Self-immolative Linkers in Prodrugs and Antibody Drug Conjugates in Cancer Treatment

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Abstract:
Background: The design of anticancer therapies with high anti-tumour efficacy and reduced toxicity continues to be challenging. Anti-cancer prodrug and antibody-drug-conjugate (ADC) strategies that can specifically and efficiently deliver cytotoxic compounds to cancer cells have been used to overcome some of the challenges. Key to the success for many of these strategies is a self-immolative linker, which after activation can release the drug payload. Various types of triggerable self-immolative linkers are used in prodrugs and ADCs to improve their efficacy and safety.

Objective: Numerous patents have reported the significance of self-immolative linkers in prodrugs and ADCs in cancer treatment. Based on the recent patent literature, we summarise methods for designing the site-specific activation of non-toxic prodrugs and ADCs in order to improve selectivity for killing cancer cells.

Methods: In this review, an integrated view of the potential use of prodrugs and ADCs in cancer treatment are provided. This review presents recent patents and related publications over the past ten years to 2020.

Results: The recent patent literature has been summarised for a wide variety of self-immolative PABC linkers which are cleaved by factors including responding to the difference between the extracellular and intracellular environments (pH, ROS, glutathione), by over-expressed enzymes (cathepsin, plasmin, β-glucuronidase) or bioorthogonal activation. The mechanism for self-immolation involves the linker undergoing a 1,4- or 1,6-elimination (via electron cascade) or intramolecular cyclisation to release cytotoxic drug at the targeted site.

Conclusion: This review provides the commonly used strategies from recent patent literature in the development of prodrugs based on targeted cancer therapy and antibody-drug conjugates which show promise in therapeutic applications.

Keywords: Self-immolative linkers, cancer, prodrugs, antibody-drug-conjugate, 1,4-elimination, 1,6-elimination, Intramolecular cyclisation

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1. Introduction

Cancer is an extensive group of diseases and the second leading cause of death globally after cardiovascular disease [1]. There are more than 100 different types of cancers categorized based on the type of cells or organs from which they originate. In 2018, the most frequently diagnosed cancers worldwide were lung (11.6%), breast (11.6%), colorectal (10.2%), prostate (7.1%), stomach (8.2%) and liver (8.2%) [2].

In order to comprehend mechanisms of the disease in its diverse manifestations, Hanahan and Weinberg proposed the ‘hallmarks of cancer’. They put forward eight hallmarks of cancer along with two emerging characteristics namely; sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis, resisting cell death, deregulating cellular energetics, in addition to tumour-promoting inflammation, as well as genome instability and mutation [3].

1.2 Causes of Cancer

There is no one single cause of cancer. The factors involved may be due to a genetic disorders, lifestyle-related factors such as tobacco smoke, diet, alcohol, UV radiation, environmental pollutants, infections, stress, obesity or physical inactivity [4].

1.3 Cancer treatments

Treatment depends upon the location and specific type of tumour, the stage of the disease as well as individual factors of the patient such as age and possible side effects. Surgery remains a mainstay in the cure and control of most solid tumours [5]. Marie Curie’s work has contributed significantly to the discovery and development of radiation treatment [6]. The conception of immunotherapy in medicine integrates the use of constituents of the immune system, including antibodies, cytokines and dendritic cells [7]. Photodynamic therapy involves the administration of photosensitizers, which utilize exogenously synthesised reactive oxygen species (ROS) to kill cancer cells [8]. Gene therapy aims to alleviate the disease by introducing genetically modified materials (e.g. DNA, RNA) [9]. A new and significant turn to the treatment of tumours took place in the 20th century in the form of chemotherapy, with the use of nitrogen mustards and folic acid based drugs [10]. Combined chemotherapy is a treatment strategy where more than one chemotherapy medication is used.

Despite the greater efficacy and better survival offered by chemotherapy, patients still face challenges associated with poor tumour selectivity, insufficient localised drug concentration, and high toxicity towards normal cells/tissue; including the lymphatic system, gut mucosa and bone marrow cells. Numerous drug delivery approaches have been developed to augment the efficacy e.g. nanoparticles, and reduce toxicity of existing anti-cancer agents. One promising area for improving tumour selectivity is prodrug therapy.

1.4 Prodrugs

In 1958, Adrien Albert introduced the term “prodrug” [11] meaning any compound that undergoes biotransformation before showing its pharmacological effects. Prodrugs have been reviewed in detail by others [12, 13], including a brief description of the types of prodrugs and their use in anti-cancer therapy. Over the last few years, there has been a growing development in the physicochemical, biopharmaceutical and/or pharmacokinetic properties of pharmacologically active compound by the application of prodrug strategies [14]. During the period 2008-2017, approximately 30 prodrugs were approved by the US Food and Drug Administration (FDA), which accounts for more than 12% of all small molecule new chemical entities [13].

1.5 Antibody drug conjugates
Drug discovery has generally focused on small-molecule anti-cancer chemotherapeutic drugs, but these can cause significant side effects such as toxicity, drug resistance and lack of selectivity against cancer cells. Antibody drug conjugates (ADCs) are an emergent class of therapies for targeted drug delivery treatment, and consist of monoclonal antibodies (mAbs) attached to biologically active cytotoxic agents through chemical linkers [15]. At the time of collating this review, there have been nine ADCs approved by the FDA namely trastuzumab deruxtecan (Enhertu®) [16], gemtuzumab ozogamicin (Mylotarg®), brentuximab vedotin (Adcetris®), trastuzumab emtansine (Kadcyla®), inotuzumab ozogamicin (Besponsa®) [17], polatuzumab vedotin-piiq (Polivy®) [18], enfortumab vedotin (Padcev®) [19], sacituzumab govitecan (Trodelvy®) [20] and belantamab mafodotin (Blenrep®) [21].

1.6 Linker design

The linker system plays a significant role in both prodrug and ADC design, where the linker connects the cytotoxic drug to the antibody or an activatable moiety [22-25]. The linker must be stable in the blood stream to avoid release of the cytotoxic drug prior to reaching the target site. Upon reaching the target cells, the linker must possess the property of releasing the drug in its active form via a specific trigger or during lysosomal degradation of the ADCs. The drug-to-antibody ratio, the number of drug molecules attached per antibody via a linker, plays an important role in stability and homogeneity of ADCs [26].

1.7 Self-immolative linkers

The para-aminobenzyloxycarbonyl (PABC) type self-immolative linker was introduced in 1981 by Katzellenbogan to resolve problems in prodrug design [27]. Since the first report, this and other self-immolative linkers have found widespread applications in the fields of prodrugs [28], antibody drug conjugates [29], drug delivery systems [30], imaging probes [31], sensors for chemicals or enzymes [32, 33], and materials science [34]. Self-immolative prodrugs consist of three components, a biologically active molecule (drug), the self-immolative linker, and a reactive trigger group that responds to a specific stimulus (e.g. nitro, amide, azide). Upon application of the specific stimulus, the covalent bond between the trigger and the self-immolative linker undergoes a cascade of disassembly reactions ultimately leading to release of the active molecule [35, 36]. From a mechanistic point of view, after the stimulus activates the trigger group, drug or payload release is thermodynamically driven and takes place via either an electron cascade for PABC-type linkers (1,4-, 1,6-, or 1,8-elimination) or an intramolecular nucleophilic cyclisation (Fig. 1) [36, 37].
Figure 1 Common types of self-immolative/elimination linkers that release payload (drug) following a PABC-type electron cascade mechanism (A-C) and a cyclisation-mediated mechanism (D-E). X = Electron-donating (A-C) or nucleophilic (E) substituent.

Drug triggering and subsequent self-immolative release, can be carried out by a number of methods including:

1. enzymatic cleavage utilising enzymes over-expressed in tumours, such as cathepsins and plasmin (which produce a newly formed amine) as well as β-glucuronidase [28],
2. enzymatic reduction of nitro substituents [38],
3. reduction of disulphide bonds by the action of glutathione or another thiol reducing agents [39],
4. oxidation of aryl boronic acid/esters [40], and
5. bioorthogonal activation strategies such as the tetrazine/trans-cyclooctene and azide/trans-cyclooctene reactions [41].

2. Patents on self-immolative linkers in cancer prodrugs

As highlighted above, self-immolative linkers have proven very useful across a number of fields, but the most significant use has been in the design of stimuli-responsive prodrug and ADC activation [42, 43].

Kratz, Warnecke and others (KTB Tumorforschungsgesellschaft mbH, Germany) have produced numerous patents covering different self-immolative cancer prodrugs. Kratz et al initially reported prodrugs comprising a dendritic polyglycerol core with a polyethylene glycol (PG) shell attached to the cytotoxic agents through a linker (Fig. 2). Cathepsin B releases the drug-PABC conjugate via amide cleavage, and a 1,6-self -
immolation of the PABC linker leads to release of free drug (e.g. Doxorubicin). Cytotoxicity studies using the prodrug were carried out on triple-negative breast cancer cells MDA-MB231 and pancreatic tumour cells AsPC1 LN and produced IC₅₀ values of 0.50 and 1.80 µM respectively for a 10 kDa dendrimer compared with the doxorubicin control giving IC₅₀ values of 0.14 and 0.36 µM respectively [44].

![Figure 2: Prodrug containing thiolated polyglycerolamine (PG) linked to the 6-maleimidopropyl-Phe-Lys-PABC-Doxorubicin [44].](image)

Kratz et al (KTB Tumorforschungsgesellschaft mbH, Germany) also synthesised a prodrug cleavable by prostate-specific antigen (PSA, a serine protease) (Fig. 3). PSA-mediated cleavage initiates a 1,6-elimination via the PABC linker. An albumin-bound form of the prodrugs showed the albumin-conjugates were hydrolysed efficiently within 3 hours releasing the free drug in LNCaP prostate tumour tissue homogenates [45, 46].

![Figure 3: A general formula for prodrugs comprising a protein binding moiety (R, e.g. maleimide), a peptide sequence, a p-aminobenzylxoycarbonyl (PABC) self-immolative linker and cytotoxic agent [45, 46]. Cytotoxic](image)
drugs used included doxorubicin and paclitaxel. The mechanism of 1,6-elimination via the p-aminobenzylloxycarbonyl linker which releases carbon dioxide along with the cytotoxic drug is shown above (in Figure 2).

Kratz et al (KTB Tumorforschungsgesellschaft mbH, Germany) went on to design a range of the bisphosphonate prodrugs incorporating a cathepsin B cleavable peptide with a PABC linker or an acid sensitive hydrazone bond used for the treatment of bone related cancer. Binding studies were performed on these prodrugs (Fig. 4) at pH 7.4 and the percentage bound to native bone was found to be 50-80% after several hours [47, 48]. Part of the work was published [49].

![Diagram of 1,6-Elimination Mechanism](image_url)

**Figure 4:** Bisphosphonate prodrugs were cleaved under acidic conditions (top – no self-immolation) or by cathepsin B with PABC 1,6-elimination to release the anti-cancer drug doxorubicin [47, 48].

Warnecke and Müller (also of KTB Tumorforschungsgesellschaft mbH, Germany) developed compounds with an imine as an acid-labile trigger group attached through double PABC linkers to a drug, where the imine bond is cleaved at acidic pH (Fig. 5). Half-lives of imine hydrolysis were determined by measuring the decrease of the concentration of the imine and the increase of the concentration of the hydrolysis products by fluorescence methods, HPLC, infrared spectroscopy and NMR spectroscopy with compounds showing minimum half-lives of 20 hours at pH 7.4 [50].
Figure 5: Aryl imine prodrugs that release the cytotoxic drugs (e.g. 5-fluorouracil and 6-mercaptopurine) at pH 5 [50]. The mechanism of elimination following acid-hydrolysis involves an initial 1,6-elimination revealing a second free aniline primed for an additional 1,6-elimination. In the case of 5-fluorouracil, an additional self-immolation would be required to release the parent drug (shown in box).

Leamon et al (Endocyte, USA, founded in the Purdue Research Park, acquired by Novartis), prepared a range of anti-folate conjugates that bind to the folate receptor (Fig. 6). A series of folate analogues were evaluated using a folate receptor positive KB (cervical carcinoma (HeLa derivative)) cell assay for their ability to compete with folate and the relative binding affinity was determined at 37°C with vinblastine and mitomycin C derivatives showing relative affinities of 0.47 and 0.59 respectively [51].

Figure 6: Anti-folate conjugates cleaved by an exogenous or endogenous nucleophile (glutathione or a bioreducing agent) releasing the anti-cancer drug (e.g. vinblastine) via a cyclisation-mediated elimination step. Other drugs reported by the inventors include mitomycin C [51].
Cohen et al (The Regents of the University of California, USA) reported prodrugs that include an anticancer moiety covalently linked to an oxidation sensitive self-immolative linker (Figure 7A) [52]. The prodrugs include a method of inhibiting the metalloprotein MMP-12 which has been found to be overexpressed in several different tumour types including non-small cell lung cancer, skin cancer, ovarian cancer, and pancreatic cancer [53]. The metal binding moiety (e.g. 1-hydroxypyridin-2(1H)-one; 1,2-HOPO-2) is formed from the reaction of the oxidation-sensitive prodrug and a ROS, e.g. H₂O₂ (Fig. 7B). The boronic ester is oxidised to the phenol followed by 1,6-elimination/immolation (Fig. 7B). IC₅₀ values of 17.8 and 12.9 µM for “proinhibitors” and 0.053 and 0.035 µM for inhibitors 1,2-HOPO-2 and PY-2 respectively were reported based on a fluorescence assay against MMP-12 [52, 54]. Compounds 1,2-HOPO and PY-2 were originally developed for ischemia–reperfusion injury (following stroke), the approach first described by Cohen et al. [54]. There has since been significant efforts to develop the arylboronic ester self-immolative prodrugs for cancer [55].

![Figure 7](https://example.com/figure7.png)

**Figure 7:** A) Arylboronic ester prodrugs of 1,2-HOPO-2 and PY-2 designed for reaction with reactive oxygen species (ROS) [52]. B) Activation of the prodrug with H₂O₂ (ROS), releasing the matrix metalloprotease inhibitor hydroxypyridinone (1,2-HOPO-2).

Bierbach and Ding (Wake Forest University, USA) reported dual action acridine cisplatin compounds that have shown superior efficacy over standard cisplatin compounds (Fig. 8). These compounds have shown enhanced cytotoxicity in several different types of cancers including NCI-H460 non-small cell lung cancer (NSCLC) cells, leukaemia, and effective tumour growth inhibition in H460 xenograft models. The compounds showed promising cytotoxic activity in a cell viability assay (IC₅₀= 0.026 – 0.35 µM against NCI-H460 cells) [56]. Parts of this work have also been published [57].
Figure 8: pH-Sensitive release of acridine cisplatin compounds where R is a targeting moiety e.g. kinase inhibitor [56].

Robillard et al (Tagworks Pharmaceuticals B.V., Netherlands) designed a drug release mechanism via bioorthogonal activation using an inverse-electron-demand Diels-Alder (IEDDA) reaction. The prodrug contains a dienophile (trans-cyclooctene) that masks the drug (doxorubicin). The drug is released upon activation by a diene (tetrazine) via a 1,4-elimination (Fig. 9) [58, 59]. The trigger and the activator undergo a fast, bioorthogonal reaction resulting in the release of the drug within 4 h. The release of drug from axial trans-cyclooctene–doxorubicin [60] was measured using LCMS with results showing up to 75% release in serum [59].

Figure 9: Tetrazine-induced release of doxorubicin from trans-cyclooctene-doxorubicin prodrug [58, 60].

Papot et al (Centre Nationale de Recherche Scientifique and Universite de Poitiers, France) prepared a self-immolative linker based on PABC that is useful for the preparation of novel anti-cancer prodrugs, including dendrimers, capable of targeting tumour cells in vivo via folate receptors (Fig. 10). The cytotoxic agent, e.g. doxorubicin, was attached at the benzylic hydroxyl group via a carbamate bond along with a folate moiety joined
via a PEG linker. Cleavage of a terminal carbohydrate by β-glucuronidase produced the active phenol which undergoes 1,6-elimination. The cytotoxicity of dendritic (2 molecules doxorubicin to one folate) prodrugs was close to that of doxorubicin (range of 0.1 < IC₅₀ < 0.15 µM against LAM type KG1 leukemia cells) [61].

Figure 10: A general formula (box) for the doxorubicin prodrug with a self-immolative linker. Targeted anti-cancer prodrug shown with doxorubicin and folate targeting moiety. Activation was via β-glucuronidase cleavage of the terminal carbohydrate [61].

Prijovic et al (Academia Sinica, Taipei, Taiwan) reported a second-generation camptothecin glucuronide prodrug (BQC-G) that showed better aqueous solubility than a camptothecin derivative of 5,6-dihydro-4H-benzo(de)quinolone-camptothecin (BQC) (Fig. 11). Cytotoxicity results were good (low nM range) against a number of cell lines in the absence of human serum albumin and were improved when activated with β-glucuronidase [IC₅₀ = 1.33 nM (CL1-5 human lung cancer cells); IC₅₀ = 1.35 nM (LS174T colon cancer cells); IC₅₀ = 1.44 nM (CaSki cervical carcinoma cells); IC₅₀ = 2.0 nM (EJ human bladder cancer cells)]. BQC-G when activated with β-glucuronidase followed by 1,6-elimination exhibited a remarkable cytotoxicity (IC₅₀ = 13.3 nM against CL1-5 cells) whereas in the presence of human serum albumin without β-glucuronidase showed lower cytotoxicity (IC₅₀ = 1080 nM) [62, 63].

Figure 11: BQC-G, a potent (IC₅₀ = 13.3 nM) tumour selective anti-cancer prodrug [62, 63].
Beria et al (Nerviano Medical Sciences S.R.L., Nerviano, Italy) have developed new functionalized thieno-indole derivatives based around duocarmycins (minor groove binding alkylating agents) that are potent against cellular proliferation disorders (Fig. 12). Their patent covers self-immolative groups including the PABC linker. In vitro cell proliferation assays were performed on the human ovarian cancer cell line A2780 and breast cancer cell line MCG7 with all compounds showing IC<sub>50</sub> < 0.5 µM [64].

![Chemical structure of functionalized thieno-indole derivatives](image)

**Figure 12:** Example structure of the functionalized thieno-indole derivatives with an enzyme activated PABC self-immolative linker [64].

Kim et al (Pharosgen Co, Ltd, Seoul, Korea) designed a series of prodrug conjugates that induce and amplify apoptosis. Upon activation of the prodrug by caspase and 1,6-elimination via a PABC linker, the cytotoxic drug (e.g. doxorubicin) was released. Cleavage studies were performed using recombinant human caspase-3 and monitored by HPLC (Table 1) [65]. A human serum albumin conjugate (Maleimide-KGDEVD-PABC-Doxorubicin) showed no noticeable cytotoxic effects up to 100 µM. However, when activated with caspase-3, it showed a similar degree of cytotoxicity to free doxorubicin against mouse squamous cell carcinoma SCC7 cells and MDA-MB-231 triple negative breast cancer cells.

**Table 1:** Chemotherapeutic prodrug conjugates comprising the caspase cleavable peptides, attached to cytotoxic drugs through a PABC linker [65].

<table>
<thead>
<tr>
<th>Example number</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maleimide-KGDEVD-PABC-Doxorubicin</td>
</tr>
<tr>
<td>2</td>
<td>Maleimide-KGDEVD-PABC-Daunorubicin</td>
</tr>
<tr>
<td>3</td>
<td>Maleimide-KGDEVD-PABC-Paclitaxel</td>
</tr>
<tr>
<td>4</td>
<td>Maleimide-KGDEVD-PABC-MMAE</td>
</tr>
<tr>
<td>5</td>
<td>Maleimide-DEV-D-PABC-Doxorubicin</td>
</tr>
<tr>
<td>6</td>
<td>Maleimide-DIED-PABC-Doxorubicin</td>
</tr>
<tr>
<td>7</td>
<td>Maleimide-DLVD-PABC-Doxorubicin</td>
</tr>
<tr>
<td>10</td>
<td>Pyridyldithiol-KGDEVD-PABC-Doxorubicin</td>
</tr>
<tr>
<td>11</td>
<td>Oleate-KGDEVD-PABC-Doxorubicin</td>
</tr>
</tbody>
</table>
3. Patents on self-immolative linkers in antibody drug conjugates

Antibody-drug conjugates have proven to be an effective method of selectively delivering a small cytotoxic payload to a targeted cell [22-24]. The incorporation of self-immolative linkers connected to the ADCs have proven to be an effective method to selectively deliver cytotoxic agents to these targeted cells.

Feng (Seattle Genetics, Inc., Bothell, WA, USA) reported on ADCs incorporating a cathepsin B peptide substrate along with heterocyclic self-immolative linkers fused to anti-cancer drugs containing amine, hydroxyl, sulphhydryl or carboxyl functional groups (Fig. 13) [66]. The heterocyclic linkers were designed to improve properties of drug-ligand conjugates by optimising the structure of the linker to overcome possible poor solubility, aggregation and optimise enzymatic substrate recognition. The detailed activity data was not available in the published patent.
Figure 13: ADC conjugate exposed to cathepsin B, resulting in cleavage at the position shown and release of the amine-containing drug (e.g. mitomycin C, doxorubicin) after 1,6-self-immolation from the amino-thiazole [66].

Beria et al (Genentech, Inc., South San Francisco, CA USA) reported the synthesis of numerous ADCs (e.g. Fig. 14) that consist of the antibody linked to a cathepsin B substrate, a PABC linker and the anti-cancer drug. The average drug/antibody ratio (DAR) of the synthesised compounds was found to be 1.25 – 3.4 [67]. Many compounds showed IC\textsubscript{50} < 0.1 µg/ml against the human breast cancer cell lines SK-BR-3, BT-474, MCF7 and doxorubicin-resistant Her2. The doxorubicin ADC in (Fig. 14) is first hydrolysed by cathepsin B, followed by two self-immolative events; a 1,6-self-immolation of the PABC group and then an intramolecular cyclisation to release the drug.

Boyd et al (Medarex, Inc., USA) designed ADCs where the drug and the antibody were linked via a peptidyl hydrazine linker that following acid-catalysed hydrolysis of the hydrazine, led to a cyclisation mediated self-immolation of the active drug (Fig. 15). The proliferation assay was performed on promyelocytic leukemia HL-60 cells, where compounds had IC\textsubscript{50} values in the range of 1 pM – 100 nM [68].
Figure 15: (A) Duocarmycin ADC examples with hydrazine linkers that undergo cyclisation mediated self-immolation via 5- or 6-membered rings [68]. ADC 1 (top) is designed to undergo a double cyclisation mediated self-immolation releasing two 5-membered rings while ADC 2 is designed to undergo a single self-immolative cyclisation to release a 6-membered ring. (B) An example self-immolative event for ADC 2 that releases the active drug and a 6-membered ring as by-product (mechanism shown following hydrolysis of hydrazine).

Dushin et al (Pfizer Inc., USA) submitted a patent application describing the synthesis of ADCs with an enhanced pharmacokinetic profile for the treatment of cancer. The antibody anti-5T4 was linked via the non-natural amino acid p-acetyl-L-phenylalanine along with a protease cleavage sequence to the drug dolastatin via the self-immolative PABC linker (Fig. 16). A cytotoxicity assay of compounds was performed on 5T4 expressing cell lines with an IC₅₀ range of 16 – 56 (Ab ng/mL) against MDA-MB-435/5T4 breast cancer cells expressing a high level of the 5T4 antigen [69]. Part of the work was published [70].
Lin et al (BioAlliance C.V., Netherlands, and Abgenomics International Inc, USA) designed ADCs in which a hydrophilic self-immolative linker (PABC) which undergoes 1,6-elimination, was attached to a cleavable peptide linker and an anti-cancer drug; e.g. dolastatin or derivatives of duocarmycins (Fig. 17). In vitro cytotoxicity activity of the antibodies c5D7 and h5F1Ca.1 functionalised with the compounds were tested in DLD-1 and COLO 205 human colorectal cancer cells, human gastric cancer SNU-16 cells, Panc02.03B pancreatic cancer cells and the % inhibition were found to be 62% (0.3 µg/mL; DLD-1) – 98% (3 µg/mL; SNU-16) [71, 72].

Morrison et al (Agensys, Inc. and Seattle Genetics, Inc., USA) synthesised ADCs consisting of the antibody 158P1D7 attached to a cleavable peptide linker incorporating a 1,6-self-immolative PABC linker and an anti-cancer drug, e.g. auristatin (Fig. 18). In vitro cytotoxicity of the compound was evaluated on the neuroblastoma CHP-212 cell line with an IC₅₀ of 0.91 nM [73].
Lyon et al (Seattle Genetics, Inc., USA) developed ADCs that can be triggered via β-glucuronidase cleavage releasing active drug following the 1,6-self-immolation of a PABC moiety (Fig. 19). They found that the positioning of a PEG unit (designed to mask the hydrophobicity of the drug) parallel to the drug improves the pharmacokinetic properties of the conjugates (Fig. 19B) [74]. In vitro cytotoxicity assays were carried out with CD30⁺ Lymphoma cell lines (Karpas299, L540cy and L428) resulting in IC₅₀ values of 0.3 – 0.4 ng/mL (Karpas299) and 2 – 8 ng/ml for the other two cell lines. Part of the work was published by the authors in 2017 [75].

Figure 18: ADC conjugate reported by Morrison et al. that releases the drug MMAE (monomethyl auristatin E) upon endogenous peptide cleavage and subsequent 1,6-self-immolation [73].

Figure 19: ADC conjugates reported by Lyon et al [74]. MMAE conjugated to the antibody and linker is released upon internalization into the cells, whereby glucuronidase hydrolysis of the carbohydrate unit exposes the PABC-
type linker. Note that in this ADC, a slight variation on the PABC linker was used whereby the hydrolysis of the glucuronide revealed a phenolic/hydroxyl group instead of an aniline/amine group.

Jeffrey et al (Seattle Genetics, Inc, USA) produced ADCs consisting of a cAC10 antibody linked to a drug via a β-glucuronide terminated linker containing the 1,6-self-immolating group that is triggered via β-glucuronidase (Fig. 20). In vitro cytotoxicity assays for the ADCs targeting the CD70 antigen resulted in IC₅₀s of 0.08 – 2.7 nM on renal cell carcinoma lines Caki-1 and 786-O (CD70⁺, CD30⁻), and IC₅₀s of 0.05 – 1.2 nM on targeting the CD30 antigen with the lymphoma Karpas 299 (CD30⁺, CD70⁻) cell line with the active drugs auristatin E, F and doxorubicin. The β-glucuronide-based ADCs displayed improved serum stability compared to similar disulphide and hydrazone-based systems [76]. Part of the work was published [77, 78].

An et al (Newbio Therapeutics, Inc., China) reported ADCs comprising an antibody attached to a tridentate linker (trisubstituted phenyl ring), further conjugated to a peptide-PABC-drug conjugate (e.g. auristatin) (Fig. 21). The antigen Her2 binding showed strong affinity with different ADC-drug combinations EC₅₀ 23.3 – 95.5 ng/mL compared with Herceptin alone (EC₅₀ = 32.5 ng/mL). The potency of the ADC-drug combinations was measured by cell proliferation inhibition assay on SK-BR-3 human breast cancer cell line (IC₅₀ = 6.7 – 15.2 ng/mL). One ADC had an IC₅₀ outside of this range at a higher concentration of 81.1 ng/mL but this lacked the peptide-PABC linker [79, 80].

Figure 20: Targeted delivery of anti-cancer drug (e.g. doxorubicin) was achieved upon activation of the ADCs by β-glucuronidase triggered self-immolation [76, 78].
Han et al (Newbio Therapeutics, Inc., China) synthesised a series of trimaleimide-linked ADCs of different sizes to study the effects on their pharmacokinetic and toxicity properties as well as their potency (Fig. 22). The trimaleimide linker was attached to a valine-citrulline enzyme cleavage site, followed by a PABC self-immolating linker designed to release various cytotoxic agents e.g. MMAE (monomethyl auristatin E). The synthesised ADCs exhibited drug/antibody ratio (DAR) in the range of 2 – 4. A cell proliferation assay using SK-BR-3 breast cancer cell lines resulted in IC50 values in the range of 5.7 – 11.0 ng/mL [81, 82].

Steinkuhler et al (Exiris S. R. L., Roma, Italy) reported ADCs comprising an antibody attached to peptide linkers and the drug cryptophycin (Fig. 23). The conjugates were activated proteolytically releasing diketopiperazine and the cryptophycin derivative. Cryptophycin-52 passed phase I clinical studies but failed phase II studies as it lacked efficacy in vivo, had high neurotoxicity, and dose-limiting toxicity at the selected doses. However when incorporated into an ADC, the cytotoxicity of the cryptophycin-55 derivative against small cell lung cancer cell lines H69 and H69Ar (adriamycin resistant) was improved with IC50 values as low as 0.09 nM (against H69) and 0.15 nM (against H69Ar) [83, 84]. Release of active drug from the ADC occurs via self-immolative cyclisation of a diglycine linker (forming diketopiperazine) or a 1,6-self-immolation via the PABC unit.

**Figure 21:** Attachment of the tridentate linker (left-hand side aromatic ring) to the antibody and the drug with a PABC-based self-immolative linker [79, 80].

**Figure 22:** Generic template of ADCs comprising the trimaleimide linker with variation in both maleimide linker length (n) and extension arm (X; different functional groups and chain length), valine – citrulline – PABC linker and the cytotoxic drug e.g. MMAE [81, 82].

**Figure 23:**
Figure 23: Upon proteolytic activation the ADCs releases the anti-cancer drug candidate cryptophycin via (A) cyclisation-mediated self-immolation or (B) 1,6-self-immolation [83, 84].

Miao et al (Sorrento Therapeutics, Inc., San Diego, CA, USA) developed ADCs (Fig. 24) that differed from those above in their use of pyridine and tetrazole heterocycles as a connector to the antibody. Several examples included the PABC linker. Compounds were tested against a range of cell types and were most active against the SBKR3 Her2+++ human breast cancer cell line with EC50 values of 0.022 and 0.031 nM (Fig. 24A and 24B respectively) [85].
Van Berkel et al (ADC Therapeutics S.A., China and Medimmune Limited, Cambridge Great Britain) reported ADCs with pyrrolobenzodiazepines (PBDs) and the antibody CD25. In these conjugates the drug was linked to the antibody via a peptide linker and a self-immolative group e.g. PABC (Fig. 25). *In vitro* cytotoxicity of the compounds was examined with lymphoma cell lines SU-DHL-1, DAUDI and Karpas299 with EC₅₀ values in the range of 0.0002 – 0.001, 0.11 – 0.27 and 0.0007 – 0.003 µg/mL, respectively. The DAR of the ADCs was found to be in the range of 1.25 – 2.19 [86].

*Figure 24:* ADCs incorporating nitrogen heterocyclic connectors with PABC as the eliminating moiety releasing a Nemorubicin derivative [85].
Dragovich et al (Genentech, Inc, CA, USA) developed pyrrolobenzodiazepine (PBD)-based ADCs whereby a peptide linker between the antibody and the drug (monomer or dimer) was incorporated with a disulphide or PABC linker. The disulphide ADCs were activated by glutathione reduction, while the PABC ADCs were activated proteolytically followed by a 1,6-self-immolation to release active drug (Fig. 26). The cytotoxicity of the synthesised compounds was evaluated against KPL-4 (best IC₅₀ = 5.0 ng/mL), SK-BR-3 (best IC₅₀ = 7.0 ng/mL), WSU-DLCL 2 (best IC₅₀ = 8.56 nM) tumour cells and resulted in EC₅₀s of 1.70 nM or lower [87, 88]. Part of the work was published and showed improved activity [89, 90].

**Figure 25:** Activation of the ADCs was performed by cathepsin to release the anti-cancer PBDs [86].
Figure 26: Anti-cancer PBD dimers are initially released via proteolytic hydrolysis releasing the boronic ester and disulphide prodrug derivatives, which are activated by glutathione and reactive oxygen species [87, 88].

Robillard et al (Tagworks Pharmaceuticals B.V., Netherlands) have produced a number of patents on ADCs that are activated by the IEDDA reaction (see also Fig. 9 for prodrug example) [91-93]. For example, they have produced ADCs consisting of the TAG72 diabody (CC49) attached to a linker comprising a trans-cyclooctene (TCO) and the drug e.g. MMAE or doxorubicin (Fig. 27 shows MMAE ADC). Activation of the TCO leads to release of drug via a 1,4-elimination [94], and more recently examples of drug attached to the tetrazine followed by addition of TCO as the activator have been reported [60]. The group have also demonstrated activation with a tetrazine in which the TCO was released from the ADC via a 1,4-elimination followed by a second 1,6-self-immolation to release the cytotoxic drug (Fig. 27) [91]. The cell proliferation assay was performed on a doxorubicin prodrug and tetrazine using carcinoma cells A431, with IC₅₀ values of 0.137 – 0.278 µM [91].
In a later publication, a CC49-MMAE ADC was activated *in vitro* and *in vivo*, providing potent pM cytotoxicity [94].

**Figure 27:** MMAE ADC conjugate and activation method reported by Robillard et al [91]. Upon activation with a tetrazine, TCO triggers the release of anticancer drug auristatin E.

Agatsuma *et al* (Daiichi Sankyo Company, Limited, Tokyo, Japan and Sapporo Medical University, Sapporo-Shi, Hokkaido, Japan) reported ADCs incorporating antibodies (hRS7 and hTINA1) covalently attached to a peptide linker and the drug (Fig. 28). They contain cleavable peptide spacer which triggers a cyclisation to release the cytotoxic agent e.g. exatecan. The ADCs exhibited a cell growth inhibitory effect value of GI₅₀ <1 nM on TROP2 antigen-positive cell lines BxPC3, NCI-H292, NIH:OVCAR-3, CFPAC-1, FaDu, Calu-3 and CaOV3. On the other hand no cell growth inhibitory effect >100 nM was observed on the TROP2 antigen-negative cell lines Calu-6 and A375 respectively [95].
Figure 28: ADCs that are covalently attached to the peptide linker masking the self-immolating group and anti-cancer drug exatecan [95].

4. Conclusion

Self-immolative linkers in prodrugs and antibody drug conjugates have been intensively studied and it is well established that these show great potential for cancer treatment. The PABC linkers have huge potential for anti-cancer therapy. They have exhibited greater systemic stability over other cleavable linkers and are favoured among the prodrugs and ADCs that are currently in clinical trials [96]. Of the nine FDA approved ADCs, three of them (brentuximab vedotin, polatuzumab vedotin, enfortumab vedotin) contain the PABC linker [97]. Our review attempts to describe the application of linkers in prodrugs as well as antibody drug conjugates that are cleaved/activated in the cancer cells specifically by cathepsins, ROS, glutathione, β-glucuronidase, prostate specific antigen, caspases and acid catalysed hydrolysis to release cytotoxic drugs at the targeted cell. Although extraordinary progress has been achieved in the design and development of these prodrugs, some of the problems such as immunogenicity, gene expression, toxicity, and poor pharmacokinetics must be addressed in the new drug applications.

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