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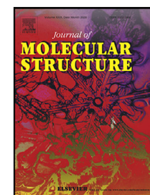

Review

A review on recent synthetic routes and computational approaches for antibody drug conjugation developments used in anti-cancer therapy

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ABSTRACT

In recent years, antibody-drug conjugates therapy (ADCs) is the most promising way for cancer treatments, which is applied for personalized cancer medication. ADCs are the combination of high cytotoxic drug (payloads) molecules, conjugated with a tumour selective monoclonal antibody (mAb). These mAbs are used to target over expressed antigens on tumour cells than on normal cells. Synthetic ADCs payloads improves the drug safety profile by increasing therapeutic index in clinical practice for its higher target selectivity. In this review, route of chemical synthesis of recently used diverse ADCs containing payloads are discussed with strategies to explore the designing part used for the development of new payload conjugates with antibody susceptibility (by reducing antigenicity) for treating cancer patients. We also focused on recent advancements using computational approaches for neo-antigen target selection and identification, web-based resources availabilities, selection and identification of cytotoxic payloads, linkers, ADC conjugations with their advantages and disadvantages and various types of molecular modelling approaches with online tools/databases available, for designing new ADCs.

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1. Introduction

Antibody Drug Conjugates (ADCs) are highly targeted biopharmaceutical agents which is a combination of monoclonal antibodies (mAbs) specific to surface antigens present on particular tumor cells and highly potent anti-cancer agents linked via a chemical linker [1,2]. In ADCs, the linkers are covalently linked with monoclonal antibodies to target surface antigens present on particular tumour cells [3]. Therefore, selective release of the drugs/pharmaceutically active agents used to target cancer cells not only improve the therapeutic effect but also reduce cytotoxic effect towards normal cells, and also avert drug resistance issue [4]. The cytotoxic drugs (payloads) are chemically linked (conjugated using disulfide or non-cleavable thioether linker chemistry) to a specific monoclonal antibody that recognizes tumour-associated antigen and exert their activity by (1) selective binding to tumor cells, (2) internalization (3) lysosomal degradation and release of the cytotoxic payload, leading to cytotoxic cell death. (Fig. 1) [5]. When ADC is administered, it binds to a target antigen, which is available on the cancer cell-surface to make a complex, further internalized into the cell. Afterwards, the internalized vesicles fuse with other one and come into the endosome-lysosome pathway. In the lysosome, in presence of mild acidic environment monoclonal antibody will be digested and release free payloads / cytotoxic agents. Finally, free payloads will cross the lysosome membrane for entering into the cytoplasm and/or the nucleus where they bind to either targeted DNA (most commonly with guanine residues) or different allosteric sites of microtubules leads to cell apoptosis [6].

At the time initial development of ADCs, researchers used clinically approved cytotoxic drugs, due to their known toxicological properties. However, these early ADCs were only moderately potent and generally less cytotoxic/active for the targeted cells than the corresponding unconjugated agents were. For this reason, researchers started to look at the compounds, which are found to be more toxic when tested as a stand-alone bioactive agent [7]. Recently, some ADCs have received market approval (Table 1) and several ADCs are currently in clinical trials (Table 2). In this review, we will discuss on narrowly specified different class of chemical compounds, which are currently used as payloads for recent developments of ADCs as an anti-cancer agent. In addition, we are reporting variant roles of computational approaches, which are used to develop potent, target specific ADCs for anti-cancer activity.

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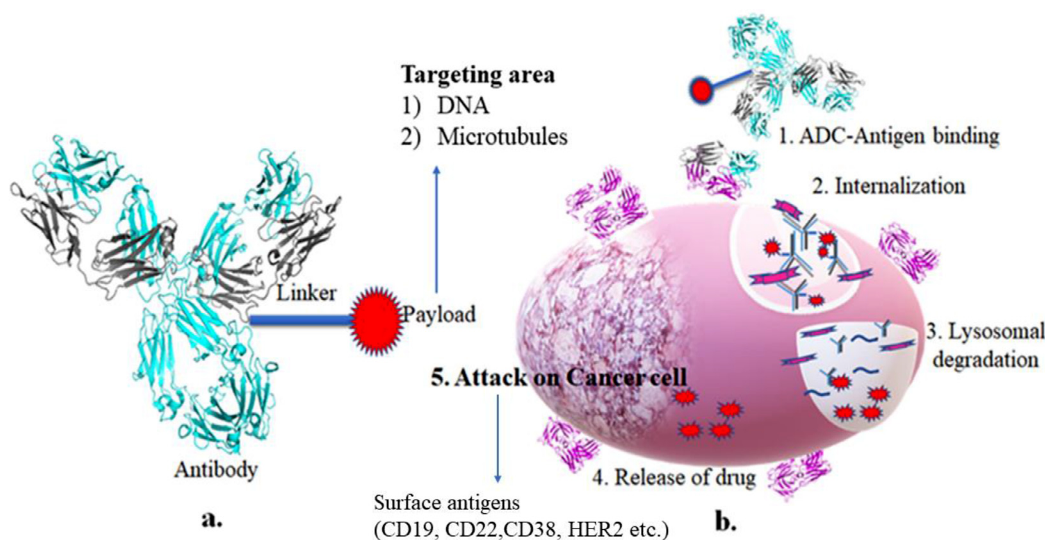

Fig. 1. ADC structure (a) and its systemic mechanism of action (b) against cancer cell.

Table 1
List of FDA approved ADCs in the market in last 5 years.

Sr No	ADC (Trade name)	Anticancer Drug	Linker	Antibody	Target antigen	Cancer type	Approval year	Company	Ref
1	Loncastuximabtesirine (Zynlonta™)	PBD dimer	VA (Cleavable)	Humanized IgG1	CD19	Large B-cell lymphoma	2021	ADC Therapeutics	[8]
2	Sacituzumabgovitecan (Trodelvy™)	SN38	Cl2A (Cleavable)	Humanized IgG1κ	TROP2	Metastatic triple –ve breast cancer	2020	Immunomedic	[9,10]
3	Belantamabmafodotin (Blenrep)	MMAF	MC (Non-cleavable)	Humanized IgG1	CD38	Relapsed or refractory multiple myeloma	2020	GlaxoSmithKline	[11,12]
4	Trastuzumabderuxtecan (Enhertu®)	DXd	mc-GGFG (Cleavable)	Humanized IgG1κ	HER2	HER2-positive breast cancer	2019	Daiichi Sankyo	[13]
5	Enfortumabvedotin (Padcev®)	MMAE	mc-vc-PABC (Cleavable)	Human IgG1κ	Nectin4	Metastatic urothelial cancer	2019	Astellas Pharma	[14,15]
6	Polatuzumabvedotin (Polivy®)	MMAE	mc-vc-PABC (Cleavable)	Humanized IgG1κ	CD79b	Diffuse large B-cell lymphoma	2019	Roche	[16]
7	Moxetumomabpasudotox (Lumoxiti®)	Pseudotox	N/A (Cleavable)	Humanized IgG1	CD22	Relapsed or refractory hairy cell leukemia (HCL)	2018	Astrazeneca	[17]
8	Inotuzumabozogamicin (Besponsa™)	Calicheamicin	AcBut (Cleavable)	Humanized IgG4	CD22	Acute lymphoblastic leukemia	2017	Pfizer	[18]
9	Gemtuzumabozogamicin (Mylotarg™)	Calicheamicin	AcBut (Cleavable)	Humanized IgG4κ	CD33	Acute myeloid leukemia	2017	Pfizer	[19,20]

2. Chemical classes of ADC Payloads for anti-cancer therapy

Recently few instances of payload classes are existing for use in ADCs. These payloads are normally used either DNA-interactive agents or tubulin inhibitors. At present, commercially available ADCs are pyrrolobenzodiazepine (for example, SGD-1882), calicheamicins (for example, calicheamicin $\gamma 1$ derivative found in Mylotarg®), maytansinoids (for example, DM1 in Kadcyla®)and auristatins (for example, monomethyl auristatin E (MMAE) in Adcetris®), Amanitin (for example, alfa-amanitin)(Fig. 2) [46]. Each of these groups are well explored in this review.

2.1. DNA alkylating/damaging compounds

2.1.1. Pyrrolobenzodiazepine

Pyrrolobenzodiazepines (PBD) (For example, SGD-1882) a class of natural products obtained from various actinomycetes, are sequence selective DNA alkylating compounds along with important antitumor properties [47] (Fig. 2). This is one of the important pharmacophores composed from 1,4-benzodiazepine ring system. It shows potent chemotherapeutic activity [48]. Some PBDs have the ability to recognize and bond to specific sequences of DNA [48,49]. Novel results revealed that PBDs could be effectively utilized for antibody-targeted therapy [50–52]. The mechanism of action of the PBDs is related with the formation of an adduct in the minor groove and interfere into the DNA processing (Fig. 3). After insertion in the minor groove, an amide bond is formed via nucleophilic attack of the N-2 of a guanine base at the electrophilic C-11 position of PBD [53]. The resulting PBD-DNA adducts cause replication forks to stall and tumor cells to arrest at the G2-M boundary, eventually resulting in apoptosis at low nanomolar to picomolar concentrations.

Chemically synthesized PBD dimers are composed of two PBD monomers, which are linked with an aromatic ring (Ring A, C8-position) via a flexible propyldioxy tether. This one is highly effective DNA minor groove cross-linking agents due to its potent cytotoxicity profile. The PBD dimers are significantly more potent than systemic chemotherapeutic drugs [48,49].The site-specific antibody conjugation technology allows PBD for uniform drug loading to the anti-CD33 antibody. PBD dimers also have been shown to

Table 2
ADCs recently used for cancer treatment under clinical trial.

1	Mirvetuximab soravtansine (IMGN853)	DM4	Sulfo-SPDB (Cleavable)	Humanized IgG1	FOLR1	Ovarian cancer, lung adeno-carcinoma	Phase 3	Immunogen	[21–23]
2	Vadastuximab talirine (SGN-CD33A)	PBD dimer	VA (Cleavable)	Humanized IgG1	CD33	Acute myeloid leukemia	Phase 3	Seattle Genetics	[24–26]
3	Trastuzumab duocarmazine (SYD985)	seco-DUBA	mc-vc-PABC-CM (Cleavable)	Humanized IgG1κ	HER2	Breast cancer	Phase 3	Synthon Biophar-maceuticals	[27,28]
4	Labetuzumab govitecan (IMMU-130)	SN-38	CL2A (Cleavable)	Humanized IgG1	CEACAM5	Metastatic colorectal cancer	Phase 3	Immunomedics	[29,30]
5	Rovalpituzumab tesirine (SC16LD6.5; Rova-T)	PBD dimer	VA (Cleavable)	Humanized IgG1	DLL3	Small cell lung cancer	Phase 3	Abbvie	[31]
6	Polatuzumab vedotin (DCDS4501A)	MMAE	VC (Cleavable)	Humanized IgG1	CD79b	Diffuse large B-cell lymphoma	Phase 3	Genentech	[32]
7	Disitamab vedotin (RC-48-ADC)	MMAE	mc-vc-PABC (Cleavable)	Humanized IgG1	HER2	Advanced gastric, urothelial carcinoma	Phase 2	RemeGen	[33]
8	Anetumab ravtansine (BAY94-9343)	DM4	SPDB (Cleavable)	Humanized IgG1λ	MSLN	Advanced pancreatic cancer	Phase 2	Bayer	[34]
9	Denintuzumab mafodotin (SGN-CD19A)	MMAF	MC (Non-cleavable)	Humanized IgG1	CD19	Lymphoma	Phase 2	Seattle Genetics	[35]
10	DS-1062a	DXd	mc-GGFG (Cleavable)	Humanized IgG1	TROP-2	Metastatic Non-small Cell Lung Cancer	Phase 2	Daiichi Sankyo	[36]
11	Glembatumumab vedotin (CDX-011)	MMAE	VC (Cleavable)	Human IgG1	gpNMB	Metastatic breast cancer and melanoma	Phase 2	Celldex	[37]
12	**Lifastuzumab vedotin** DNIB0600A	MMAE	mc-vc-PABC (Cleavable)	Humanized IgG1	NaPi2b	Platinum-resistant ovarian cancer	Phase 2	Genentech	[38]
13	Trastuzumab deruxtecan (DS-8201a)	DXd	mc-GGFG (Cleavable)	Humanized IgG1κ	HER2	Advanced solid tumors	Phase 1	Daiichi Sankyo	[39]
14	Samrotamab vedotin (ABBV-085)	MMAE	mc-vc-PABC (Cleavable)	Humanized IgG1κ	LRRC15	Solid tumors	Phase 1	Abbvie	[40,41]
15	IMGN-779	DGN462	Sulfo-SPDB (Cleavable)	Humanized IgG1	CD33	Acute myeloid leukemia	Phase 1	ImmunoGen	[42,43]
16	Losatuxizumab vedotin/ABBV-221	MMAE	Mc-Val-Cit-PABC (Cleavable)	Humanized IgG1κ	EGFR	Breast cancer, colorectal cancer, Lung cancer	Phase 1	AbbVie	[44]
17	Trastuzumab deruxtecan (DS-8201A, T-Dxd)	DXd; DX-8951 derivative	Gly-Phe-Leu-Gly (Cleavable)	Humanized IgG1	HER2	Non-Small Cell Lung Cancer, Advanced Solid Tumors	Phase 1	Daiichi Sankyo Inc.	[39,45]

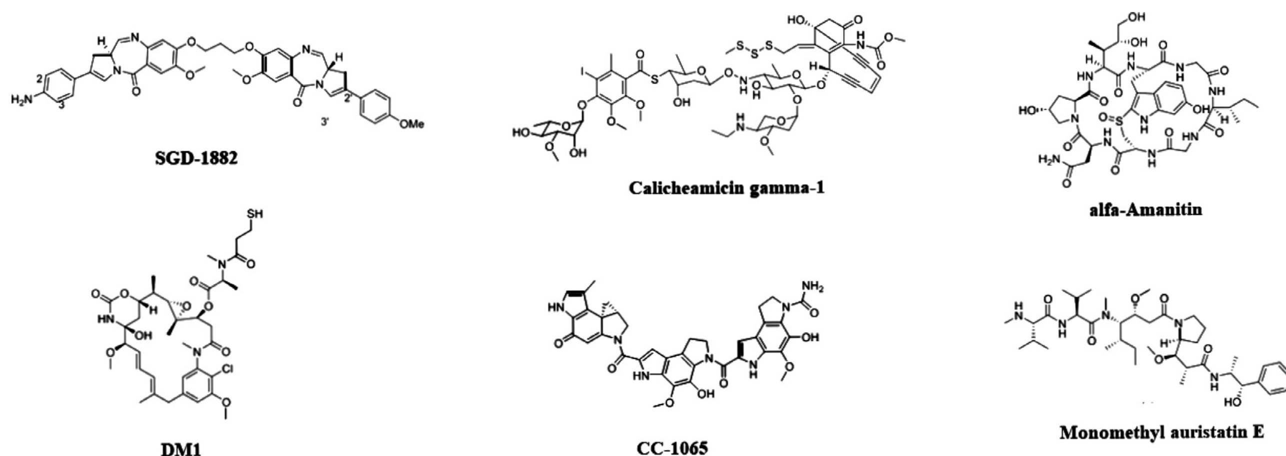

Fig. 2. Structures of representative members of the various payload classes used in ADCs.

have broad-spectrum anti-tumour activity in vivo. PBD dimers bind in the minor groove of DNA and form covalent aminal cross- links between the N-2 of guanine and the C-11 position of the PBD. For that reason, apoptosis will form at lower nanomolar to picomolar concentrations. Due to the potency of PBD dimers and similar cell cycle–independent payloads, normal tissues accessed by such potently armed ADCs must be devoid of target expression [53]. PBD blocks the cancer cells' division without distorting its DNA helix, thus potentially avoiding the common phenomenon of emergent drug resistance.

2.1.2. Synthetic route development of Pyrrolobenzodiazepines

PBDs contain the tricyclic ring system made by an anthranilate (A), a diazepine (B), and a hydropyrrole (C) rings, shown in Fig. 4. Different types of chemical modifications in the side chains at the A- and C-rings offered chemical diversity among PBDs.

These can be found in different arrangements based on the storage conditions (Fig. 4). For an example, in aqueous solution, the imine form is in equilibrium with the carbinolamine form and is stabilized due to the presence of electron-withdrawing groups at C8 position (Fig. 4) [54–56].

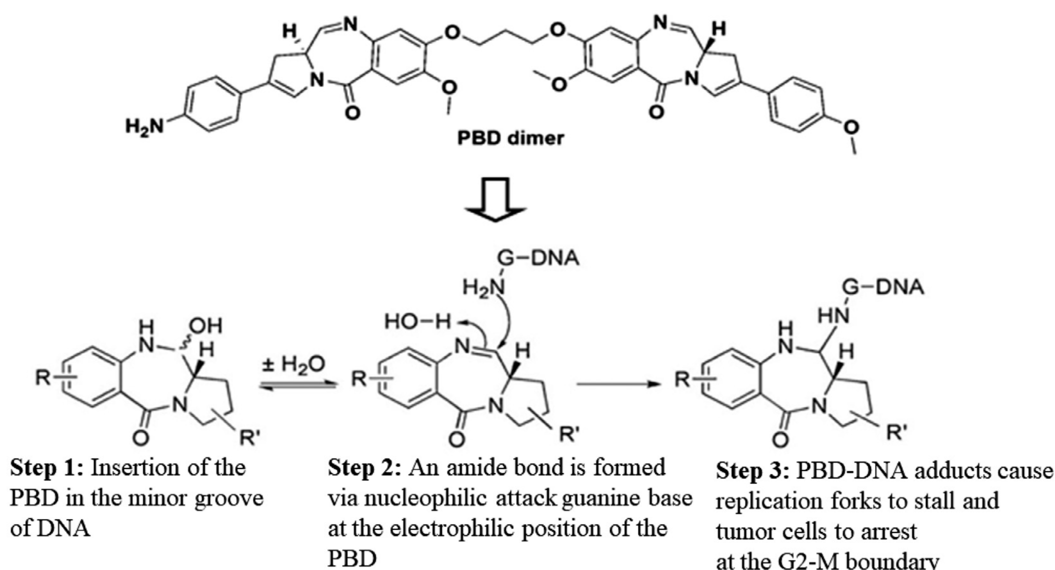

Fig. 3. PBD-DNA complex formation.

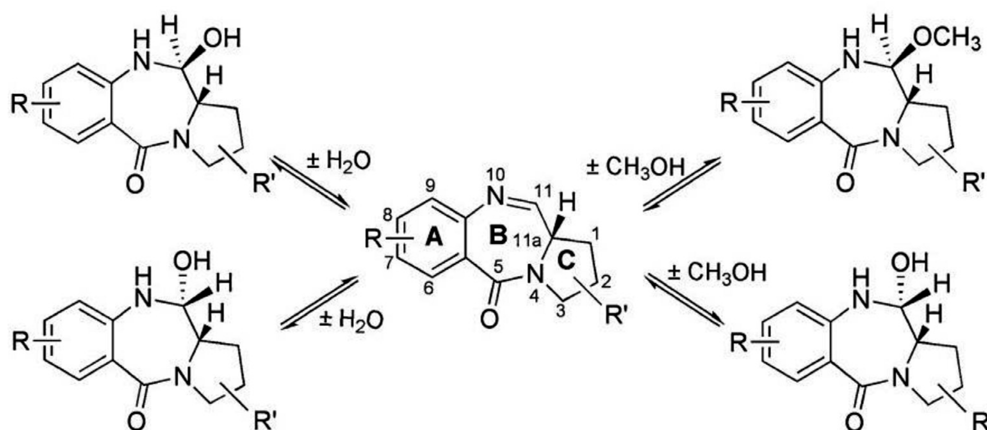

Fig. 4. Epimerization at C-11 of PBDs in presence of protic solvents.

2.1.3. Monomeric form of PBDs

Chemical syntheses of naturally occurring PBDs, such as anthramycin [57–59], DC-81 [60–62],tomaymycin [63]and chicaymycin [64] have been reported. Although, chemical synthesis of naturally occurring PBDs and their derivatives is vulnerable for the higher stability of the N10-C11 imine bond, retaining of the stereochemistry at C11a position (Fig. 4), loss of unsaturation in the C-ring under reductive cyclization and dependence on ring A modifications for the diazepine ring closure [65,66]. Hence, chemical synthesis of PBDs was highly challenging to overcome all these issues.

A common route of synthetic synthesis has been performed for most of the compounds (Fig. 5) where the C5–N4 amide bond is formed first followed by intramolecular cyclization viaaza-Witting reaction of azidocarbonyl compounds [67], catalytic hydrogenation of nitro carbonyl compounds [68,69]. or Swern-oxidation of amino hydroxyl compounds [70–72]. The originalities in this PBD chemical synthesis are the use of solid-supported palladium catalyst and diethanolamine in the Suzuki coupling reactions respectively. The microwave conditions during the Suzuki coupling were also modified. Both modifications reduce the total time of reaction and purification.

2.1.4. Dimeric form of PBDs

Monomeric DNA alkylating agent PBDs are fail to modify target double stranded DNA more specifically due to its short length. Thus, to get more DNA base specificity of PBDs, Thurston's group designed a PBD dimer with two DC-81 unit linked together by 1,3-propanediyldioxy ether linkage named DSB-120 (Fig. 6) [73]. The diether linker was optimized by changing its length to 3 methylenes by synthesis of DSB-120 derivative with 3, 4, 5, and 6 methylenes [74,75] and by evaluation of their biological activities [76,77]. It has been reported that C7-linked dimers are less biologically active than C8-linked dimers [78,79]. The most remarkable PBD dimer is SJG-136.

It is more important to show the types of PBDs that are currently being used as ADC payloads and the follow this up with a table containing information and stages of developments of PBD containing payloads.

2.1.5. Derivatives of Pyrrolobenzodiazepines

Anthramycin is the first PBDsantitumour antibiotic, anthramycin, produced by *Streptomyces refuineus*, was discovered in 1965. Since then, a number of naturally occurring PBDs have been reported, and over 10 synthetic routes have been devel-

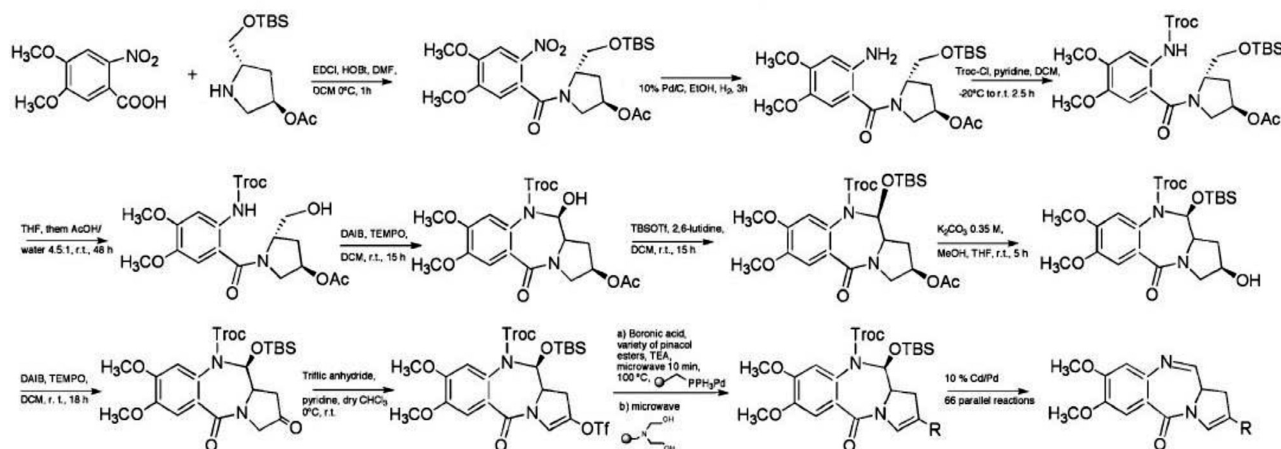

Fig. 5. Synthetic route for monomeric form of PBDs.

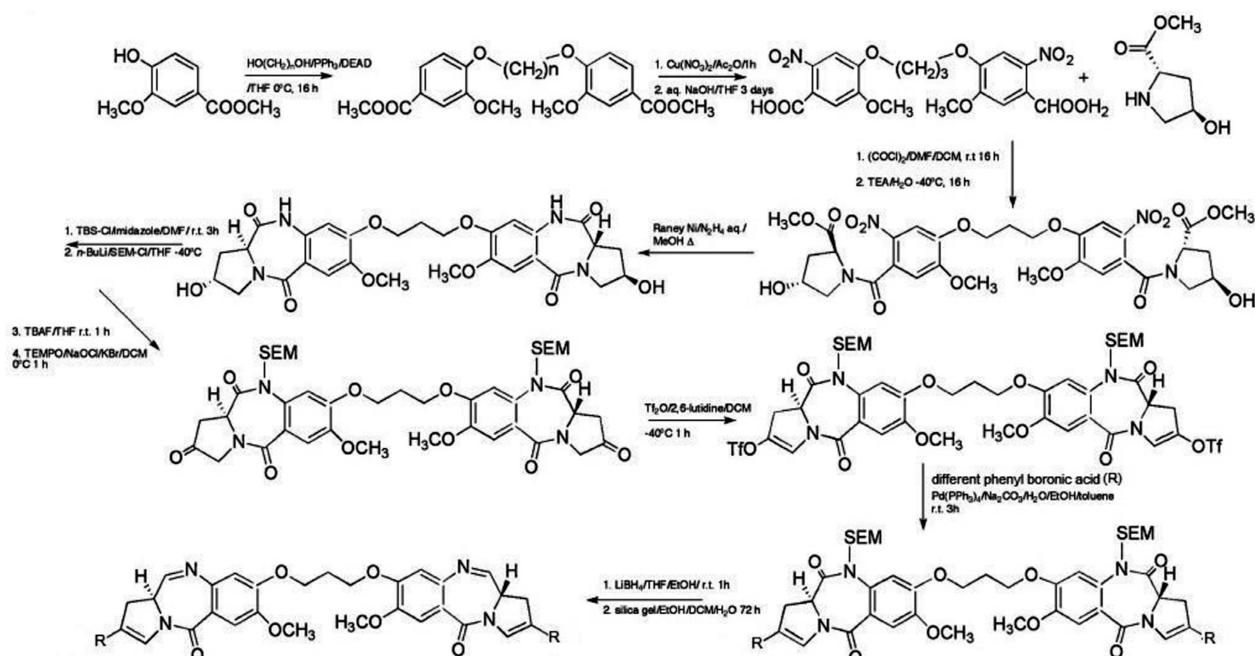

Fig. 6. Synthetic route for Dimeric form of PBDs.

oped to a variety of analogues. Other agents belonging to the pyrrolo(1,4)benzodiazepine antibiotic group include abbeymycin, chicamycin, DC-81, mazethramycin, neothramycins A and B , porothramycinprothracarcin, sibanomicin (DC-102) sibiromycin and tomamycin [80].Presentlythere are several PBD dimer-containing ADCs available in different stages of clinical studies like camidanlumabtesirine, loncastuximabtesirine, epratuzumab-cys-tesirine and vadastuximabtalirine.

2.1.6. Calicheamicins

Calicheamicins are a class of enediyne antitumor antibiotics (Fig. 2) and isolated from the bacterium *Micromonosporaechinospora* [81] (+). N-acetyl dimethyl hydrazide calicheamicin, was developed as a targeted therapy for non-solid tumor cancer acute myeloid leukemia (AML) which is CD33 antigen specific. Calicheamicins (Fig. 7) binds to the minor grove of the dsDNA and reduced by cellular thiols to form a 1,4-dehydrobenzene radical intermediate, followed by removing hydrogen from the deoxyribose ring and breaks the DNA strand [82] through a Bergman cyclization reaction (Fig. 8) [83]. Calicheamicin has several derivatives with different chemical modifications, among which $\gamma 1$ is the most well-characterized. Calicheamicin $\gamma 1$ contains an aglycon consisting of a bicycle tridec-9-ene-2,6-diyne system along with a labile methyl trisulfide group and an aryl tetra-saccharide chain. Calicheamicins also contain enediyne moieties that are structurally similar to other enediynes, such as esperamicins, neocarzinostatin, and kedarcidin, etc. After reduction by cellular thi-

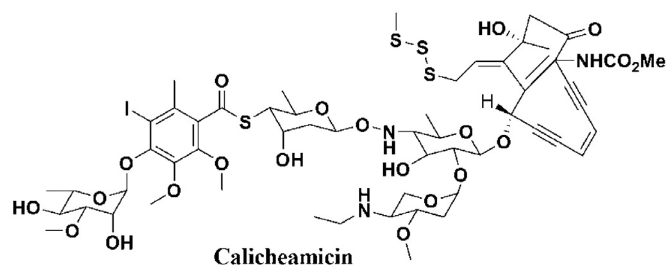

Fig. 7. Structure of Calicheamicin.

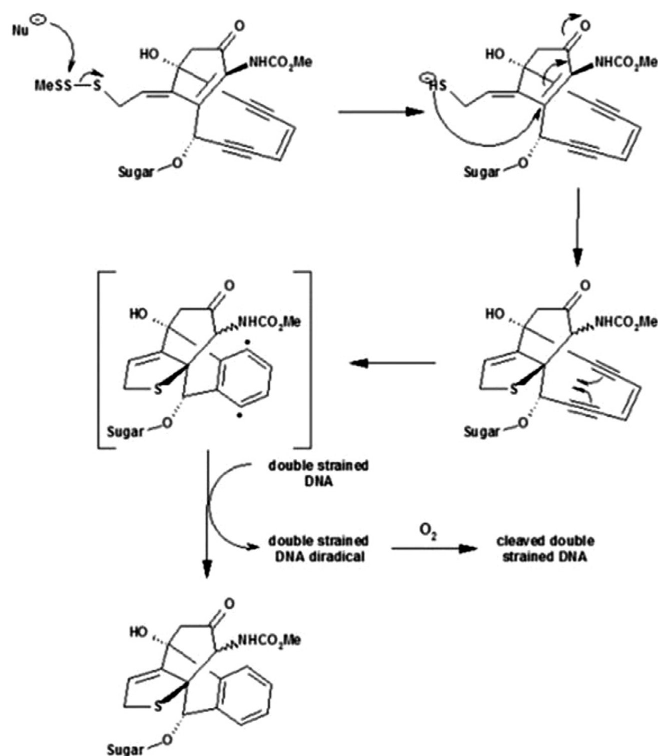

Fig. 8. Mechanism of DNA cleavagebyCalicheamicins.

ols, the calicheamicinenediyne moieties rearrange to produce a 1, 4-benzenoid diradical.

2.1.7. Development of the synthesis of Calicheamicins for ADC

This synthesis condenses theaglycone and the saccharide with the trichloroacetimidate method. As in the Danishefsky synthesis shown in Fig. 9 [84], the coupling between glycosyl trichloroacetimidate and enediyne was achieved under the action of AgOTf, providing the desired β-glycoside in a decent 34% yield. The α-glycoside was not detected; this is attributable to the role of the double stereo-differentiation effect in the glycosylation. Removal of the ketal in (with CSA) followed by cleavage of the silyl groups (with TBAF) furnished calicheamicin in 32% yield (for two steps).

2.1.8. Synthetic route of Calicheamicin -mAb conjugates

ADCs using calicheamicin derivatives as payloads are under active development and evaluation. In 2019, Nicolaou and co-workers developed the synthetic route of mABs Calicheamicin as a payload (Fig. 10) [85].Calicheamicin molecule was investigated as payload for several antibody drug conjugated therapies. Among all, gemtuzuma bozogamicin and inotuzuma bozogamicin are the most significant ADC molecules. Gemtuzuma bozogamicin is used as ADC which contain N-acetyl γ calicheamicin dimethyl hydrazide group as payload and it is used for acute myelogenous leukemia treatment targeting the CD33 surface antigen. Inotuzuma bozogamicin is a distinctive ADC composed of a humanized monoclonal antibody, inotuzumab, which targets CD22, and a cytotoxic calicheamicin derivative. CD22 is a cell surface antigen showing high level of expression on nearly 90% of B-cell malignancies. Upon CD22 binding, inotuzumabozogamicin is internalized via receptor mediated endocytosis and the calicheamicin payload is released to accomplish its cytotoxic effects [86].Marketed ADCs Mylotarg® and Besponsa^{TM} both contain calicheamicin payloads.

2.1.9. Duocarmycin

Duocarmycins are a series of natural products obtained from *Streptomyces* bacteria in 1988. They showed anti-tumour activity due to its notably high cytotoxicity. CC-1065 (Fig. 2) and duocarmycin SA are the most widely used as duocarmycin derivatives. These are extremely powerful anti-neoplastic compounds that display high cytotoxicity against the growing cancer cells in culture. Duocarmycin derivatives are DNA minor groove binding agents that also exert adenine-N3 alkylation activity and an AT-sequence selectivity. Duocarmycinand its derivatives bind to the minor groove of DNA and then induce irreversible DNA alkylation, which is used to hinder DNA architecture and structural integrity. The alkylation of DNA ultimately leads to DNA cleavage followed by apoptosis of the cells.

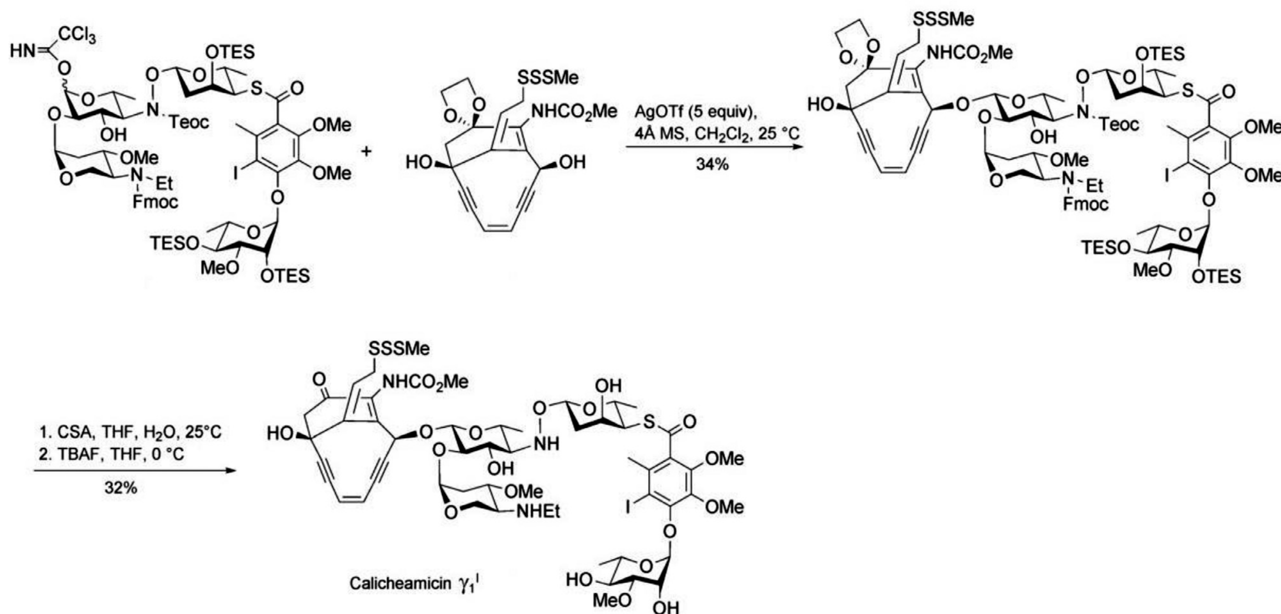

Fig. 9. O-glycosylation method used for Calicheamicins synthesis.

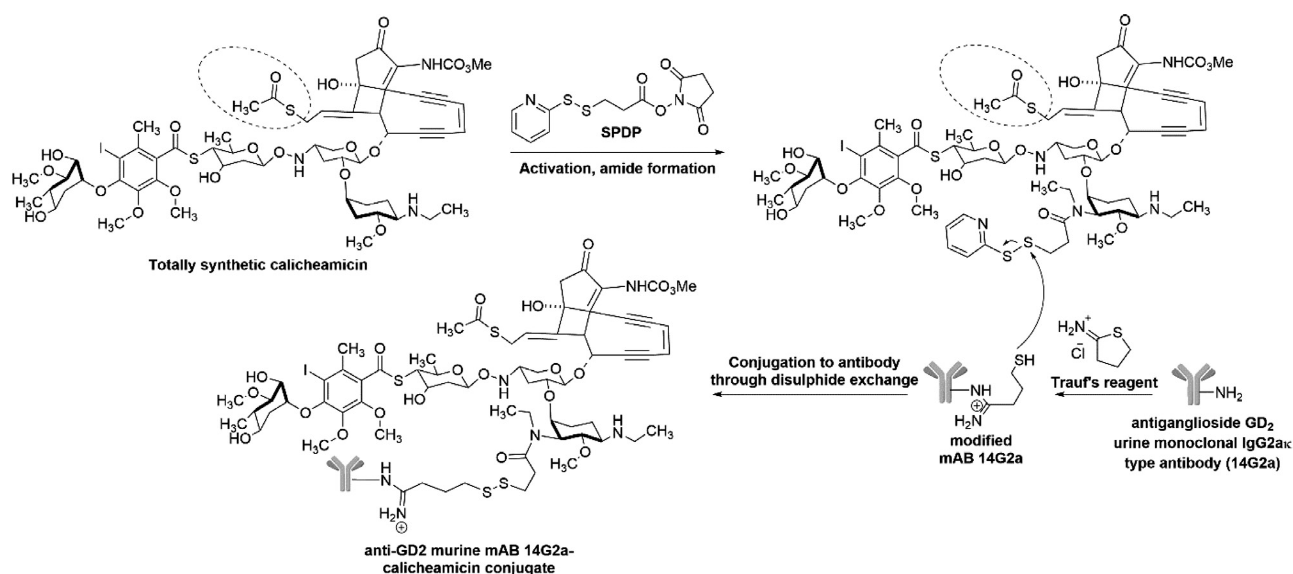

Fig. 10. Synthetic route of mABs Calicheamicin as a payload (Nicolaou et al. 2019).

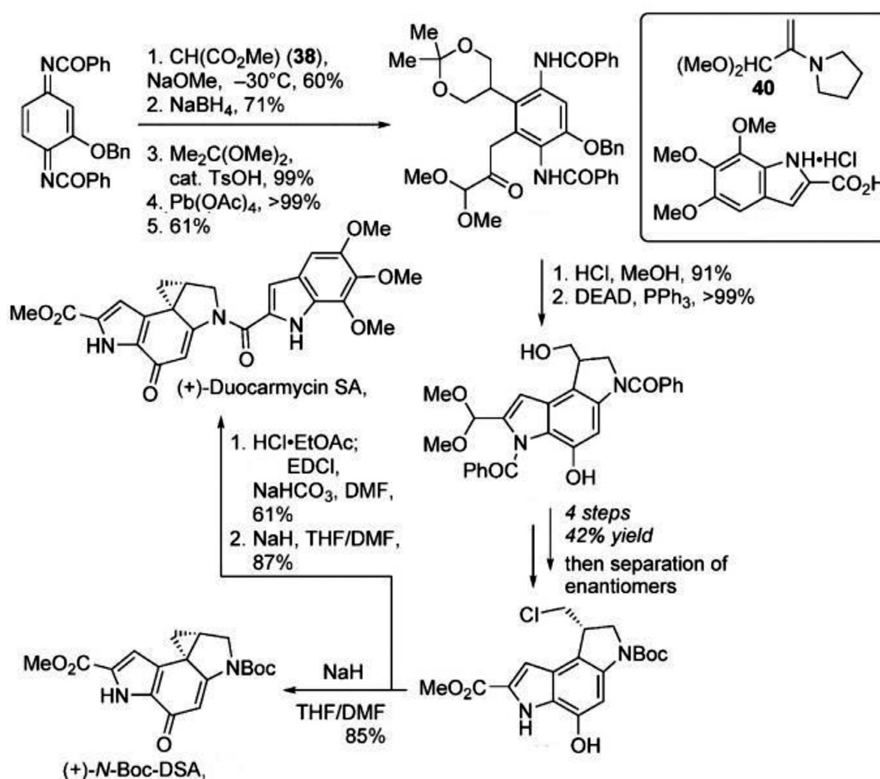

Fig. 11. Synthetic pathway of (+) Duocarmycin and (+)-N-Boc-DSA.

2.1.10. Synthetic route of Duocarmycin-mAb conjugates

Two successive regioselective alkylations of substituted diimidequinone (synthesized in 5 steps and 38% yield) [87] (Fig. 11) were performed at C5 with dimethyl malonate, and then at C6 with pyrrolidine enamine of pyruvaldehyde dimethyl acetal [88] to afford aniline. Acidic conditions removed the acetal group and promoted indole formation, which was followed by cyclization of the resulting diol to complete the dihydropyrroloindole core. Eight additional steps, including chiral resolution of the bis- (R)-O-acetylmandelate esters, provided (+)-dihydropyrroloindole. This intermediate could then be directly transformed into (+)-N-Boc-DSA analogue in 85% yield, or to the natural antipode of (+)-duocarmycin SA by coupling with the 5,6,7-trimethoxyindole-2-carboxylic acid salt (made in 3 steps and 74% yield from 3,4,5-trimethoxybenzaldehyde) [67]in 53% yield over the two steps. The unnatural (-)-N-Boc-DSA and (-)-duocarmycin SA enantiomers were also synthesized from (-)-ent-[89].

Adozelesin, bizelesin and carzelesin are artificially synthesized analogues of duocarmycins and are members of the cyclopropyl pyrroloindole family [90,91]. These drugs have high research and clinical values and all of them have advanced into clinical trials for cancer treatments. Adozelesin is an alkylating small groove DNA

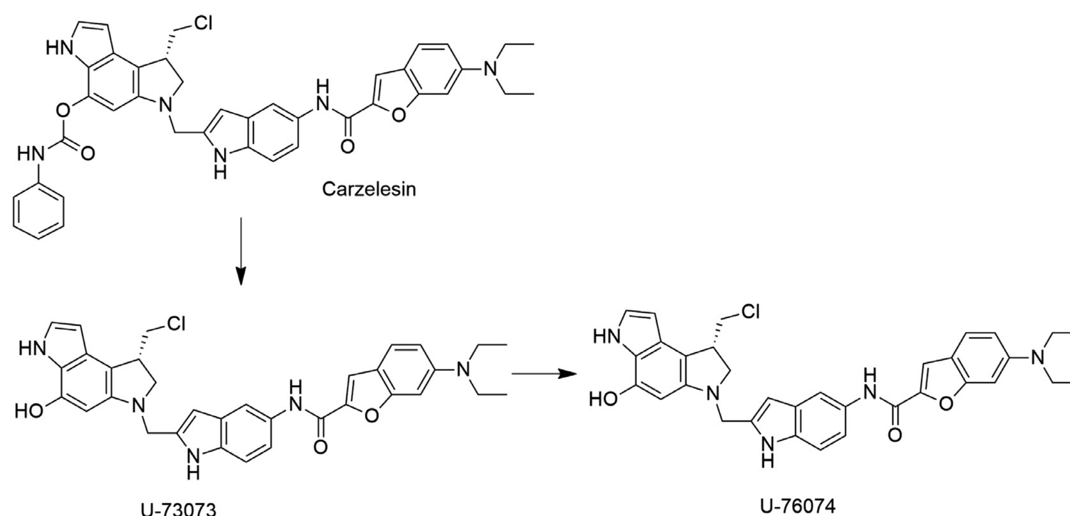

Fig. 12. Synthesis of Duocarmycin analogue (U-76074).

binder, which quickly restrains DNA replication in treated cells via a trans-acting mechanism. Adozelesin primarily arrests cells in S phase. Bizelesin targets the small groove of DNA and causes DNA cross-linking, thereby restraining DNA replication and RNA synthesis. It also strengthens p21 and p53 induction and induces G2/M cell-cycle arrest, leading to slow cell death but without apoptosis.

Carzelesin is a cyclopropylpyrroloindole prodrug containing a nonreactive chloromethyl fore body that is functional upon activation [92]. It is activated by hydrolysis of the phenylurethane substituent to generate U-76073 and a subsequent ring closure step yields the cyclopropyl-containing U-76074 form that is active in DNA binding (Fig. 12).

Duocarmycin analogues are active in picomolar range [93–95]. They are exceptional candidates as payloads, which yield ADCs with maximized cell-killing potency for regular and more importantly, multi-drug resistant cancer cells. A linker-drug platform containing a cleavable linker and a, DUBA (final active drug form), has been reported and developed into several new duocarmycin analogue generation ADCs for *in vitro* or *in vivo* efficacy evaluations. SYD983, a HER2-targeting ADC based on trastuzumab, is a leading ADC derived from this platform [96]. SYD983 results in decreased tumor growth in a BT-474 mouse xenograft *in vivo* and is stable in human and cynomolgus monkey plasma. Byondis' trastuzumabduocarmazine, a HER2-targeting ADC currently in phase 3 clinical trials, uses one such derivative, seco-DUBA, as its payload.

2.2. Tubulin Polymerization Inhibitors

2.2.1. Auristatins

Auristatins are water-soluble synthetic derivatives. This is a marine natural product (dolastatin 10) obtained from the extract of a sea hare called *Dolabellaauricularia* (Fig. 2,Fig. 13). The mother compound was originated in cyanobacteria *Symplocahydnoides* and *Lyngbyamajuscula*, which are nourishment to the sea hare [97,98]. Dolastatin 10 is a series of linear peptides which includes chemical constituents like dolavaline, valine, dolaisoleuine, dolaproine amino acid residues and a complex primary amine (dolaphenine). It is found to be potent against a various cancer cell lines at very lower concentrations (average IC_{50} value is below sub nanomolar range) [99–101]. It shows the similar mechanism of action related to tubulin-binding activity as vinca alkaloids. Also, it inhibits tubulin polymerization and tubulin-dependent GTP hydrolysis that causes cell cycle arrest in the G2/M phase, eventually leading to

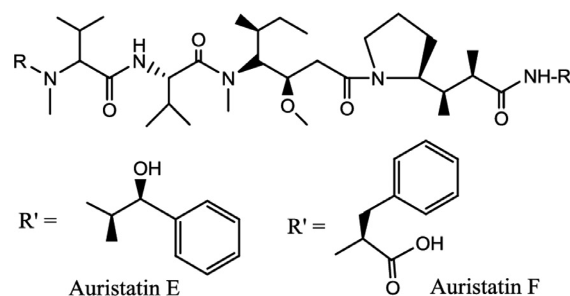

Fig. 13. Structure of Auristatins.

cell apoptosis [102]. There are two auristatin derivatives (MMAE and MMAF) developed by Seattle Genetics currently being used as payloads in several ADCs by making a linker with the cysteine residues of the mAb [103–105]. Bentuximabvedotin is a derivative of Auristatins, which was approved by FDA as ADC. It incorporates MMAE which bind with the cysteine residues of anti-CD30 antibody via protease sensitive valine-citrulline dipeptide linker [97]. Cell-mediated endocytosis process is used to carry Bentuximabvedotin into the cytosol. Here, the linker is selectively slashed in the presence of elevated lysosomal protease cathepsin B [100]. Now, MMAE can easily penetrate the cell membrane, which can prompt bystander killing where it diffuses through nearby cells independent of antigen expression [101]. Usually, MMAF is impermeable to cell membrane due to the presence of C-terminal carboxylic acid group i.e., a charged phenylalanine moiety which perturbs its cell membrane permeability [99].Although MMAF is also more hydrophilic. It has a poor tendency to increase systemic toxicity than MMAE because of its lower ability to cross cell membranes than MMAE [106].The marketed ADCs containing MMAE payloads areAdcetris®, Padcev® and Polivy®.

2.2.2. Synthetic route of Auristatins-mAb conjugates

In 2017, Wang and his co-workers already reported a synthetic route to make Auristatins-mAb conjugates.[104] (Fig. 14). Maleic anhydride (1) was used to make an interaction with amino acid solutions (2a-2b) to produce an intermediate 3-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl) propanoic acid (3a-3b). It was again utilized to form a linker (4a-4b). Beside this, (((9H-Fluoren-9-yl)methoxy)carbonyl)-l-valyl-l-alanine was synthesized. This product was further used to develop(S)-2-Amino-N-((S)-1-((4-(hydroxymethyl)-phenyl)amino)-1-oxopropan-2-yl)-3 substi-

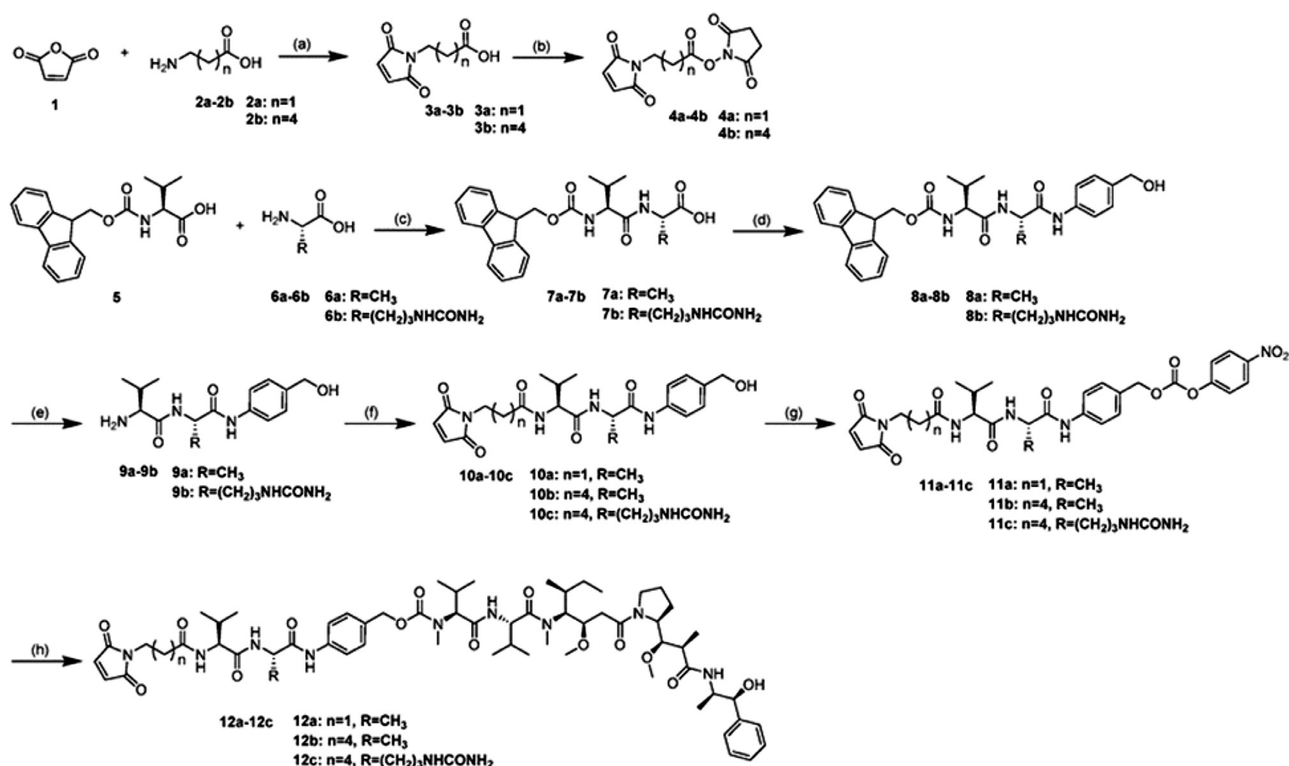

Fig. 14. Synthetic route of Auristatins-mAb conjugates.

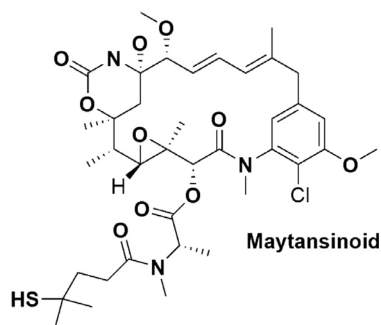

Fig. 15. Structure of Maytansinoids.

tuted butanamide (9a-9b). Finally, final products (10a-10c) were formed by using stranded protocols considering 9a and 9b as starting materials.

2.2.3. Maytansinoids

Maytansiniod derivatives (DM1 and DM4) are the microtubule polymerization inhibitors that are developed by Immunogen. It is isolated from the bark of Ethiopian shrubs *Maytenusovatus* and *Maytenusserrata*. These derivatives are having similar type of tubulin binding affinity and follow same mechanism of action likevinca alkaloids and destabilizes microtubule assembly resulting cell cycle arrest in G2/M phase (Fig. 15) [107–109]. DM1 and DM4 are the major derivatives of maytansinoid with methyl disulfide substitutions at the C-3 (N-acyl-N-methyl-L-alanyl ester) side chain position of maytansine [109]. Kadcyla® is a clinically approved drug where DM1 (Fig. 2) is used as a payload with an average DAR of 3.5 for treatment of HER2+ metastatic breast cancers [110]. SAR3419 is another ADC where DM4 is used as payload to target CD-19 tumour cell. It is now in phase II clinical trial for the treatment of B-cell malignancies. In SAR3419, DM4 payload is linked to the lysine residues of the mAbs. Here, thiol sensitive N-succinimidyl-4-(2-pyridyldithio) butyrate (SPDB) linker was utilized with an average DAR of 3.5 [111]. While it is reported that numerous chemical groups are not crucial for preventing the microtubule assembly [112], thus ester side chain is less explored (Fig. 16).

2.2.4. Synthesis of Maytansinoid-mAb conjugates

Maytansine-analogues were conventionally synthesized by linking with mAbs. During synthesis ansamitocins was used as starting material which was obtained from fermentation of the microorganism *Actinosynnemapretiosum*. Afterwards, two steps synthesis were carried out (through maytansinol and the disulfide ester) and reactive thiol-containing maytansinoids were obtained, as follows. It gives the alpha-beta-unsaturated maytansinoid maysine as the C-3 ester group in maytansinoids is liable for elimination under mild basic (pH $>$ 9) conditions. Ester hydrolysis process was performed through a reductive cleavage mechanism. Primarily, a strong reducing agent like lithium aluminumhydride (LAH) was applied to get the C-3 alcohol maytansinol [113]with lower yield value. Successively, the ester group was cleaved by using the mild reducing agent lithium trimethoxyaluminum hydride (LTAMH) , under controlled temperature (−30 to −40 °C) to get the alcohol withmaximum yield value [109]. Finally, esterification for Maytansinol was conducted with N-methyl-N-(methyldithiopropanoyl)-L-alanine in the presence of dicyclohexylcarbodiimide (DCCI) and zinc chloride ($ZnCl_2$), to give DM1 (Fig. 17) [113].

Various ADCs possessing the maytansinoid payloads batansine (Bio-Thera Solutions' BAT8001) [89], DM1 (ImmunoGen'slorvotuzumabmertansine) [90] and DM4 (ImmunoGen's SAR566658) [90] are currently undergoing phase 2 or phase 3 clinical evaluation [46].

2.2.5. Amanitins

The amatoxins are a group of naturally occurring bicyclic octapeptides that take their name from the genus of mushroom in which they are found from *Amanita phalloides* [46,114]

Fig. 16. Structure-activity relationship (SAR) of Maytansine.

Fig. 17. Synthesis of Maytansinoid-mAb conjugates.

Fig. 18. General structure of Amanitins.

(Fig. 2,Fig. 18). Amatoxinsare highly selective allosteric inhibitors against RNA polymerase II, which disrupts transcription and protein synthesis. Out of all the chemical constituents of amatoxins, α-amanitin and β-amanitin revealed to have best application as ADC payloads. Due to the presence of high potency and plasma stability, hydrophilicity, metabolism-disruption, toleration of a linker moiety without loss of cytotoxicity and, most importantly, the ability to kill dormant as well as dividing ones tumour cells [91]. The most advanced ADC utilising an amanitin-type payload is Heidelberg Pharma's HDP-101, a preclinical candidate that combines a B cell maturation antigen-targeting mAb with α-amanitin via a cleavable valine–alanine dipeptide linker [92]. The synthesised linker dissolved in HOBt in DMF along with amine followed with DIPEA attached with the designed octapeptide dissolved in pyridine/DMF. The reaction performed at RT for 23 hrs [114].

3. Advantages and disadvantagesof synthetic ADC payloads

The general advantages of synthetic ADCs payloads with having cell-killing potential of monoclonal antibodies; higher cancer cell selectivity, better tolerability with a limited systemic exposure [115,116]. The disadvantage of the synthetic payloads relied

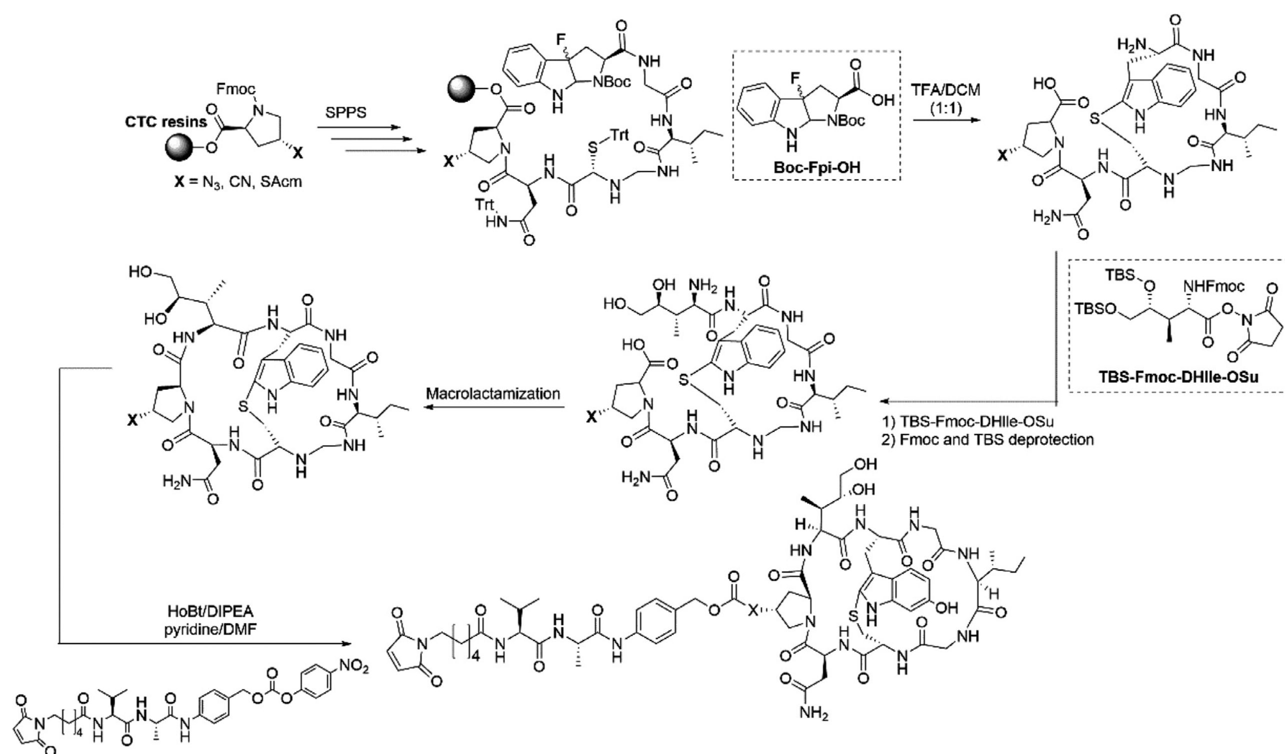

Fig. 19. Synthesis of Amanitins derivatives-mAB conjugates (azido, cyano and SAcm-prolines).

on linker development because of remarkable bio-functional challenges [117]. Ideally, linkers should be systemic stable, should not disrupt antigen binding, after the engulfment, it should efficiently degrades or release the synthetic payloads in lysozymes. For example, disulfides (SPDB-DM4) linker attached with the antibody cysteine [118,119]. Another example, pH sensitive linkers hydrazone based linkers can hydrolyze more in acidic environment such as CL2A linker of SN38 [120].The disadvantage that they de-conjugate during circulation and leads to toxicity [121].

4. Recent advances in Computational Approaches

ADC is a multicomponent complex drug molecule [1,7,116]. Rational design and discovery of optimum ADCs against cancer disease is a very challenging process. In view of this, several computational studies have been done to optimize ADCs against cancer treatment [117–119]. The concepts and working of these studies are critically discussed and updated here to arouse interest and to advance the drug research in area of ADCs therapy.

4.1. Neoantigen target selection and identification

Generally, immune system tries to eliminate tumor cells [120]. The antigen presenting cells (APCs) detect the antigens from tumor exposed cell surface and present to T cells. The activated T cell then binds and destroy the tumor cell. These antigens work as markers to detect tumor and cancerous cells. Preferential antigen selection to the cancerous cell and to normal cell by ADCs is the key step in strategic plan to design ADCs. Rationally good target sites (antigens) for ADCs are those, which are having extracellular domain as an immunizing agent, good internalization capacity, no shedding in circulation and overexpressed on the cancer cells in comparison to normal cells [122]. The selection/identification of an appropriate neoantigen is an important factor that affects the pharmacological and toxicological profile of ADCs. The antigenicity is due to the antigenic determinant part (an epitope) of the antigen and an antigen can have different epitopes – linear (Primary/linear structure of protein) as well as conformational (3-D structure of protein) – on its surface [121]. In antibody-antigen reaction, most of the antibodies recognize conformational epitopes. The physio-chemical characteristic features and importance of conformational epitopes are crucial parts in designing of ADCs, vaccine and diagnostics kit [123]. Neoantigen is difficult to locate in the human genome. In this backdrop, various online databases are available to search the antigen target for ADCs development (Table 3). The obtained structural information (Table 3) from online databases of antigen is required for the design of optimal payload delivery and promote the development of potential ADCs for the treatment of cancer.

The human major histocompatibility complex (MHC)/ human leukocyte antigen complex (HLA), which can be divided into three classes MHC-I (HLA-I), MHC-II and MHC-III. Among these MHC-I and MHC-II are directly involved in tumor antigenic presentation. Both include antigenic peptide binding pocket/grooves, which are responsible for antigen presentation pathway selection [124]. Thus, the prediction of peptide binding interaction to MHC is an important aspect to predict the possible specificity of a T-cell immune response. Considering this, several machine learning-based epitope prediction tools are developed, such as NetMHCpan4.1 [125], NetMHCIIpan4.0 [126], NetCTLpan [127], MHCflurry [128], MHCflurry 2.00 [129], ConvMHC [130] and PLAtEAU [131] to predict binding interaction between peptides and MHC-I/MHC-II.

Machine learning is an extensively used approach to train models that predict data with different features [132]. Generally, the machine-learning approach requires more data for the generation of a better model [126]. However, data is limited in immunotherapy development. The feature selection method in machine learning is to find the best set of features that allows one to build a good model in absence of more data [133]. Fauteux *et al.* applied feature selection and classification methods to identify target anti-

Table 3
Some web-based databases and tools for antigen information retrieval.

S No	Databases	Input	Output	Web Link	Ref.
1	NCBI	Keyword*	Antigen information	http://www.ncbi.nlm.nih.gov/	[137]
2	UNIPROT	Keyword*	Antigen information	http://www.uniprot.org/	[138]
3	dbPepNeo	Keyword	Neoantigen information	http://www.biostatistics.online/dbPepNeo/	[139]
4	Immune Epitope Database (IEDB)	Keyword*	Antigen information	https://www.iedb.org/	[140]
5	TSNAdb Specific NeoAntigen	Keyword*	Neoantigen information	http://biopharm.zju.edu.cn/tsnadb.	[141]
6	NeoPeptide	Keyword*	Neoantigen information	http://www.neopeptide.cn/	[142]
7	Cancer Antigenic Peptide	Keyword*	Neoantigen information	https://caped.icp.ucl.ac.be/Peptide/list	[143]
8	TANTIGEN 2.0: Tumor T-cell Antigen	Keyword*	Neoantigen information	http://projects.met-hilab.org/tadb/	[144]
9	BEPITOPE	Sequence	Predicting the location of continuous epitopes and patterns in proteins	http://bepitope.ibs.fr/	[145]
10	varDB	Keyword*	Antigenic variation database.	http://www.vardb.org/vardb/homepage.html	[146]
11	Bcepred	Sequence	Prediction of linear B-cell epitopes, using physico-chemical properties.	http://www.imtech.res.in/raghava/bcepred/	[147]
12	Bcipep	Sequence	A database of B-cell epitopes.	http://bioinformatics.uams.edu/mirror/bcipep/	[148]
13	Abcpred	Sequence	Artificial neural network-based Prediction of B-cell epitopes.	http://www.imtech.res.in/raghava/abcpred/ABC_submission.html	[149]
14	CBTOPE	Sequence	Prediction of conformational B-cell Epitopes.	http://www.imtech.res.in/raghava/cbtope/submit.php	[150]
15	LBtope	Sequence	Prediction of Linear B-cell Epitopes.	http://www.imtech.res.in/raghava/lbtope/	[151]
16	IgPred	Sequence	Prediction of Antibody-specific B-cell epitopes.	http://crdd.osdd.net/raghava/igpred/	[152]
17	BepiPred 1.0	Sequence	Prediction of linear B-cell epitopes, using a Markov model and a propensity scale method.	http://www.cbs.dtu.dk/services/BepiPred/	[153]
18	LEPS	Sequence	Prediction of B-cell linear epitopes based on propensity scale and SVM.	http://leps.cs.ntou.edu.tw/	[154]
19	B-Pred	Structure	Prediction of B-cell epitopes.	http://immuno.bio.uniroma2.it/bpred/	[155]
20	BCEP	Structure	Prediction of B-cell epitopes.	http://curie.utmb.edu/B-Cell.html	[156]
21	SVM-TriP	Sequence	Prediction of antigenic epitopes using support vector machine to integrate tri-peptide similarity.	http://sysbio.unl.edu/SVMTriP/prediction.php	[157]
22	EpiSearch	Sequence	Mapping of Conformational Epitopes.	http://curie.utmb.edu/episearch.html	[158]
23	CTLPred	Sequence	Prediction of cytotoxic T lymphocyte (CTL) epitope based on SVM and ANN.	http://www.imtech.res.in/raghava/ctlpred/index.html	[159]
24	NetCTL 1.2	Sequence	Prediction of CTL (cytotoxic T-lymphocyte) epitopes.	http://www.cbs.dtu.dk/services/NetCTL/	[160]
25	SYFPEITHI	Sequence	Database for MHC ligands and peptide motifs	http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm	[161]
26	NetMHC 3.4	Sequence	Predictions of human mouse and monkey MHC class I affinities for peptides of length 8-11.	http://www.cbs.dtu.dk/services/NetMHC/	[162]
27	B-cell Epitope prediction using Support vector machine Tool (BEST)	Sequence	Epitope	http://biomine.cs.vcu.edu/datasets/BEST/	[163]
28	LBEEP		Epitope	https://github.com/brsaran/LBEEP	[164]
29	COBEpro	Sequence	Epitope	http://scratch.proteomics.ics.uci.edu./	[165]
30	TCR3d	Keyword	key information on antigen binding mode, interface features, loop sequences and germline gene usage, 3-D model	https://tcr3d.ibbr.umd.edu/cancerseqs	[166]
31	Adapt	Sequence of antibody and antigen	Antibody-specific epitope prediction	https://sysimm.org/adapt/	[167]

* Keyword; Antigen and epitopes name, species, biomarkers, etc.

gen in breast cancer [134]. Recently, Lo *et al*. designed two sequential methods: (1) matching and (2) prediction for prediction of the conformational epitope [135]. The first method assists to search matching protein sequences and surface patches to quickly find homologous antigenic epitope regions from a presented epitope database. The second method, spiral vector search (SVS) assists a novel surface spiral feature vector for large-scale surface patch detection when queried against a comprehensive epitope database. The proposed strategy advances the traditional epitope prediction method [117]. Currently, Molecular docking and molecular dynamic simulation (MDS) experiment has become popular for assembly of ADCs and investigation of interactions between ADCs and cancer antigen [136]. The traditional approach for antigenic target selection is unguided, laborious and resource-intensive. *In silico* antigenic target prediction along with experimental validation is critical to advancement in the ADCs development. Indeed, antigen structural information provides a basis for the selection and identification of appropriate antibodies for the development of ADCs.

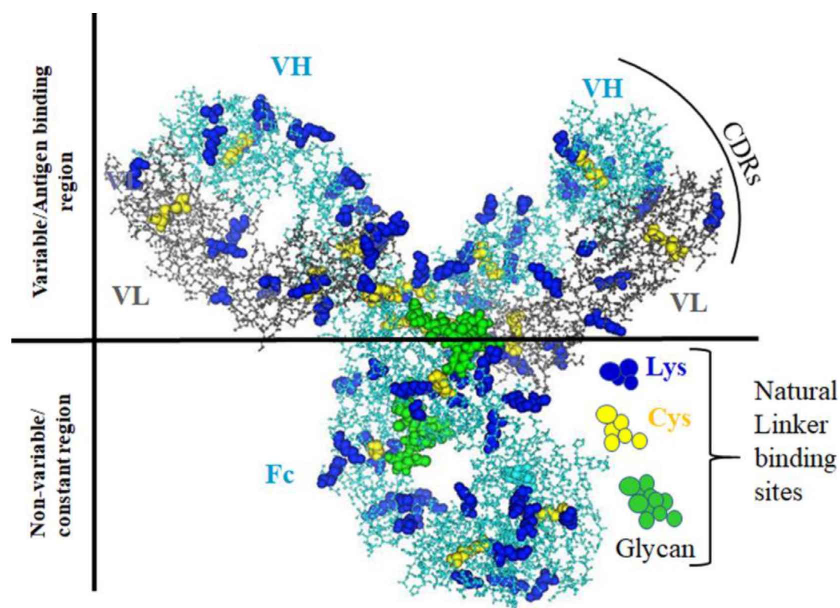

Fig. 20. Ball and stick model of antibody (PDB: 5DK3). Figure generated by MOE Software.

4.2. Antibody selection and identification

Selection and Identification of appropriate antibody takes precedence over the design/discovery of ADCs. In cancer disease, the antibody is selected in such a way that modulation of their activity causes minimal adverse effects to the normal cells. Antibody act as carrier for delivery of drug in ADCs treatment [168]. The efficacy of the antibody relies on the binding of the ADC to the specific cancer antigen and on its internalization capacity [169].

3-D structure attributes and dynamic flexibility profile of antibody greatly help in the selection of antibody in ADCs development [170]. 3-D structure of Antibody molecule has Y-shaped structure (PDB: 5DK3) consisting of roughly three equal-sized portions (Fig. 20) [171]. Its structure contains two heavy (HC, 50 kDa) and two light (LC, 25 kDa) chains. The structure of antibody having N terminal (Fab/Fv/variable region/ VH, VL) and C terminal (Fc/constant region) [172]. The variable domain of both chains having complementarity determining regions (CDRs: CDR1, CDR2, CDR3). A set of CDRs (paratope/binding site) responsible for binding interaction between antibody-antigen and directly command the antibody specificity and affinity for a specific antigen [173]. The lysine (approxly 80-100), cysteine (16 pairs of cysteine residues exist as 12 intra-chain and four inter-chain disulfide bonds) and glycan are natural linker binding sites in antibody structure responsible for payload conjugation [7]. The lysine and cysteine residues in the variable region of antibody are occupied for antigen binding site therefore not conjugated with payloads [7]. Conjugation chemistry is discussed in the later part of the review.

To date number of 3-D structures of antibodies have been reported. There are various databases available for procurement of 3-D structure of antibodies namely protein data bank (PDB) [173], Structural Antibody Database (SabDAb) [174], Therapeutic SAbDab [175], Abysis (commercial) [176], AbDb [177], international Immunogenetics information system (IMGT) [178] and Tabs - Therapeutic Antibody Database [179] (commercial) (Table 4). These databases track all reported antibodies and their structural information, which help to design ADCs.

Antibody structure and function are the key part of ADCs. The harmonizing relationship between shape, size and physicochemical properties of the binding sites of antibody and the antigen epitope conformation is the guiding principle for the designing of potential ADCs. X-ray and NMR data of antibody and antigen provide the direct structural information of antigen and antibody to design appropriate ADCs. In the absence of 3-D structural information, structural modeling methods have come up to predict the 3-D structure of targets. Among these methods, homology, fragment based and *ab initio* modeling are applied for the prediction of 3-D structures of targets (Table 5).

The previous reports [180] have convincingly shown the scope of antibody structural modeling where the 3-D structures of antibodies are not available Table 5. represents the list of various online tools/servers useful in antibody modeling. SAbPred is an assembly of online computational toolbox (ABodyBuilder [181]: Fv/ Fab modeling, PEARS: Side chain prediction, Sphinx and FREAD: Loop modelling, ANARCI: Sequence numbering, Antibody i-Patch: Paratope prediction, EpiPred: Epitope prediction, SCALOP, Developability prediction, TAP: Canonical form prediction from sequence, ABangle: VH-VL orientation calculation) that make predictions about the different properties of antibodies structures. Antibody informatics tools can help to design and engineering of ADCs molecules.

Recently some engineered antibody mimicking agents came into light as good conjugated drugs against cancer disease [188]. Sachdeva et al exploit Knob-Socket model for mapping protein-protein interaction between Cetuximab and EGFR (PDB id: 1YY9) [189]. EGFR interacting peptides (mimicking antibody) were designed and selected based on geometry, the probability of the mapped knob-sockets pairs and docking energy analysis [189]. Kadonosono et al computationally design small antibody mimetics, named fluctuation-regulated affinity proteins (FLAPs) [190]. The 3-D structures of linear CDR peptides, model linear peptides, model hexapeptide-grafting scFv molecules, model hexapeptide-grafting scaffold candidates and CDR peptide-grafting scaffolds were generated using Discovery studio 3.1 with the help of fragment/scaffold retrieved from the protein data bank. The generated models were further optimized by MDS experiment [191].

As we know antibody has stability or high levels of aggregation issues in antibody-based therapeutics designing and developability issue [191]. Lipinski's rule of five assists in designing of small molecules with appropriate biophysical parameters [192]. Raybould

Table 4
Some databases for antibody structure retrieval.

S. No.	Name	Input	Output	Link	Ref.
1	PDB	PDB code, keywords	3-D Structure information	https://www.rcsb.org/	[173]
3	SabDAb	PDB code, keywords	3-D Structure information	http://opig.stats.ox.ac.uk/webapps/newsabdab/sabdab/search/	[174]
4	Thera-SAbDab	Therapeutic INN name and attributes	Therapeutic, 1/3-D structure information	http://opig.stats.ox.ac.uk/webapps/newsabdab/therasabdab/search/	[175]
5	Abysis	Variable keyword attributes	1/3-D structure information	http://www.abysis.org./abysis/	[176]
6	AbDb	PDB code, keywords	3-D Structure information	http://www.abybank.org/abdb/	[177]
7	IMGT/3Dstructure-DB	PDB code, keywords	2D and 3-D Structure information	http://www.imgt.org/3Dstructure-DB/	[178]
8	Tabs	Variable keyword attributes	Therapeutic, 1/3-D structure information	https://tabs.craic.com/users/sign_in	[179]

Table 5
Online tools for antibody structural modelling.

S. No.	Name	Input	Output	Method	Link	Ref.
1	SAbPred-ABodyBuilder	Heavy and light chain sequences	Prediction of antibody Fv region, or nanobodies.	homology modeling	http://opig.stats.ox.ac.uk/webapps/newsabdab/sabpred/abodybuilder/	[174]
2	FREAD and ConFREAD	Protein fragment	Prediction of CDR structure	knowledge-based	opig.stats.ox.ac.uk/webapps/fread/php	[182]
3	PIGSPro	Heavy and light chain sequences	Prediction of antibody Fv region	homology modeling	https://rosie.rosettacommons.org/antibody/submit	[183]
4	Repertoire Builde/ Kotai Antibody Builder	Heavy and light chain sequences	Prediction of antibody Fv and H3 loop region region	homology modelling/ ab initio approach/ MDS	https://sysimm.ifrec.osaka-u.ac.jp/rep_builder/	[184]
5	RosettaAntibody	Heavy and light chain sequences	Prediction of antibody Fv region	homology /ab initio modeling	https://www.rosettacommons.org/docs/latest/application_documentation/antibody/antibody-applications	[185]
6	NovaFold Antibody	Heavy and light chain sequences	Prediction of antibody model and CDRs loop location	homology modeling for framework regions and fragment-based or ab initio modeling for hypervariable loop regions,	https://www.dnastar.com/software/nova-protein-modeling/novafold-antibody/	[186]
6	Profacgen	Heavy and light chain sequences	Prediction of antibody Fv region, or nanobodies	homology modeling	https://www.profacgen.com/Antibody-Modeling.htm	[187]

first time introduced five computational developability guidelines for therapeutic antibody profiling in absence of in silico analog for designing of antibody [193]. They modeled the variable domain light and heavy chain sequences of 137 post-phase-I clinical-stage antibody therapeutics (CSTs) and calculate in silico metrics to estimate their biophysical properties. These properties include total CDR length, patches of surface hydrophobicity (PSH) metric across the CDR vicinity, patches of positive charge (PPC) metric across the CDR vicinity, patches of negative charge (PNC) metric across the CDR vicinity and structural Fv charge symmetry parameter (SFvCSP) [188].

High-affinity antibody against cancer antigen is the basic requirement for ADC-based cancer therapy. Generally, antibodies with higher antigen binding affinity showed greater uptake and internalization capacity. Yamashita et al. investigate promising single amino acid substitutions that can improve antigen-antibody interactions [194]. In this experiment, Alanine mutagenesis scanning of the interfacial amino acid of a cancer-targeted antibody (B5209B) was performed based on X-ray crystallography analysis. Some substitutions (ex; Y30L) were shown to potentially enhance the binding affinity for the antigen. MDS experiment strongly supports that the enthalpic improvement could be assigned to the stabilization of distant salt bridges placed at the periphery of the antigen-antibody interface. This strategy can be helpful to design high-affinity antibodies for ADC development [194].

4.3. Cytotoxic payload

In ADCs, payload is the crucial component showing cytotoxic activity against disease targets. It can be divided in either microtubule inhibitors or DNA damaging payloads which is further subdivided into other categories such as DNA alkylating agents, radionuclides (For example I-131, Lutetium-177 etc.) [195–197]. Different payloads have their unique mechanism of action to target or kill cancer cells. The designed payload should be more potent (sub-nano-molecular range) and have good internalization power because limited target antigen molecules available on the cell surface ($<10^5$ copy numbers per cell) of diseased cells [198]. Among the available payloads, small molecules account for a majority of ADCs developed against cancer.

Recently, many companies (MedChemExpress, Biochempeg, Abbvie, Creative Biolabs, BroadPharm, ADC Express) manufacturing and discovering high quality ADCs and their components. They can provide high quality service to customers in academia and industry fields all over the world. Among these, Creative Biolabs providing various types of payloads including toxins Targeting -Tubulin Filaments, DNA, RNA; Nanocarriers; Protein Toxins; Enzymes [199].

4.4. Linker selection and identification

A stable linking between the antibody and the payload is a crucial aspect in the design of ADCs. During the design and selection of linkers, we have to ensure that more of the payload is bioavailable at the cancer cell and less falls off in circulation [200–203]. This will increase the safety and efficacy profile of ADCs. ADCs linker and their conjugation are based on different techniques including cleavable (chemically labile: acid-cleavable hydrazone linker, reducible disulfide linker; enzyme cleavable linkers: peptide-based, β-glucuronide), non-cleavable (maleimidocaproyl, thioethers) [200–203]. The selection of linkers is reliant on the 3-D structure of the targets (antibody, payloads) and their mechanism (internalization, cleaving, degradation). Moreover, the choice of linker is also influenced by preclinical *(in vitro* and *in vivo* activity) data comparison of available conjugates [203]. Many pharmaceutical/ biotechnology-oriented companies (MedChemExpress, Broadpharm, Biochempeg, Novasep) manufacturing high purity linkers for academic/research/commercial use. From their databases, researchers can easily explore the different combinations of linker, antibody and payloads with the help of molecular docking/simulation experiments.

4.5. Conjugation methods

Linkers are covalently bonded to naturally occurring amino acids lysine (ado-trastuzumab emtansine) or the inter-chain cysteine residues by conjugation chemistry (Brentuximab vedotin) on the antibody (Table 6) [203–205]. Various new methods, summarized in Table 6, came into light namely site specific unnatural amino acid engineered antibodies chemical conjugation, chemo- enzymatic conjugation, glycan engineering and photoactive molecular conjugation [206–214]. Natural conjugation methods have considerable limitations. Dose optimization is the major challenge in ADC development. Recently, Bhatnagar and group designed a mechanistic model (Physiologically based pharmacokinetic (PBPK) modeling/ Krogh cylinder model) for intra-tumor spatial distribution of ADC [215]. In this study, they tried to understood the relationship between ADC tumor penetration and efficacy. This strategy described a mechanistic basis of ADC efficacy in solid tumors and help to optimize dosing techniques to improve patient outcomes [215].

The lysine conjugation method produces a heterogeneous mixture of ADC species with a variable drug-to-antibody ratio (DAR), resulting in unpredictable pharmacokinetics and safety profiles [203]. Recently, Hwang et al. developed site-specific lysine bioconjugate based ADC against cancer [216]. They have done structural modeling on Lys99Arg of h38C2 Fab region of the antibody (PDB ID 6U85), Arg99 was replaced with an azido-(PEG)-4-PODA-derivatized Lys residue and subsequently stabilized by MDS [216].

In practice, cysteine is majorly used for conjugation as it is a less abundant amino acid in comparison to lysine and can afford greater selectivity [203,217]. But the drawback of cysteine conjugation is that instability in plasma and causes off-target toxicity. Chemical modification of cysteine at specific sites in antibodies produced virtuous ADCs for the treatment of cancer [217–221]. *In*

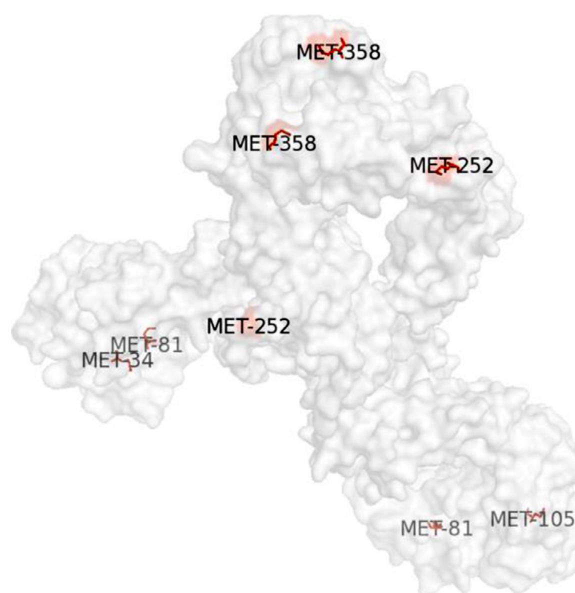

Fig. 21. Methionine binding site (red stick model) position in surface model of antibody (White) structure (PDB: 5DK3). Figure generated by Pymol Software

silico virtual screening can provide in-depth knowledge for the selection of an optimal binding site for the different linkers. Considering this, Coumans *et al.* developed an *in-silico* docking screening experiment for selection of optimal binding sites (cysteine mutants) in the Fab region of antibody to where a hydrophobic linker–drug can be easily conjugate [222]. Conjugation of a linker–drug to site specific binding sites confirmed the ability of the antibody to shield the hydrophobic nature of the linker–drug. The tumor targeted ADCs also maintained their cytotoxic potency. The site-specific ADCs improved properties over randomly conjugated ones [222]. Engineering/modification of natural amino acids in antibodies is an old story for the formation of anticancer ADCs, but largely is limited to modification of amines and thiols with various electrophiles.

Recently, a methionine specific conjugation chemistry has been developed, called redox activated chemical tagging (ReACT) [223]. Methionine is the second least abundant amino acid in antibodies after tryptophan, making it a potentially ideal binding site for site-specific conjugation (Fig. 21) [223].

In follow up this strategy, Elledge *et al.* systematically tested oxaziridines and examined accessible and buried sites of methionines in antibody (trastuzumab) structure against breast cancer [224]. They assumed that site-specific modification of methionine by ReACT technology has great possibility for ADC conjugation. The expression of the methionine mutants is relatively high. Therefore, the number of methionines can easily be introduced in antibodies. This method is simple and fast and not required premodification of antibodies. The strategy provided good flexibility for binding site selection for linker conjugation and the resulting conjugates were stable at biological temperature. In this technology, simple homology modeling was used for stable binding sites instead of complete methionine surface scan [201]. Sadiki *et al.* summarised site-specific conjugation studies and their advantages. In their review report they discussed, site-specific methods are better performed in terms of biological parameters, such as improved binding efficiency, improved plasma stability, less variability in dose-dependent studies, as well as increased tumor uptake [207]. Among site specific methods, microbial transglutaminase-based technology highlighted the promising results in the formation of ADCs [225]. Cornelissen and group

Table 6
ADC Conjugation mechanism and their advantages and disadvantages.

S. No.	Type of Conjugation	Antibody functional group	Linker/conjugate functional group	Conjugation Mechanism	DAR Range	Advantages	Disadvantages	Ref.
1	Natural chemical Conjugation	Nucleophilic Amine (NH) of Lys	Carboxylic acid, ester-based/ bi-functional reagent (containing amine- and thiol-reactive functional groups)/ electrophilic linker: MCC, SPDB, AcBut, MCC-DM1, SPDB-DM4	Amidation (-CONH) of Lys	0-7	-Flexible conjugation at any site (Fab/Fc) of Ab-Flexible for development of non-specific/ site-specific ADCs.	-Pre-modification of Ab required-High heterogenicity-24 h processing time-More than 40 binding sites	[203–205]
		Thiol (SH) of Cys	Maleimidocaproyl (MC), MCC	-Cys alkylation	0~8	-Simple and economic process	-Pre-modification of Ab required-Medium heterogenicity-Loss of 3-D structure integrity-4.5 h processing time-Drug exchange with albumin-Unstable in plasma	[194,195]
			Dibromo/ Disulfonate reagent	Reduction of interchain Cys, disulfide, Cys rebridging				
			Aryl-palladium complex reagents	-Cys-Aryl coupling				
	Engineered cysteine technology, THIOMAB-drug conjugate (TDC)	Site-directed mutagenesis to incorporate Cys into the antibody	Maleimide linker	Reduction and oxidation	2	-Highly stable, potent and near homogeneous	Requires genetic engineering	[214,215]
	Engineered methionine, ReACT	Incorporation of Met	Oxaziridine analogs	ReACT	-	-Excellent DAR-ADCompatible with biological environments		[217,218]
2	Site specific unnatural amino acid engineered antibodies chemical conjugation	pAcPhe	Alkoxyamine-functionalized linkers	Oxime	2		-Complex approach,-Require the introduction of artificial amino acids into the antibody	[182–184]
		pAMF/ AzK	Alkyne functionalized linker drugs	Copper-catalyzed				
				Strain-promoted (copper-free) azide-alkyne cyclization.				

(*continued on next page*)

Table 6 (*continued*)

S. No.	Type of Conjugation	Antibody functional group	Linker/conjugate functional group	Conjugation Mechanism	DAR Range	Advantages	Disadvantages	Ref.
3	Site specific Chemo-enzymaticConjugation	GETPL and LPETG tagged mAb	Oligoglycine functionalized linkers	Transpeptidation (SMAC-Technology™)		-Homogeneity	Complex process	[203,231]
		Amide of Q295 of deglycosylated mAb	Primary amine-containing linker	Transpeptidation (Microbial transglutaminase-mediated conjugation)	1.8-2	-Homogeneity	-Requires removal of N-glycan on N297	[203,209]
	Site specific Chemo-enzymatic Conjugation and Glycan engineering;	N-glycan of N297- Fc of IgG	Alkoxyamine-functionalized linkers	1.Introduction of sialic acid by GalT and SialT. 2. sialic acid converted into aldehyde by oxidation	1.6	-Homogeneity	-Requires multiple steps	[188,232]
		Modification of N-glycan of N297- Fc of IgG	Strained cyclooctyne-functionalized linkers	1.Glyco Connect technology (endoglycosidase/ galactosyltransferase/ N-azidoacetylgalactosamine). 2.Strain-promoted click reaction with payload	2			
4	Photo-active molecule conjugation	Fc domain of IgG1	UV-active-cross-linker/ Protein Z-BPA	1.Incorporation of Protein Z into Fc domain with payload via ligation (EPL, STPAL). 2.Both moity conjugated via exposure (15 min to 2 h) of UV light (365 nm)	-	-Compatible with nearly all native IgGs, -Generates site-specific, homogeneous ADCs, -Simple and reproducible process without the requirement for genetic engineering or pre-activated scaffolds	-	[213,214]
5	MAGNET-based conjugation	Conserved high-affinity binding sites of IgG1	MEP–PEG construct (MAGNET linker)	Computational docking and all-atomistic large-scale MDS	6	-No modification of antibodies is required. Defined sites-Low heterogeneity -Preservation of higher-order structure Stable in the presence of albumin. Less than 1.5 h process	Low DAR	[119]

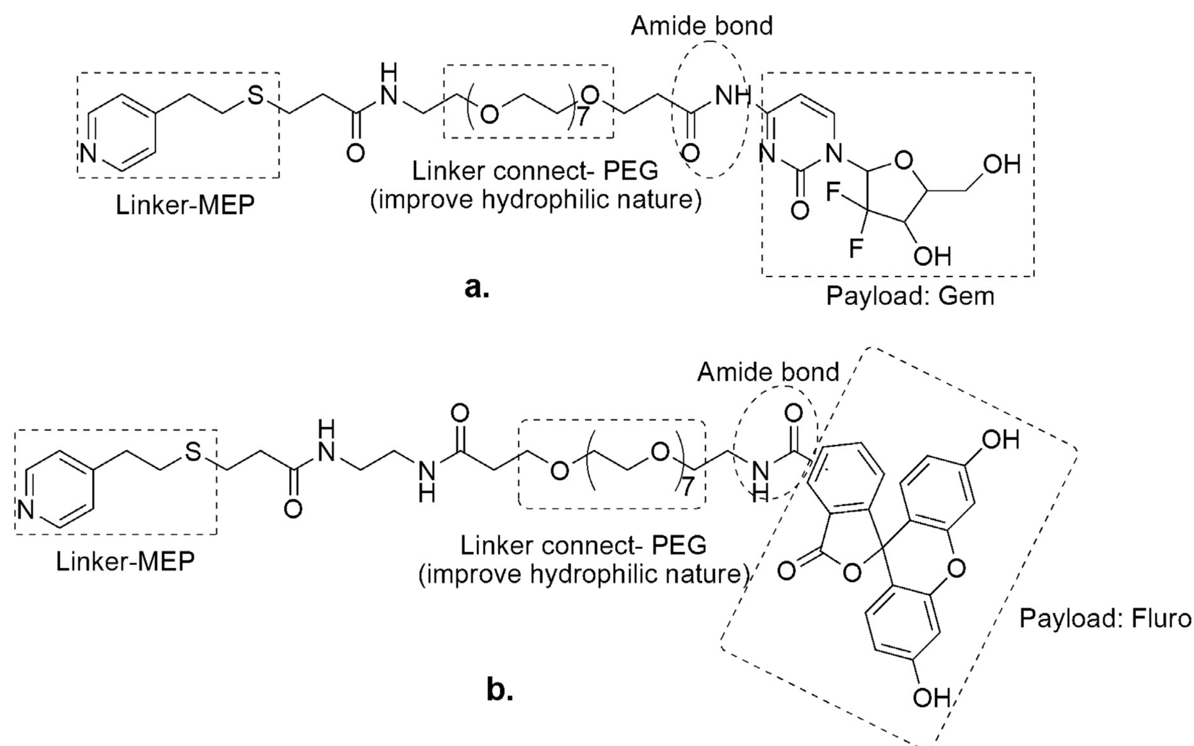

Fig. 22. 2D-Structure of MAGNET–Gem (a) and MAGNET–Fluro (b)

validated the industrially-applied chemoenzymatic Q-tag method based on transglutaminase-mediated amide-bond formation in the development of Zirconium-89 radiolabelled antibody conjugates [226]. They showed Q-tag transglutaminase-mediated Antibody conjugation yields less homogeneous conjugates than previously thought. In very recent, Wang and group presented a simple one spot reaction method to produce dual payload conjugates with the site-specifically engineered cysteine and p-acetyl-phenylalanine (pAcPhe) using anti-HER2 antibody drug trastuzumab [227]. The developed ADC showed better efficacy and was able to overcome drug resistance [227,228].

Recently, Gupta *et al* gave a new direction in designing and discovery of ADCs [229]. They used molecular docking/ dynamics simulation experiments for assembly of Antibody, linker and payloads. They synthetically extended MEP linker with hydrophilic spacer PEG called as MAGNET linker (Table 6). Produced MAGNET linker further conjugated with GEM and Fluro payload with the help of amide bond conjugation (Fig. 22). Then MAGNET-GEM/fluro linked at conserved binding sites of Fab and Fc Region of antibodies with the help of molecular docking/ dynamics simulation experiments (Fig. 23). Developed ADCs retain their structure and antibody-antigen binding properties. It's biological specificity, is stable in plasma and improves anti-tumour efficacy in mice with non-small cell lung tumor xenografts [211].

Very recently, Verdoes and group described a Clustered Regularly Interspaced Short Palindromic Repeats/Homology Directed Repair (CRISPR/HDR) hybridoma genomic engineering technique [230]. In this study, they modified the HC and LC portion of the mouse IgG1 hybridoma with the help of the genetic engineering method. The dual tagged Fab region of engineered hybridoma is laced with two different sortags enzyme. These enzymes allow the ligation of payload (amino-terminal polyglycine motif) onto the target protein. The developed strategy will be helpful in the development next-generation ADCs [230].

5. Limitations and Prospects

A substantial rise in this field has been detected following the success and FDA approval for Adcetris® in 2011, Kadcyla® in 2013, Besponsa™ in 2017, Lumoxiti® in 2018, Polivy® in 2019, Trodelvy™ in 2020 and Zynlonta™ in 2021. These recent achievements have boosted ADC developments and presently ~50 ADCs are in pipeline for the treatment of hematologic and solid tumor malignancies.

There are several factors and components involved in designing of ADC. A well understanding of the role of linker and method of conjugation to the clinical profile of the ADC have led to development of several state of art site-specific conjugation methods for homogenous antibody conjugate invention. The major limitation of ADCs development is heterogeneity of structure, low payload potency, instability in plasma, unusual off-target toxicity, DAR optimization and processing time. Another important challenge is to develop cost effective and affordable ADC medications. At present ADCs are quite expansive, for example yearly brentuximab vedotin treatment regimen costs ~$100,000 [204]. *In silico* approaches in combination of *in situ* helps the researchers to get optimum structural details of antibody, linker and payloads for development of economic and potential ADCs. The necessity of appropriate structure of antibody is at the forefront. The availability of 3-D structure of antibody is an initiative to design other desirable parts (linker, payloads) of ADC. The antibody structure from computational (homology/ fragment based/ *ab initio*) modeling is a substitute for X-ray experimental modeling in the designing process of ADC. Homology method is the template (known experimental 3-D structure of reference protein) based method and there is barely any *de novo* facility to compile innate folding of protein/antibody structure. In this case, *ab initio* method helps to provide a better-folded target structure. Although, these methods hold scope for further advancement of molecular forcefields and other parameters needed to predict inherent and accurate antibody structure.

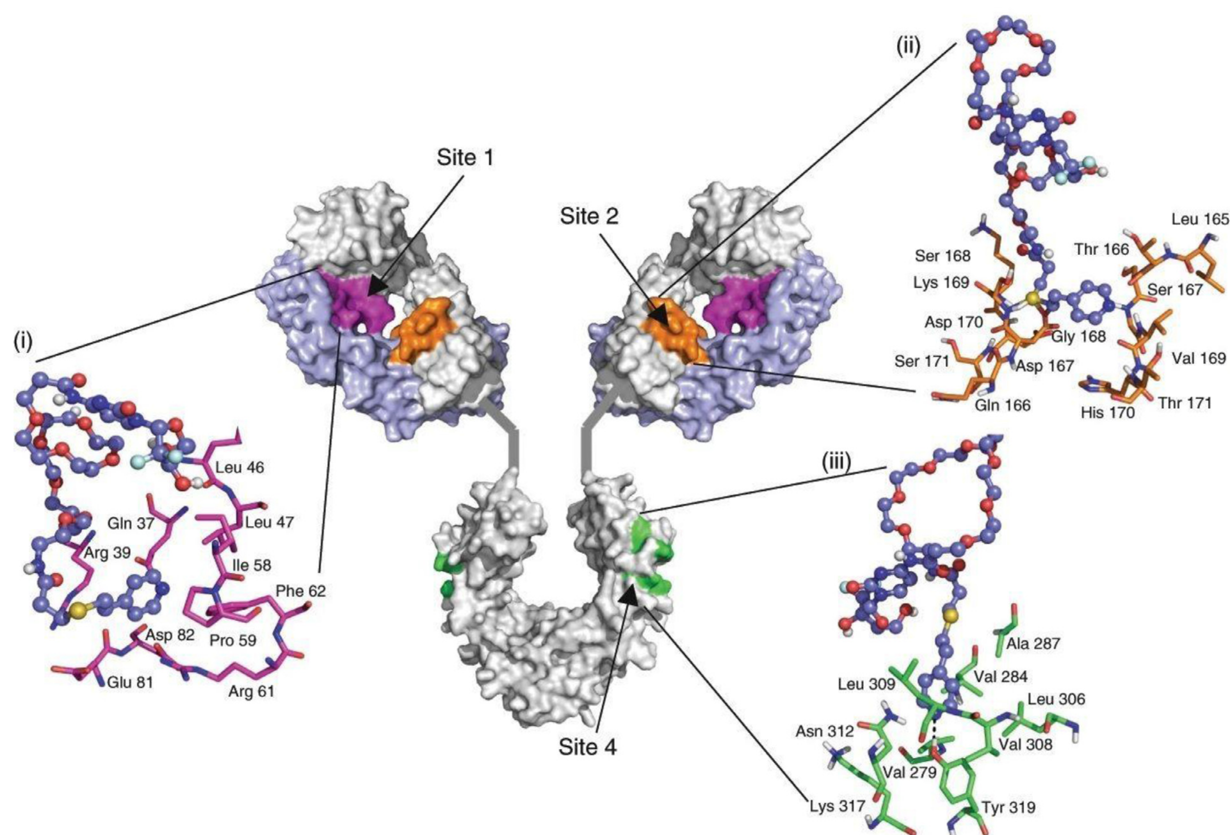

Fig. 23. Conserved binding site location of MAGNET linkers on an antibody identified by MDS. Docking conformation of MAGNET–Gem to site 1 (i; magenta) and site 2 (ii; orange) of the Ctx Fab and site 4 (iii; green) of the Fc. Amino acids(that form the binding sites shown in stick and the linker are shown in ball and stick model; MAGNET–Gem is indicated in blue [98].

Molecular docking and MDS with experimental/ modeled antibody provide more details for designing of targeted ADC. The structural conformational knowledge of each component of ADCs will be a big leap in the design of potential targeted ADC. However, MDS method also required advanced parameters for molecular force fields and physicochemical environment of selected targets (antibody/antigen/protein) in a biological system to mimic the *in situ* experimental conditions. Along with this, required high-end computational power to overcome the computational time constraints.

The aforesaid three, Antibody, linker and payload are the interlocking building blocks of ADC. The appropriate selection and binding optimization of these three is a complex job for researchers. The various available databases/tools will help directly or indirectly in design of ADCs and influence their outcome. The coalition of both *in silico* and *in situ* approaches can lower the barrier of design and discovery of ADC.

5. Concluding remarks

ADCs is a very hopeful therapeutic strategy for cancer treatment. Almost > 80 ADCs are presently under investigation and are in various stages of clinical development for cancer treatment. This article highlights the recent advancement in synthetic payloads and *in silico* techniques in maneuvering the development of ADCs. Progression in synthetic procedures of payloads provide all required information for the facile production of various potent cytotoxic payloads conjugates with different site-specific mechanism of actions. Furthermore, computational approaches and online databases/tool discussed in this review can play a major role in selection of optimum fit components of ADC (mAbs, linker, payload) for targeted cancer antigen. The shifting paradigm of conjugation techniques will help the researcher to understand the different conjugation methods and their advantages and disadvantage. We hope this review will improve the understanding and encourage the practicing of research in anticancer ADCs development.

Footnote

DAR: Drug antibody ratio; MCC: maleimidomethyl cyclohexane1-carboxylate; SPDB: N-succinimidyl-4-(2-pyridyldithio) butanoate; AcBut: 4-(4-acetylphenoxy)butanoic acid; DM1: N2′ -Deacetyl-N2′ -(3-mercapto1-oxopropyl)maytansine; DM4: N2′ -Deacetyl-N2′ -(4-mercapto-4- methyl-1-oxopentyl)maytansine); MC: Maleimidocaproyl; pAcPhe: p-acetylphenylalanine; AzK: N6-((2-azidoethoxy) carbonyl)-L-lysine; SMA: Sortase-mediated antibody conjugation; GalT: β-1,4-galactosyltransferase; SialT: α-2,6-sialyltransferase BPA: benzoylphenylalanine; EPL: Expressed protein ligation; STEPL: sortase-tag expressed protein ligation; MAGNET: multivalent and affinity-guided antibody empowerment technology; PEG: polyethylene glycol; MEP: 4-mercaptoethylpyridine

Declaration of competing interest

Authors have no potential conflict of interest to report.

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