence of the SECIS element and supplemental 1 μ M sodium selenite (Na_2SeO_3) in the cell culture medium), the TGA codon (UGA in the mRNA), which is normally a stop codon, instructs the incorporation of a selenocysteine residue during translation [26]. As selenocysteine insertion at the TGA codon competes with termination, this expression cassette yields both Fc with and without U234. By virtue of the hexa-histidine encoding sequence that follows the TGA codon, Fc-stop and Fc-U-H₆ proteins can be efficiently separated by immobilized metal affinity chromatography (IMAC) [7,25,27]. However, as termination dominates selenocysteine insertion, Fc-U-H₆ is produced at approximately five-fold lower levels than Fc-stop protein, and the majority of dimeric Fc-U-H₆ protein is composed of a hinge-C_H2-C_H3-stop and a hinge-C_H2-C_H3-U-H₆ half, resulting in the display of only one unique reactivity center in an asymmetric Fc fragment (Figure 1C). Interestingly, without further loss in yield, a second unique reactivity center, U240, can be added by placing a tetra-glycine-serine (G₄S) encoding sequence followed by a second TGA codon between the first TGA codon and the hexa-histidine encoding sequence [28]. Due to the larger size of selenium (34 electrons) compared to sulfur (16 electrons), the selenol group of selenocysteine (pKa 5.2) is more nucleophilic than the thiol group of cysteine (pKa 8.3) and can be selectively and rapidly conjugated to maleimide (Figure 3C) and iodoacetamide derivatives of small molecules under mildly acidic (pH 5.2) and mildly reducing (0.1 mM dithiothreitol) conditions without modifying any other amino acid residues in the antibody molecule [25]. Although requiring complex translation machinery [29], all parts necessary for selenocysteine insertion preexist in mammalian cells and can be exploited for antibody engineering. Endogenous antibodies do not contain

selenocysteine residues, providing an opportunity for a unique reactivity center in engineered antibodies.

In addition to the Fc format, mAbs in IgG, scFv-Fc, and Fab format have been engineered with C-terminal selenocysteine residues [7,27,28]. mAbs with a C-terminal selenocysteine can also be expressed in bacteria as demonstrated for the scFv format [30]. Although using the same codon, TGA, the prokaryotic selenocysteine insertion machinery differs from its eukaryotic counterpart in several respects, including the location of its SECIS element immediately downstream of the TGA codon, which confines the location of the selenocysteine residue to the C-terminus if amino acid sequence mutations are to be avoided. By contrast, the location of its SECIS element in the 3' UTR theoretically allows placing the selenocysteine residue at various locations without compromising downstream amino acid sequences. Nonetheless, a C-terminal location (upstream of a His₆ tag) ensures that even larger synthetic components in cpAbs do not interfere with Fc fragment interactions that govern circulatory half-life and effector functions. Regardless of whether utilizing prokaryotic or eukaryotic selenocysteine insertion machineries, strategies for increasing the ratio of selenocysteine insertion over termination [31,32] may prove important for translating cpAbs with a selenocysteine interface from preclinical studies to clinical trials.

Other unique reactivity centers—Site-specific and covalent conjugation of a synthetic component to an antibody component is also relevant in the generation of antibody-drug conjugates (ADCs) [33]. In contrast to cpAbs, ADCs use the antibody component for mediating antigen binding and the synthetic component for mediating cytotoxicity. Nonetheless, defined molecular assemblies involving unique reactivity centers are relevant for both ADCs and cpAbs. In addition to natural amino acids, such as engineered cysteines [34], genetically encoded unnatural amino acids, such as pAcPhe [35], have been used as unique reactivity centers in ADCs. Inserting genetically encoded unnatural amino acids via termination codons (typically TAG) requires an exogenous translation machinery with an orthogonal tRNA/aminoacyl-tRNA synthetase pair [36]. This strategy allows for the insertion of nucleophilic or electrophilic unnatural amino acids, affording a variety of conjugation chemistries.

mAbs with unique reactivity centers can also be generated enzymatically through a genetically encoded, typically C-terminal peptide tag [37,38]. For example, formylglycine-generating enzyme converts the cysteine residue (thiol group) in the consensus pentapeptide sequence CXPXR to a formylglycine residue (aldehyde group). The electrophilic aldehyde group is then reacted with small molecules derivatized with nucleophilic aminoxy or hydrazide groups [39]. Other enzymes that have been used for site-specific and covalent conjugation of a synthetic component to an antibody component include sortase [40] and transglutaminase [41]. For therapeutic applications that necessitate repeated dosing, traceless or near traceless strategies, which replace all or most of the peptide tag with the small molecule, are preferred because they minimize the potential of immunogenicity [42]. Collectively, the strategies described in this paragraph provide a rich assortment of alternative routes to antibody components with unique reactivity centers.

Features of the synthetic component of cpAbs

In order to serve as synthetic components for cpAbs, small molecules must bind these antigens with high specificity and affinity. Suitable pharmacophores for the synthetic component of cpAbs were created by rational design or selected from chemical libraries. The ability to use one-bead-one-compound (OBOC) and other chemical libraries for developing synthetic components is a key incentive for cpAbs because it may allow

identifying pharmacophores for targeting extracellular antigens with unknown high affinity binding sites for small molecules [43,44]. In addition, the selection of peptide libraries by phage display has yielded pharmacophores for synthetic components of cpAbs [45]. In fact, peptides derivatized with suitable linkers and reactive groups are used as synthetic components of first-generation cpAbs that have reached clinical trials (Table 2). An advantage these assemblies have over peptibodies [46], where a peptide is genetically fused to a Fc fragment, is that the linkage is not confined to the *N*- or the *C*-terminus of the peptide but can involve amino acid residues in the middle, affording antibody-peptide conjugates with superior pharmacological properties [47]. In addition, whereas peptibodies are limited to peptides that consist of L-amino acids, cpAbs can also accommodate peptides with D-amino acids, which have demonstrated stronger resistance to proteolytic degradation.

IgG-based and symmetric Fc-based cpAbs dimerize the pharmacophore by virtue of their two unique reactivity centers (Figure 1B). The ability to bind extracellular antigens bivalently rather than monovalently can offset shortcomings of pharmacophores that bind their target molecules with suprananomolar to submicromolar affinity. An avidity gain of asymmetric Fc-based cpAbs and Fab-based cpAbs with one unique reactivity center can be achieved by synthesizing synthetic components with homodimeric pharmacophores [7,48]. Notably, neither approach substantially increases the size of cpAbs. In addition to homodimeric pharmacophores, synthetic components with heterodimeric pharmacophores engaging two different extracellular antigens have been deployed to afford bispecific cpAbs as discussed below. It is conceivable that synthetic components of cpAbs could incorporate additional moieties that augment the pharmacological properties of cpAbs. These may include cytotoxic drugs or radioisotopes for indications that require efficient target cell destruction or peptides, peptidomimetics, or other small molecules that extend the circulatory half-life of cpAbs by binding to serum proteins. For certain applications it may be advantageous to add these moieties to the already assembled cpAb by click chemistry [49]. In addition to small synthetic molecules, the synthetic component in cpAbs can comprise large synthetic molecules, such as RNA or DNA aptamers [9,50] (Table 2), which can be selected for high specificity and affinity from oligonucleotide libraries. Collectively, the ability to tailor specificity, valence, potency, and circulatory half-life through chemical synthesis is a distinctive asset of cpAbs.

Bispecific cpAbs

Bispecific antibodies are a class of next-generation antibody pharmaceuticals enabling simultaneous engagement of two antigens or epitopes. Bispecific binding can augment specificity or potency or both. For example, through acquiring bispecificity, mAbs can be made more specific for target cells by requiring the presence of two different cell surface antigens and can be made more potent by simultaneously antagonizing two different signaling pathways [51]. Furthermore, bispecific antibodies can exert cytotoxicity by recruiting and activating endogenous immune cells [51]. This is achieved by combining specificities for target and effector cells in one molecule. Bispecific cpAbs, in which one or both specificities are mediated by a synthetic component, have also been described [7,10,11,48,52]. There are many possible architectures for bispecific cpAbs (Figure 4). A first group among these utilizes two different monospecific synthetic components. These are either conjugated to two identical unique reactivity centers (resulting in mixtures of bivalent monospecific and monovalent bispecific cpAbs) or to two orthogonal unique reactivity centers. For example, an antibody component that pairs a reactive lysine residue in the paratope with a *C*-terminal selenocysteine residue affords two orthogonal unique reactivity centers that can be simultaneously or sequentially conjugated to two different synthetic components. A second group of bispecific cpAbs utilizes a bispecific synthetic component. Published examples include trifunctional synthetic components that combine specificities for

two different extracellular antigens with a reactive group [48,52]. A third group of bispecific cpAbs make use of antibody components with a paratope that remains untouched by chemical programming. Conjugating a monospecific synthetic component to a unique reactivity center in these antibody components affords two antigen binding sites, one provided by the antibody component and one provided by the synthetic component. With a molecular weight of 50 kDa, Fab-based bispecific cpAbs [7,10,11] share the same size with a newer class of conventional bispecific antibodies that brings target and effector cells into close proximity to enable cytolytic synapses and avoids systemic effector cell activation through monovalent rather than bivalent engagement in the absence of target cells [53,54]. In addition to the above discussed economic and pharmacological advantages cpAbs have over mAbs (Table 1), bispecific cpAbs may be more adept at reaching concealed epitopes in membrane proximal locations for the formation of cytolytic synapses.

Comparison of cpAbs with ARMs

Chemical programming of a unique reactivity center in the paratope of an IgG can be achieved by either covalent or non-covalent conjugation. In the latter case, the reactive moiety in the synthetic component is replaced with a hapten that binds with high affinity to the paratope. For example, fluorescein can serve as a hapten for the non-covalent conjugation of a synthetic component to an anti-fluorescein mAb. Notably, non-covalent conjugations enable the chemical programming of endogenous antibodies. In this concept [55], which has been reviewed elsewhere [56], the synthetic component serves as an antibody-recruiting molecule (ARM). ARMs can recruit preexisting endogenous antibodies that recognize certain haptens, such as galactosyl- α -(1,3)-galactose (α -Gal) or 2,4-dinitrophenol (DNP). Alternatively, ARMs can recruit endogenous antibodies that were triggered by immunization with hapten-carrier conjugates. For example, in a phase I clinical trial [57], patients with metastatic renal cell carcinoma were first vaccinated with fluorescein isothiocyanate (FITC; hapten) conjugated to keyhole limpet hemocyanin (KLH; carrier) and were then treated with a folate-FITC ARM. Folate (vitamin B9) binds with high affinity to folate receptor 1 (FOLR1), which is expressed on the cell surface of tumor cells. Thus, the folate-FITC ARM decorates tumor cells and recruits endogenous anti-FITC antibodies that kill the tumor cells by mediating CDC, ADCC, and ADCP. An advantage that ARMs have over cpAbs is that they can be made orally available. Nonetheless, compared to ARMs that assemble with endogenous antibodies of varying quantity and quality *in vivo*, preassembled cpAbs afford pharmacological control over the antibody component. In case of IgG-based cpAbs, which can be preassembled covalently or non-covalently, covalent conjugation of the synthetic component has the advantage that the resulting cpAb is a single molecule rather than a mixture of two molecules, which generally expedites the approval process of Investigational New Drug (IND) applications by regulatory agencies prior to commencing clinical trials.

Applications of cpAbs

Targeting integrins

Several integrins, which are expressed on tumor cells and tumor endothelial cells, have high affinity binding sites for tripeptide motifs, such as RGD (integrin α v β 3, α v β 5, α v β 6, and α 5 β 1) and LDV (integrins α 4 β 1 and α 4 β 7). These tripeptide motifs are present in extracellular matrix and cell surface proteins that bind to integrins. As linear or cyclic peptides, they potently antagonize these interactions and induce apoptosis. To improve the pharmacological properties of small molecules that target and antagonize integrins, a large variety of peptidomimetics have been developed in academia and industry [58]. Tapping into this resource of extensive chemical diversity, the concept of cpAbs was established with synthetic components having an integrin-targeting and antagonizing peptidomimetic as

pharmacophore (Table 2). The pharmacophore used in the first cpAbs was developed by rational design as orally available RGD peptidomimetic that antagonizes integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [59].

In the first reports of cpAbs, the anti-1,3-diketone hapten mAb 38C2 [12,15], and subsequently its humanized version mAb h38C2 [60], were conjugated to 1,3-diketone derivatives of proapoptotic peptidomimetics that bind with high specificity and affinity to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [6,61]. Chemical programming endowed mAb 38C2 with the ability to bind and kill tumor cells and tumor endothelial cells expressing these integrins. In mouse models, the cpAb prolonged the circulatory half-life of the peptidomimetic >100-fold and increased its cytotoxicity >1000-fold, with effector functions, such as CDC and ADCC, playing contributing roles [6,62].

Notably, in addition to the preassembled cpAb, which was generated *in vitro* by simply incubating mAb 38C2 with a twice equimolar concentration of the 1,3-diketone derivative for two hours at room temperature, antibody component (given intravenously) and synthetic component (given intraperitoneally) also spontaneously assembled *in vivo* [6]. Prompted by this finding, 1,3-diketone derivatives were also shown to serve as ARMs for endogenous antibodies triggered by immunization [63]. With the preassembled cpAb being a preferred IND entity, however, subsequent studies with mAbs 38C2 and h38C2 switched the electrophilic group of the synthetic component from 1,3-diketone to 2-azetidinone (β -lactam), which affords irreversible covalent conjugation to K99 [64]. Irreversible covalent conjugation to mAb 38C2 and other aldolase mAbs was also achieved with a vinylketone released from its stable acetone aldol adduct by the catalytic activity of the reactive lysine residue [65]. Validating the notion of broad utility of a single antibody component, an increasing number of preclinical studies have used chemically programmed mAbs 38C2 and h38C2 to target a multitude of extracellular antigens involved in cancer and other human diseases (Table 2).

Fc fragments with an engineered C1 were expressed in yeast by inserting the cleavage site of an endogenous yeast protease, Kex2, immediately upstream of the cysteine residue and downstream of a yeast secretion signal peptide [23,66]. For proof of concept, C1 was then reacted with a thioester derivative of the cyclic RGDfK peptide [23]. The resulting cpAb was shown to target tumor cells expressing integrin $\alpha_v\beta_3$. Antibody fragments with *N*-terminal cysteine residues have also been expressed in bacteria [67] and mammalian cells [68], and these were reacted with small molecules derivatized with an aldehyde group to yield thiazolidine heterocycles.

For initial proof of concept of Fc fragments with an engineered C-terminal selenocysteine, U234 was reacted with a maleimide derivative of the peptidomimetic LLP2A. LLP2A is an orally available LDV-mimicking peptidomimetic that had been selected from an OBOC chemical library for binding to integrin $\alpha_4\beta_1$ with picomolar affinity [69]. Chemical programming endowed both antibody component and synthetic component with pharmacological advantages. Fc-U-H6 acquired the ability to target tumor cells expressing integrin $\alpha_4\beta_1$ and to block the interaction of integrin $\alpha_4\beta_1$ with vascular cell adhesion molecule 1 (VCAM1). LLP2A acquired a prolonged circulatory half-life and the ability to be delivered via the lung to the blood by FcRn-mediated transcytosis [25] (Table 1).

Targeting other membrane-bound antigens

In addition to integrins, a number of other cell surface receptors with high affinity binding sites for small molecules have been targeted by cpAbs (Table 2). These include receptors with natural small molecule ligands, such as FOLR1 [7,11], and receptors with natural peptide ligands, such as endothelin receptor type A [70], luteinizing hormone receptor [48]

and kappa opioid receptor [71]. Other non-integrin cell surface receptors with high affinity binding sites for small molecules that were derived by rational design or selected from chemical libraries have also been described as targets of cpAbs. This list includes prostate-specific membrane antigen (PSMA) [11], CCR5 [72], gp120 of HIV-1 [73], and neuraminidase of the influenza virus [74]. In addition, protein tyrosine kinase-7 and cell surface IgM have been targeted with cpAbs that deploy DNA aptamers as synthetic component [9]. Furthermore, cpAbs have been used to extend the short circulatory half-lives of small therapeutic peptidomimetics, peptides, and proteins, including analogs of thrombospondin-1 [75,76], glucagon-like peptide-1 (GLP-1), human growth hormone [77], and FGF-21 [78].

Simultaneous targeting of two different cell surface receptors was demonstrated for bispecific cpAbs in Fab format that engage CD3 on T cells via their antibody component and integrin $\alpha 4\beta 1$ [7], FOLR1 [7,11], or PSMA [10] on target cells via their synthetic component. Bispecific cpAbs can also engage two different cell surface receptors on the same target cell [48].

Targeting secreted antigens

An important application of cpAbs has been the neutralization of growth factors promoting tumor growth and angiogenesis, including angiopoietin-2 [47,52,79], VEGF [52], and placental growth factor-1 [45]. Simultaneous neutralization of two growth factors was demonstrated for a bispecific cpAb in IgG format. Specifically, the ϵ -amino group in a lysine residue of a peptide neutralizing angiopoietin-2 was linked to the ϵ -amino group in a lysine residue of a peptide neutralizing VEGF via a polyethylene glycol linker displaying a branched out 2-azetidinone for chemical programming of mAb h38C2 [52]. The resulting bispecific cpAb was shown to simultaneously bind angiopoietin-2 and VEGF and to potently inhibit tumor growth and angiogenesis in mouse xenograft models.

Clinical trials with cpAbs

The versatility of cpAbs, which is the result of generic molecular assembly of a variable synthetic component with an invariable antibody component (Figure 2), along with the ability to produce the antibody component by Good Manufacturing Practice (GMP) at kilogram scale, has facilitated the rapid expansion of the preclinical and clinical pipeline of cpAbs based on mAb h38C8, also known as CovX-Body platform. CovX, Inc. (San Diego, CA), which was acquired by Pfizer, Inc. (New York, NY) in 2008, advanced several CovX-Bodies to clinical trials. The antibody component, mAb h38C2 (CVX-2000), was expressed in mammalian cells and purified by sequential Protein A and ion exchange chromatography [52]. Four CovX-Bodies have been investigated in eight clinical trials registered at ClinicalTrials.gov (Table 2). As reported on abstracts published by the American Society of Clinical Oncology (ASCO), CVX-045, which mimics thrombospondin-1, CVX-060, which neutralizes angiopoietin-2, and CVX-241, which is a bispecific cpAb that neutralizes angiopoietin-2 and VEGF, were generally found to be well tolerated and to permit dosing once a week. Human anti-cpAb antibodies were detected at low titers in a portion of treated patients without clear effects on pharmacokinetics and safety. Following completion of the phase I clinical trial, CVX-060 was tried in combination with already approved multi-targeting receptor tyrosine kinase inhibitors for the treatment of renal cell carcinoma. This includes an active multicenter phase I/II clinical trial (NCT00982657) for CVX-060 plus sunitinib and a terminated multicenter phase II clinical trial (NCT01441414) for CVX-060 plus axitinib. As reported on ClinicalTrials.gov, the latter was prematurely discontinued due to an unexpected frequency of arterial and venous thrombotic events. Also terminated was a phase I clinical trial with the bispecific cpAb CVX-241. Despite a very promising PK and PD profile in extensive preclinical studies [52], it was reported on ClinicalTrials.gov that the

circulatory half-life of at least one of its two chemically programmed specificities was shorter than expected. There were no safety concerns with CVX-241. CovX-Bodies have also been developed for indications other than cancer. CVX-096, which mimics GLP-1, was evaluated in three phase I clinical trials for the treatment of type II diabetes mellitus, which collectively enrolled approximately 250 patients. No data showing the clinical safety and activity of CVX-096 has been published yet. CVX-096 was recently discontinued from the Pfizer pipeline along with CVX-045, CVX-060, and CXV-241. At this time it is not known when and if other CovX-Bodies with promising PK and PD profiles [45,71,78] will be translated from preclinical studies to clinical trials. Notably, all clinically investigated CovX-Body used peptides as synthetic components (Table 2), not taking full advantage of the unlimited chemical diversity of small molecules that can provide superior specificity, affinity, and stability. Medicinal chemistry approaches toward strengthening the PK and PD profiles of peptides [80] may also help improve the pharmacological performance of corresponding cpAbs.

Concluding remarks

A key aspect driving the preclinical and clinical development of cpAbs is their versatility. It only requires the cloning, expression, and purification of a single protein (*i.e.*, the antibody component), which is then chemically programmed with small molecules (*i.e.*, the synthetic components) for targeting a virtually unlimited number and variety of extracellular antigens. Both targeting and carrying moieties of cpAbs are experiencing rapid progress with respect to optimizing their pharmacological properties and broadening their scope for the treatment and prevention of human diseases. Several hundred patients treated with first-generation cpAbs constitute a robust platform for further advancing this new class of pharmaceuticals at the interface of chemistry and biology.

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Glossary

Antibody-dependent cellular cytotoxicity (ADCC)	lysis of an antibody-decorated target cell by an Fc γ receptor-expressing effector cell, such as an NK cell
Antibody-dependent cellular phagocytosis (ADCP)	engulfment of an antibody-decorated target cell by an Fc γ receptor-expressing effector cell, such as a macrophage
Bispecific antibody	engineered antibody that binds to two different epitopes monovalently or multivalently
C_{H1}	first constant domain of the heavy chain
C_{H2}	second constant domain of the heavy chain
C_{H3}	third constant domain of the heavy chain
C_L	constant domain of the light chain
Chemically programmed antibody (cpAb)	molecularly defined covalent composition of a variable synthetic component that serves as targeting moiety with an invariable antibody component that serves as carrying moiety

Complement-dependent cytotoxicity (CDC)	lysis of an antibody-decorated target cell by triggering the complement cascade
Complementarity-determining region (CDR)	peptide loop in the V _H or V _L domain that is involved in antigen binding
Epitope	antibody binding site of an antigen
Fab	antigen binding fragment of an antibody containing the V _H , C _{H1} , V _L , and C _L domains
Fc	crystallizable fragment of an antibody containing two C _{H2} and two C _{H3} domains
Fcγ receptor	cell surface receptor of IgG including CD16, CD32, and CD64
Hapten	small molecule that elicits an immune response only when conjugated to a macromolecular carrier such as a protein
Immunoglobulin G (IgG)	most common antibody isotype in the blood
Monoclonal antibody (mAb)	natural or engineered antibody that binds one epitope monovalently or multivalently
Neonatal Fc receptor (FcRn)	cell surface receptor of IgG that mediates IgG transcytosis and salvage
Paratope	antigen or hapten binding site of an antibody
Peptidomimetic	small molecule that mimics a peptide
Pharmacophore	moiety of a small molecule that binds to a target molecule
Reactive immunization	immunization with a hapten-carrier conjugate that elicits antibodies that covalently bind to the hapten
Unique reactivity center	amino acid residue(s) in the antibody mediating site-specific and covalent conjugation of a small molecule
V_H	variable domain of the heavy chain
V_L	variable domain of the light chain

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Highlights

- cpAbs are a new class of pharmaceuticals.
- Small molecules are equipped with the pharmacological properties of antibodies.
- The small molecule serves as navigator and the antibody serves as carrier.
- Several hundred patients have been treated with cpAbs in clinical trials.

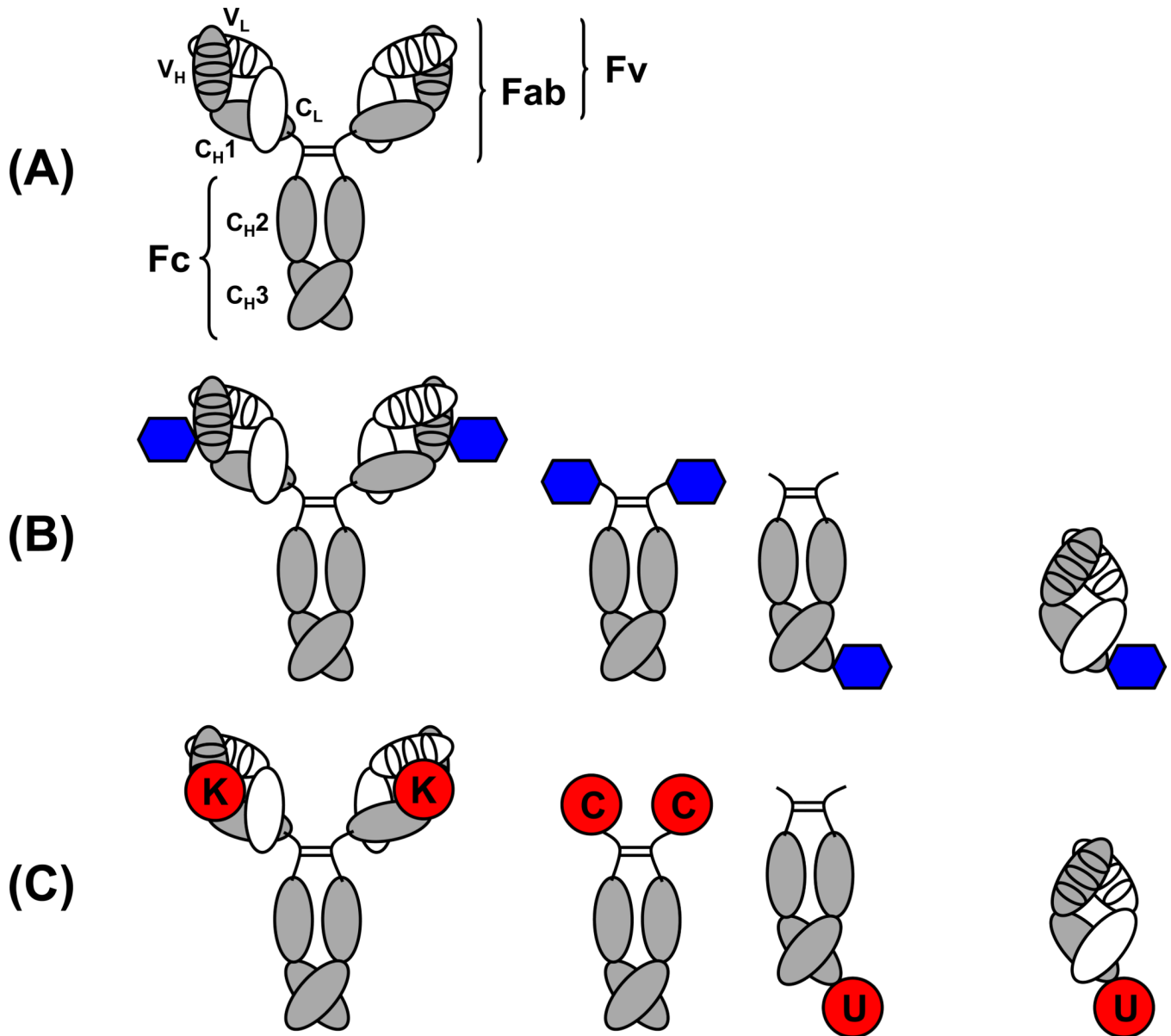


Figure 1. Comparison of the architecture of conventional mAbs and cpAbs

(A) The 150-kDa IgG1 molecule is the dominantly used pharmaceutical format of mAbs. It contains two identical light chains (*white*) and two identical heavy chains (*gray*). The light chains consist of an *N*-terminal variable domain (V_L) followed by one constant domain (C_L). The heavy chains consist of an *N*-terminal variable domain (V_H) followed by three constant domains (C_{H1} , C_{H2} , and C_{H3}). C_{H1} and C_{H2} are linked through a flexible hinge region (*bent lines*) that anchors four interchain disulfide bridges of the IgG1 molecule, one for each of the two light and heavy chain pairs (*not shown*) and two for the heavy chain pair (*straight lines*). The antigen binding site, or paratope, is formed by six CDRs (*ovals*), three provided by each variable domain. Fv, Fab, and Fc fragments of the IgG1 molecule are indicated. (B) Antigen binding in cpAbs is mediated by a synthetic component (*blue hexagon*) that is site-specifically and covalently attached to an antibody component. The antibody component of cpAbs typically is a whole IgG1 molecule, which displays two synthetic components (*left*), or a symmetric or asymmetric Fc fragment, which displays two or one, respectively,

synthetic components on either its *N*- or *C*-terminus (*center*). In contrast to these IgG-based and Fc-based cpAbs, Fab-based cpAbs (*right*) bind one antigen with their synthetic component and another antigen with their antibody component. (C) Unique reactivity centers in the antibody component of cpAbs include a reactive lysine (K) residue in the paratopes of an IgG (*left*), engineered *N*-terminal cysteine (C) or engineered *C*-terminal selenocysteine (U) residues of a Fc fragment (*center*), and an engineered *C*-terminal selenocysteine (U) of a Fab fragment (*right*).

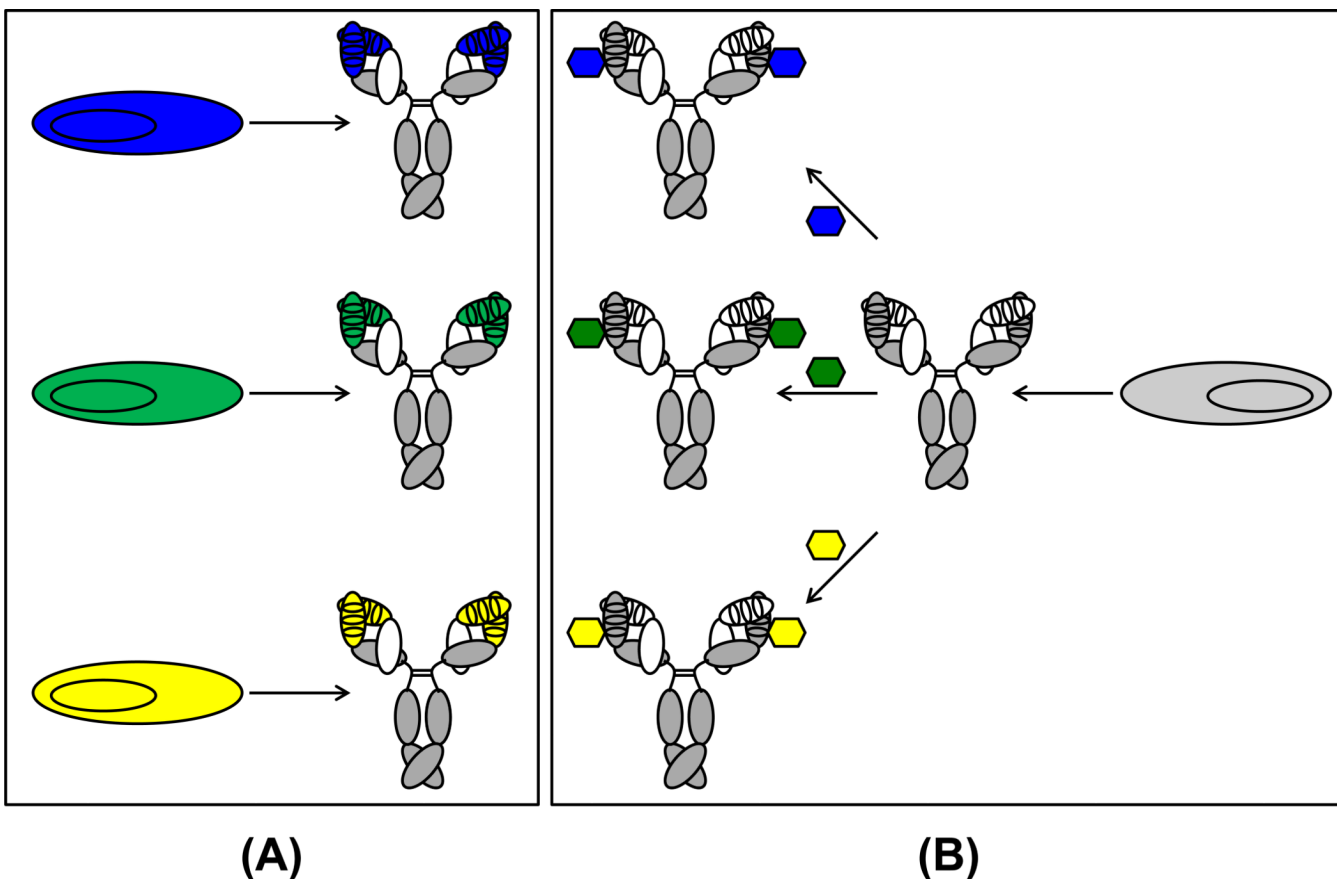


Figure 2. Comparison of the production of conventional mAbs and cpAbs

(A) In conventional mAbs, antigen binding is mediated by the paratope, requiring the cloning, expression, and purification of different proteins for different specificities (*blue, green, and yellow*). (B) By contrast, cpAbs only require the manufacturing of a single protein (*i.e.*, the antibody component), which is then chemically programmed with different small molecules (*i.e.*, the synthetic components) for targeting different specificities. Since GMP manufacturing costs are substantially lower for small molecules compared to proteins, cpAbs are economically more attractive than conventional mAbs. Using the same protein for a range of specificities and indications also shortens drug discovery and development time.

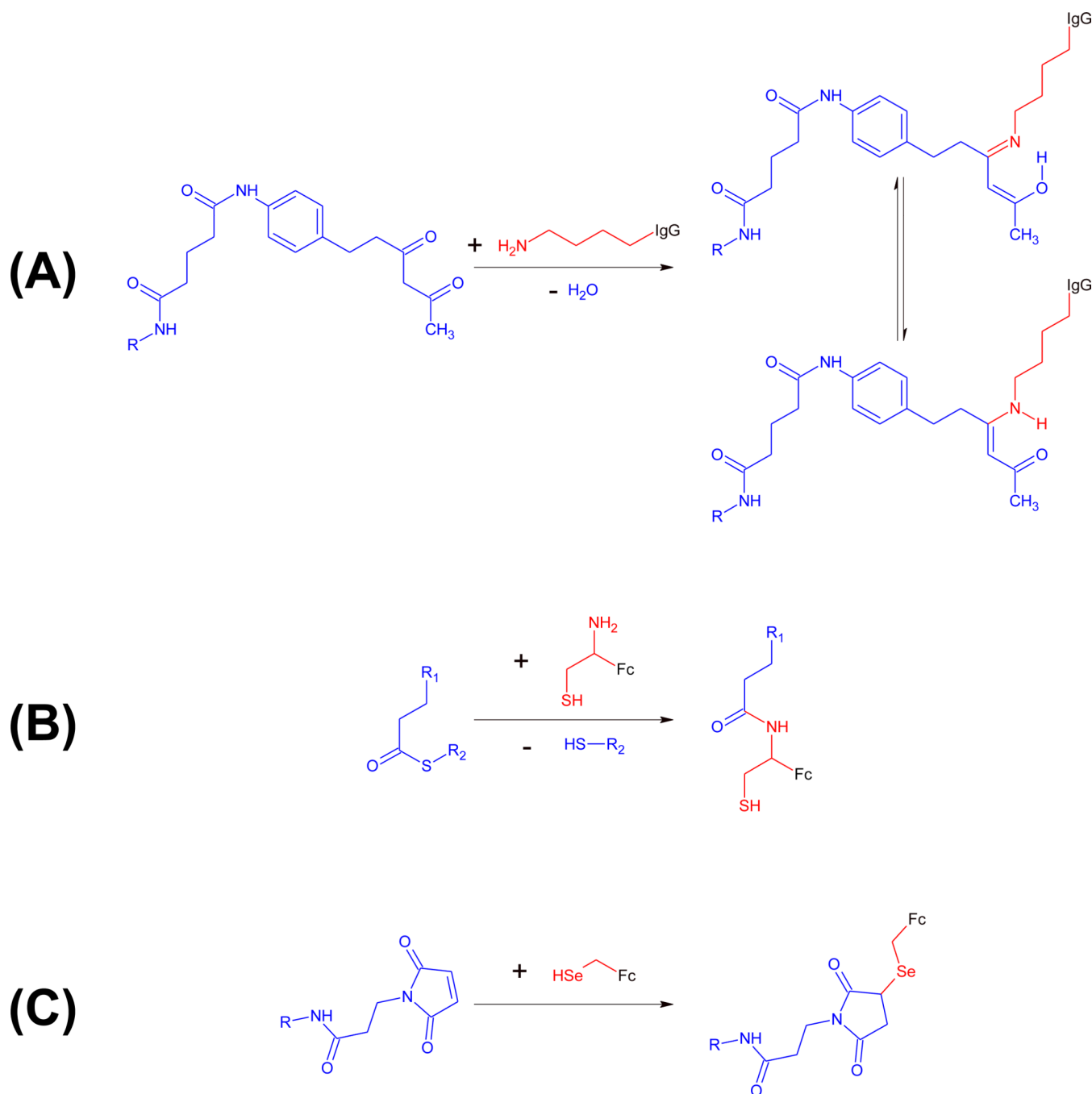


Figure 3. Covalent conjugation in cpAbs

Unique reactivity centers of antibody components are shown in red, synthetic components are shown in blue. **(A)** mAbs 38C2 and h38C2 harbor a reactive lysine residue (*red*) at the base of a deep hydrophobic cleft. The nucleophilic ϵ -amino group of this reactive lysine residue can be covalently conjugated to small molecules derivatized with an electrophilic 1,3-diketone group (*blue*). The covalent adduct is stabilized by imine-enamine tautomerism. **(B)** Fc fragments with an engineered *N*-terminal cysteine display a naturally occurring 1,2-aminothiol group (*red*), which reacts with small molecules derivatized with a thioester group (*blue*) in the presence of sodium 2-mercaptoethanesulfonate (MESNA) by first undergoing

transthioesterification and then *S*-to-*N* acyl shift rearrangement under formation of an amide bond. (C) Fc fragments with an engineered C-terminal selenocysteine display a selenol group (*red*), which reacts with maleimide (*blue*) or iodoacetamide derivatives of small molecules.

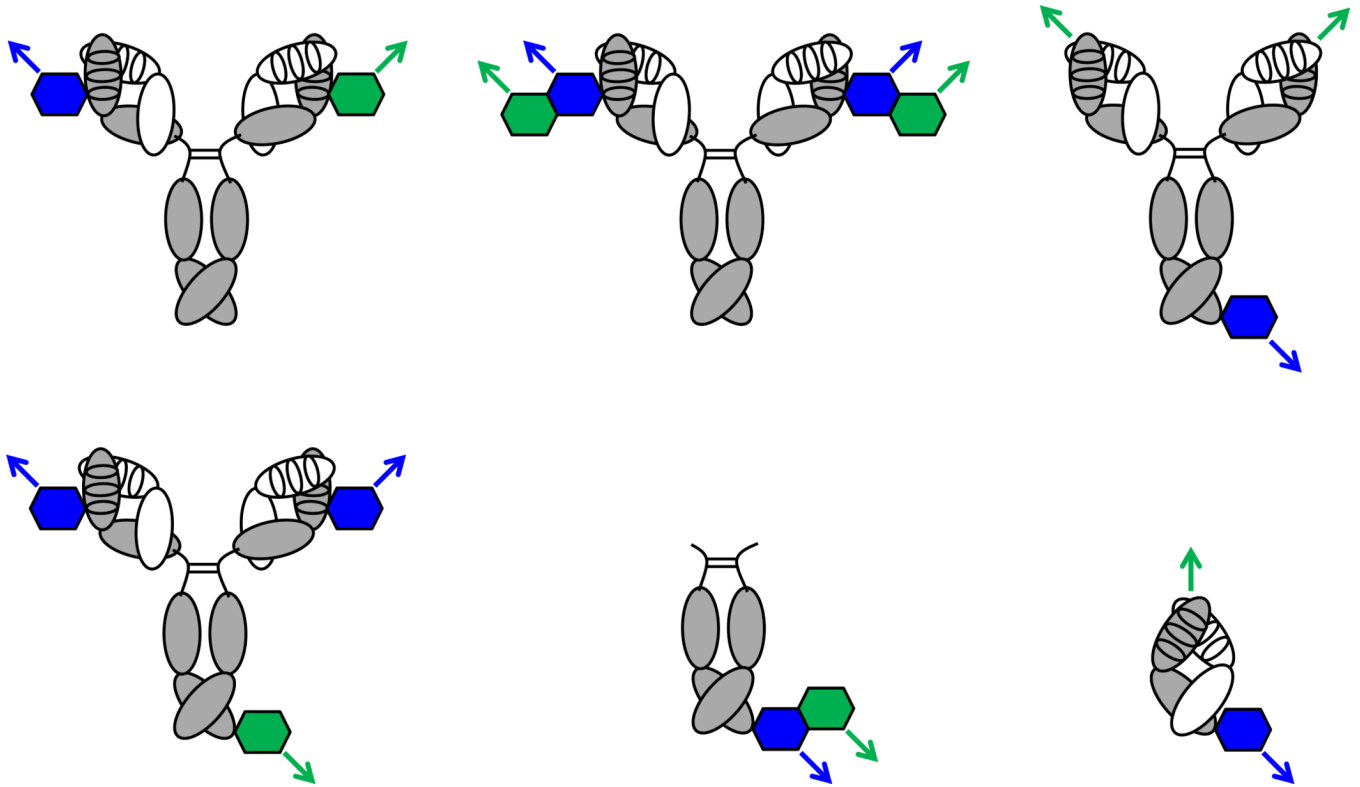


Figure 4. Bispecific cpAbs

Three conceptually different groups of bispecific cpAbs are depicted on the *left*, in the *center*, and on the *right*. All can simultaneously engage antigen-1 (*blue arrow*) and antigen-2 (*green arrow*). Bispecific cpAbs are generated by using two different synthetic components (*blue and green hexagons*) to chemically program an antibody component with two identical (*top left*) or two orthogonal (*bottom left*) unique reactivity centers. Bispecific bivalent cpAbs (*top center*) or bispecific monovalent cpAbs (*bottom center*) are generated by merging a bispecific synthetic component with an antibody component having two identical or one unique reactivity center, respectively. In cases where the antibody component of cpAbs harbors an antigen binding site that remains untouched by chemical programming, IgG-based (*top right*) or Fab-based (*bottom right*) bispecific cpAbs that bind to antigen-1 via their synthetic component and to antigen-2 via their antibody component can be generated.

Table 1

Comparison of small molecules, mAbs, and cpAbs.

	Small molecules	mAbs	cpAbs
Chemical diversity	unlimited	limited	unlimited
Molecular weight	<1 kDa	150 kDa	50–150 kDa
Target binding sites	1	2	1–2
Target binding specificity	medium to high	high	high
Target binding affinity	μM to nM	nM	nM
Target location	intracellular and extracellular	extracellular	extracellular
Target accessibility	unlimited	limited	limited
Circulatory half-life	minutes to hours	weeks	days to weeks
FcRn-mediated transcytosis	no	yes	yes
Effector functions	no	yes	yes
Route of administration	topical, enteral, and parenteral	parenteral	parenteral
Manufacturing costs	low	high	medium

Table 2

Components, targets, and indications of cpAbs.

Antibody component	Synthetic component	Mechanism of action	Indication	References	Clinical trials/
38C2 IgG	peptidomimetics	target and antagonize integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$	cancer	[6,61,62, 65,81–83]	
38C2 IgG	peptide	targets and antagonizes endothelin receptor type A	cancer	[70]	
38C2 IgG	peptidomimetic	mimics thrombospondin-1	cancer	[75]	
38C2 IgG	cyclic peptide	targets and antagonizes integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$	cancer	[64]	
38C2 IgG	peptide	targets luteinizing hormone receptor	cancer	[48]	
38C2 IgG	peptidomimetic	targets and antagonizes integrin $\alpha_5\beta_1$	cancer	[84]	
38C2 IgG	small molecule	blocks CCR5-mediated HIV-1 entry	AIDS	[72]	
38C2 IgG	small molecule	blocks CD4-mediated HIV-1 entry	AIDS	[73]	
38C2 IgG	small molecule	inhibits neuraminidase	influenza	[74]	
38C2 IgG	RNA aptamer	neutralizes VEGF	cancer	[50]	
84G3, 85H6, and 90G8 IgG	peptidomimetics	target and antagonize integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$	cancer	[65]	
h38C2 IgG	peptidomimetic	targets and antagonizes integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$	cancer	[60]	
h38C2 IgG	peptide	neutralizes angiopoietin-2	cancer	[47,79]	NCT00879684 ^c NCT00982657 ^a NCT01441414 ^f (CVX-060)
h38C2 IgG	peptide	neutralizes placental growth factor-1	cancer	[45]	
h38C2 IgG	peptide	mimics thrombospondin-1	cancer	[76]	NCT00879554 ^e (CVX-045)
h38C2 IgG	peptide	agonizes kappa opioid receptor	pain	[71]	
h38C2 IgG	bispecific peptide	neutralizes angiopoietin-2 and VEGF	cancer	[52]	NCT01004822 ⁱ (CVX-241)
h38C2 IgG	peptide	mimics GLP-1	diabetes mellitus type II		NCT00886821 ^c NCT01301456 ^e NCT01637285 ^e (CVX-096)
C-Fc	cyclic peptide	targets and antagonizes integrin $\alpha_v\beta_3$	cancer	[23]	
AKT-Fc	DNA aptamers	target protein tyrosine kinase-7 and cell surface IgM	cancer	[9]	
Fc-U	peptidomimetic	targets and antagonizes integrin $\alpha_4\beta_1$	cancer	[25,85]	

Antibody component	Synthetic component	Mechanism of action	Indication	References	Clinical trials [/]
Fc-U	folate	targets FOLR1	cancer	[49]	
anti-CD3 Fab-U	monovalent and bivalent peptidomimetics	simultaneously targets CD3 and integrin $\alpha_4\beta_1$	cancer	[7]	
anti-CD3 Fab-U	folate	simultaneously targets CD3 and FOLR1	cancer	[7]	
anti-CD3 Fab-pAcPhe	folate	simultaneously targets CD3 and FOLR1	cancer	[11]	
anti-CD3 Fab-pAcPhe	small molecule	simultaneously targets CD3 and PSMA	cancer	[10]	

[/] Status of clinical trials: a, active; c, completed; t, terminated.