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Review

Recent advances in tumor-targeting anticancer drug conjugates

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This article is dedicated to Professor Koji Nakanishi for his truly outstanding contributions to organic, bioorganic, and natural products' chemistry on the occasion of his receiving the 2004 Tetrahedron Prize and his 80th birthday.

Abstract—Traditional cancer chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing severe undesirable side effects. Therefore, various drug delivery protocols and systems have been explored in the last three decades. Tumor cells overexpress many receptors and biomarkers, which can be used as targets to deliver cytotoxic agents into tumors. In general, a tumor-targeting drug delivery system consists of a tumor recognition moiety and a cytotoxic warhead connected directly or through a suitable linker to form a conjugate. The conjugate, which can be regarded as 'prodrug', should be systemically non-toxic. This means that the linker must be stable in circulation. Upon internalization into the cancer cell the conjugate should be readily cleaved to regenerate the active cytotoxic agent. Tumor-targeting conjugates bearing cytotoxic agents can be classified into several groups based on the type of cancer recognition moieties. This review describes recent advances in tumor-targeting drug conjugates including monoclonal antibodies, polyunsaturated fatty acids, folic acid, hyaluronic acid, and oligopeptides as tumor-targeting moieties.

Contents

1. Introduction 5043
2. Monoclonal antibody-based tumor-targeting 5044
3. Tumor-targeting with polyunsaturated fatty acids 5046
4. Tumor-targeting with folic acid 5047
5. Tumor-targeting with hyaluronic acid 5048
6. Tumor-targeting with peptides. 5049
7. Conclusion 5051
References and notes 5051

1. Introduction

Cancer is the second major cause of death in the U.S. Despite the significant progress in the development of anticancer technology, there is still no common cure for patients with malignant diseases. In addition, the long-standing problem of chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects such as hair loss, damages to liver, kidney, and bone marrow. Therefore, various drug delivery protocols and systems have been explored in the last three decades.

In general, a tumor-targeting drug delivery system consists of a tumor recognition moiety and a cytotoxic

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warhead connected directly or through a suitable linker to form a conjugate. The conjugate, which can be regarded as 'prodrug', should be systemically non-toxic. This means that the linker must be stable in circulation. Upon internalization into the cancer cell the conjugate should be readily cleaved to regenerate the active cytotoxic agent.

To achieve effective tumor-specific drug delivery, it is important to recognize the morphological and physiological differences between malignant and normal tissues. Rapidly growing cancer cells require quick formation of new blood vessels. The tumor vasculature has many defects, which allows large molecules and lipids to easily enter the extravascular space in tumors. On the other hand, the lymphatic drainage in cancer cells is undeveloped so that the large molecules and lipids cannot be released from the tumor. This phenomenon, termed 'enhanced permeability and retention' (EPR) effect, was first described by Maeda et al. in 1986.[1,2] Since the accumulation of macromolecules, including plasma proteins in tumors, does not require a specific receptor, the EPR effect is passive in nature.

The main physiological characteristic of cancer cells is their enhanced metabolic rate, which causes hypoxicity. This induces anaerobic metabolism, resulting in the formation of lactate and consequently lowering the intracellular pH.[3,4] For this reason, various conjugates have been designed to release the cytotoxic agent upon acidification. A rapidly growing tumor requires various nutrients and vitamins. Therefore, tumor cells overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors. Tumor-targeting conjugates bearing cytotoxic agents can be classified into several groups based on the type of cancer recognition moieties. Some of these conjugates have been receiving increased attention for efficacious cancer chemotherapy. This review describes recent advances in tumor-targeting drug conjugates including monoclonal antibodies, polyunsaturated fatty acids, folic acid, hyaluronic acid, and oligopeptides as tumor-targeting moieties.

2. Monoclonal antibody-based tumor-targeting

The discovery of antigens that are particularly overexpressed on the surface of cancer cells suggests that by using certain antibodies (mAbs) to selectively 'mark' tumor cells, malignant tissues could be distinguished from normal tissues.[5] mAbs, which have shown high binding specificity to tumor-specific antigens, could fulfill this task. These mAbs could be used as vehicles to deliver cytotoxic drugs selectively to tumor cells.[5–7] The mAb moiety then binds to the antigens on cancer cells and the conjugate is internalized via receptor-mediated endocytosis followed by release of the parent drug to restore its original activity.

The concept of mAb–drug conjugates for treatment of malignant diseases can be traced back to early 1900s when Ehrlich suggested the use of an antibody conjugated to diphtheria toxin.[8,9] This attempt was not successful due to the technical difficulty in obtaining appropriate antibodies. In 1975, Kohler and Milstein[10] described their pioneering work on monoclonal antibody by hydridoma technology. This discovery substantially accelerated the progress in mAb-based anticancer therapy. In 2000, Mylotarg® (gemtuzumab-ozogamicin)[11] was approved by the Food and Drug Administration (FDA) for the treatment of acute myelogenous leukemia (AML), providing the first mAb–drug immunoconjugate for the treatment of cancer in clinic. Several other mAb–drug conjugates, including maytansinoid-bearing huC242-DM1,[5] huN901-DM1,[12] MLN2704-DM1,[12] herceptin-DM1,[12] anti-CD44v6 antibody-DM1,[12] BR96–doxorubicin[13] and, CTM01-calicheamicin,[14,15] are currently in the human clinical trials.

The efficacy of mAb–drug immunoconjugates as chemotherapeutic drugs heavily depends on the tumor specificity of mAb, the potency of the cytotoxic agent, and the efficiency of the linker, which connects a mAb to a warhead. A mAb (150 kDA) has a characteristic general structure, consisting of two light chains (25 kDa each) and two heavy chains (50 kDa each) that are linked by disulfide bonds (Fig. 1). The high molecular weight of mAb–drug immunoconjugates might have an additional benefit by bringing in the EPR effect. Early mAb–drug conjugates used monoclonal antibodies derived from murine hybridomas. Unfortunately, the therapeutic effects were severely impaired due to the human anti-mouse antibody (HAMA) response, resulting in the rapid clearance of the immunoconjugates from the bloodstream. Consequently, a recombinant DNA protocol was developed, which produced generations of chimeric and humanized mAbs with decreased immunogenicity.[16] Humanized mAbs are generated by grafting the complementarity determining region (CDR) from a mouse mAb into a human immunoglobulin G (IgG). The binding affinity of the murine CDR in the humanized mAb can be fully preserved.[17] The large molecular size of mAb–drug immunoconjugates often results in poor penetration into solid tumors. To overcome this problem, truncated mAb fragments have been prepared and examined for their efficacy in immunoconjugates. It has also been shown that a truncated mAb can penetrate tumors much faster than the original mAb, but it is cleared in circulation more rapidly by

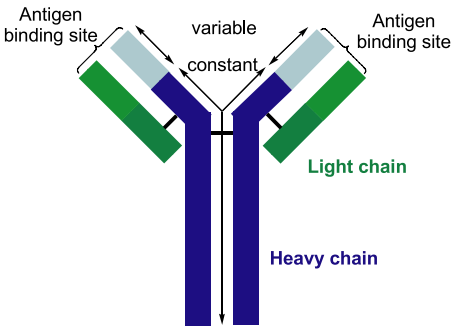

Figure 1. Antibody structure.

kidneys.[18] Nevertheless, chA7Fab-neocarzinostatin, bearing a truncated mAb fragment, has exhibited significantly better antitumor activity than A7-neocarzinostatin with whole mAb against human pancreatic carcinoma xenograft in nude mice.[19]

The cytotoxic agents used in the immunoconjugates should be highly potent because only a limited number of molecules can be loaded on each mAb without diminishing the binding affinity of the mAb moiety[6] and increasing the immunogenicity of the conjugate.[20] Another reason for the necessity of using a highly potent cytotoxic drug is that there is only a limited amount of antigens overexpressed on the tumor cell surface. In the early development phase of mAb–drug immunoconjugates, mitomycin C,[21] methotrexate,[22] vinca alkaloids,[23] and vinblastine[24] were linked to mAbs. However, none of these conjugates showed appreciable antitumor efficacy in vivo. The failure can be attributed to the moderate cytotoxicity of the cytotoxic agents employed. Attention then shifted towards the use of highly toxic compounds. Immunoconjugates containing extremely cytotoxic warheads, such as calicheamicin,[11] maytansine derivative DM1,[5] CC-1065,[7] monomethylauristatin E (MMAE),[25] and a second-generation taxoid[26] (Fig. 2), exhibited promising antitumor activity in vivo. Protein toxins, such as diphtheria toxin,[27–29] pseudomonas exotoxin A[30–32], and ricin,[33] belong to another class of highly cytotoxic agents suitable for mAb-based tumor-targeting therapy. Interestingly, doxorubicin (DOX), with moderate cytotoxicity, is still widely used in mAb–drug conjugates (Fig. 2). For example, BR96–DOX conjugate containing a hydrazone linker entered phase II clinical trial.[34] An *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-based conjugate bearing DOX and human immunoglobulin (H_uI_g) through a biodegradable Gly-Phe-Leu-Gly linker has been developed and clinically studied.[35] Use of the HPMA polymer could add the EPR effect to the conjugate and also increase the drug-loading without diminishing the binding affinity of the mAb moiety.

In addition to the mAb and cytotoxic agent components, the linker moiety has a critical significance in the efficacy of the immunoconjugates. An ideal linker must be stable in circulation, while being efficiently cleaved inside the cancer cells. The most frequently used linkers can be categorized into three classes, that is, (i) hydrazone linker, (ii) peptide linker, and (iii) disulfide linker. Selection of an appropriate linker depends on the type of cancer and the required cytotoxic agent. None of the linkers is universal and each of them has advantages and disadvantages.

The hydrazone linker takes advantage of the acidic (~pH 5) conditions in lysosome to release the cytotoxic drug via non-enzymatic hydrolysis. This linker has been successfully used in Mylotarg®, comprising a humanized mAb P67.6 conjugated to a highly potent cytotoxic agent, *N*-acetyl-γ-calicheamicin. The linker contains two cleavable bonds, a hydrazone and a sterically hindered disulfide. It has been shown that the hydrazone linker is the actual cleavage site.[36] Premature cleavage of the hydrazone linker at physiological pH was observed in the BR96–DOX conjugate,[37] which indicates the instability of this linker during circulation. Nevertheless, this linker in Mylotarg® has proven to be suitable for the treatment of AML, which could be attributed to the easy access to the target leukemia T-cells.

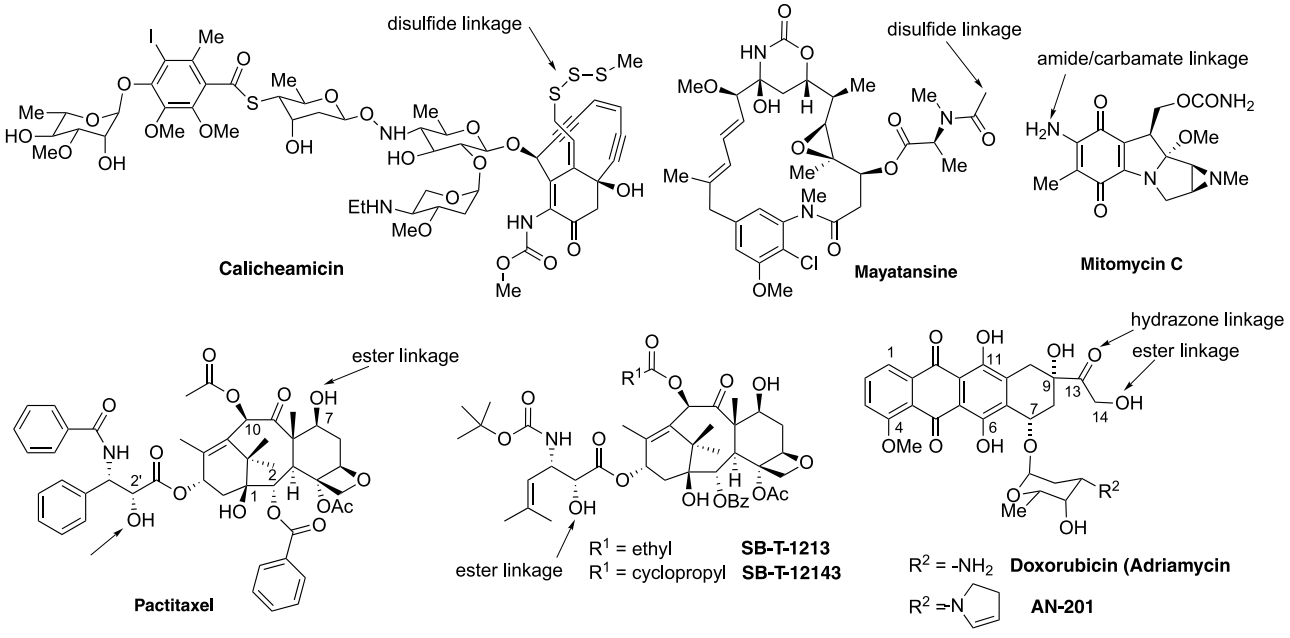

Figure 2. Cytotoxic agents.

Peptide linkers are designed for rapid lysosomal hydrolysis and high serum stability. Examples of peptide linkers include tetrapeptide Gly-Phe-Leu-Gly[38] as well as dipeptides Phe-Lys and Val-Cit (Cit = citrulline).[39] Dipeptide linkers Phe-Lys and Val-Cit have been employed for the immunoconjugates of doxorubicin with mAb BR96[40] as well as monomethylauristatin E (MMAE) with mAb cAC10.[25] In both cases, the corresponding immunoconjugates bearing a hydrazone linker were prepared earlier. It appears that the peptide linkage is much more stable than a hydrazone linker.

Disulfide linkers are cleaved inside the tumor cells through disulfide exchange with an intracellular thiol such as glutathione. The use of disulfide linkers is attractive by taking into account the fact that the concentration of glutathione is much higher in tumor cells as compared to normal cells.[41] The disulfide linkers have exhibited superior efficacy to other linkers against several tumor xenografts in preclinical models.[5,12] For example, a maytansine derivative DM1 (Fig. 2) bearing a methyldisulfanyl (MDS) group was linked to humanized mAbs, including HuC242,[42] HuN901, MLN2704, Herceptin, and anti-CD44v6.[12] These mAb–drug immunoconjugates are currently in phase I or II clinical studies.[12,43,44] Ojima et al.[26] developed mAb-SB-T-1213 (second-generation taxoid) conjugates with a disulfide linker (Fig. 3). The 10-methyldisulfanyl (MDS)-alkanoyl analog of SB-T-1213 was linked to mAbs, KS-61 and KS-77, targeting the human epidermal growth factor receptor (EGFR) that is overexpressed in several human squamous cancers such as head, neck, lung, and breast cancers. Both immunoconjugates showed remarkable antitumor activity against human squamous cancer (A431) xenografts in SCID mice, resulting in complete inhibition of tumor growth in all the treated animals without any systemic toxicity for the duration of the experiment. Necropsy on day 75, followed by histopathological examination, showed residual calcified material at the tumor site but no evidence of tumor cells. In sharp contrast, free taxoid SB-T-12136 at the same dose showed no therapeutic effect, indicating a highly efficient tumor-targeting delivery of the taxoid by its mAb conjugate.

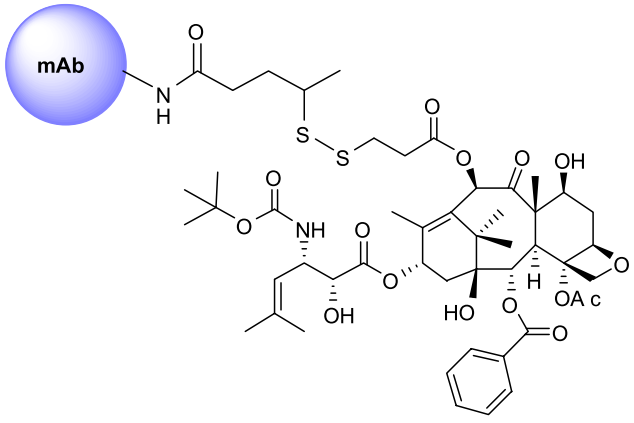

Figure 3. mAb–SB-T-12136 conjugate.

3. Tumor-targeting with polyunsaturated fatty acids

Essential fatty acids are polyunsaturated fatty acids (PUFAs) that can be obtained only from the diet. There are several known PUFAs having 18, 20, and 22 carbons, and 2–6 unconjugated cis-double bonds separated by one methylene. Vegetable oils are the source of α-linolenic acid (LNA), linoleic acid (LA), and arachidonic acid (AA), while cold-water fish is the supply for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AA can be obtained also from meat[45,46] (Fig. 4).

PUFAs have exhibited anticancer activity against CFPAC, PANC-1, and Mia-Pa-Ca-2 pancreatic and HL-60 leukemia cell lines, and their antitumor activities have been evaluated in preclinical and clinical studies.[47,48] Moreover, it has been shown that PUFAs are taken up greedily by tumor cells, presumably for use as biochemical precursors and energy sources.[49,50] In addition, PUFAs are readily incorporated into the lipid bilayer of cells, which results in disruption of membrane structure and fluidity.[51,52] This has been suggested to influence the chemosensitivity of tumor cells.[53] These findings strongly suggest the benefit in the use of PUFAs for tumor-targeting drug delivery.

A pharmaceutical composition containing daunomycin (Fig. 2) and arachidonic acid (AA) displayed enhanced cytotoxicity against hepatoma cell line, AH66 as compared to that by the drug alone. In contrast, the replacement of arachidonic acid with the corresponding saturated arachidic acid in the composition failed to show any enhancement of the potency of the drug.[54]

Esterification of 4′-demethyldeoxypodophyllotoxin (DDPT) with various PUFAs has shown enhanced in vivo antitumor activity as compared with DDPT itself,

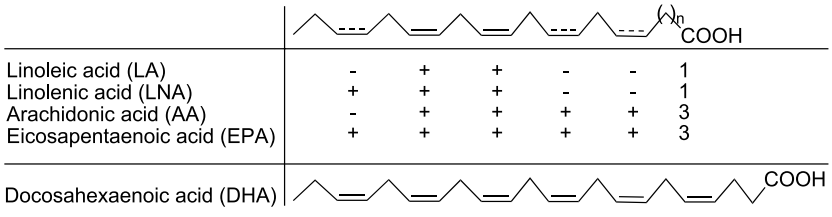

Figure 4. Polyunsaturated fatty acids (PUFAs).

which can be attributed to delayed metabolic excretion and increased tumor selectivity.[55]

Bradley et al. (Protarga Inc.)[56] prepared the docosahexaenoic acid (DHA) conjugate of paclitaxel (Taxoprexin®) by linking DHA to the C-2′ position of paclitaxel. The conjugate exhibited substantially increased antitumor activity and reduced systemic toxicity against the M109 lung tumor xenograft in mice as compared to paclitaxel. Furthermore, the conjugate is stable in blood plasma and high concentrations in tumor cells are maintained for a long period of time, slowly releasing the active cytotoxic agent. Consequently, the conjugate is more effective in killing slowly cycling or residual tumor cells and can reduce side effects. Taxoprexin® was selected as a first-track development drug candidate by FDA and has advanced to human phase III clinical trials.[57]

Although Taxoprexin® exhibits an impressive antitumor activity against drug sensitive tumors, this conjugate would not be effective against multidrug-resistant (MDR) tumors. The MDR tumor cells overexpress P-glycoprotein (Pgp), an effective ATP-binding cassette (ABC) transporter, which effluxes out hydrophobic anticancer agents including paclitaxel and docetaxel.[58] Although Taxoprexin® was found to be a weaker substrate of Pgp than paclitaxel, the released paclitaxel would be caught by the Pgp efflux pump and eliminated from the cancer cells. Many of the second-generation taxoids developed by Ojima and co-workers showed 2–3 orders of magnitude higher activity against drug-resistant cancer cells and tumor xenografts in mice, expressing MDR phenotypes.[59–61] Accordingly, PUFAs were conjugated to selected second-generation taxoids (Fig. 2) that possess built-in Pgp modulating ability. For example, DHA and α-linolenic acid (LNA) were coupled to the C-2′ OH group of the potent second-generation taxoids to give the corresponding conjugates.[62] The antitumor activities of these PUFA–taxoid conjugates were evaluated against the drug-sensitive A121 human ovarian tumor xenograft and the drug-resistant DLD-1 human colon tumor xenograft in SCID mice (Fig. 5). Taxoprexin® was very effective against the drug-sensitive A121 ovarian tumor xenograft, but DHA–SB-T-1213 and DHA–SB-T-1216 showed even better antitumor activity.[62] However, as anticipated, paclitaxel and Taxoprexin® were totally ineffective against the drug-resistant DLD-1 human colon tumor xenograft. In contrast, DHA–SB-T-1214 caused complete regression of the tumor (iv administration on days 5, 8, and 11) for the duration of experiment (201 days) in all treated animals.[62] LNA-SB-T-1213 also exhibited excellent efficacy against the DLD-1 tumor xenografts although the systemic toxicity of this conjugate was higher than that of DHA–SB-T-1214. Accordingly, LNA could be a good substitute of DHA in PUFA–taxoid conjugates.[62]

As described above, PUFA–taxoid conjugates have a high potential to become efficacious tumor-targeting chemotherapeutic agents in cancer therapy. However, further detailed study deems necessary to clarify the tumor-targeting mechanism of these conjugates.

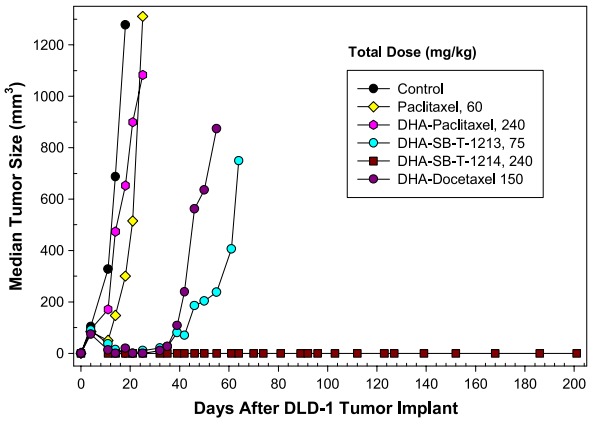

Figure 5. Effect of DHA–taxoid conjugates on human colon tumor xenograft (Pgp+) DLD-1.

4. Tumor-targeting with folic acid

Folic acid (Fig. 6) belongs to the vitamin B family. It is important in the formation of new cells by participating in the biosynthesis of nucleotide bases. Therefore, this vitamin is required for children, while adults need only minimal amount. Folic acid taken from food is delivered to epithelial cells through a kind of receptor-mediated endocytosis, 'potocytosis'.[63] There are two membrane-bound folic acid receptors, FR-α and FR-β. Both of the 38 kDa FR isoforms bind folic acid with a high affinity (K_D < 1 nM).[64] The expression of FR in normal tissues is low and restricted to various epithelial cells such as placenta, choroid plexus, lungs, thyroid, and kidneys. In contrast, FR is overexpressed in several tumors, particularly in ovarian and endometrial cancers. Radiolabeling study has shown that [^{3}H]folic acid binds to tumor cells 20-times more than normal epithelial cells or fibroblasts.[65] Thus, extensive studies have been performed on the tumor-targeting conjugates by linking folic acid to cytotoxic agents as well as imaging agents.

The relationship between cancer and folic acid was first recognized by Leuchtenberger et al. in 1944,[66] when an extract from *L. casei* factor inhibited the growth of murine sarcoma. A few years later, it was found that the active principle in this extract was not folic acid but a peptidic conjugate of folic acid with two glutamic acid residues.[67] Since then, considerable efforts have been made to develop suitable folic acid antagonists as cytotoxic agents, with methotrexate being the most recognized representative in this class of anticancer agents.[68]

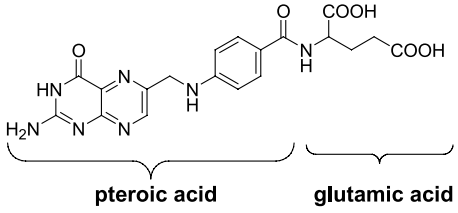

Figure 6. Folic acid.

As for the use of folic acid in tumor-targeting drug delivery, studies have mainly focused on its macromolecular conjugates bearing anticancer agents. Proteins,[69] polymeric micelles,[70] liposomes,[71] synthetic polymers,[72] and nanoparticles[73] have been employed as macromolecular carriers for these conjugates. Although the expression of FR in kidneys is not as significant as that in tumor cells, any damage to kidneys would cause undesirable side effects. The use of a macromolecular carrier bearing folic acid molecules as tumor-targeting moieties has an advantage because the FRs in kidneys are expressed in the region which macromolecules cannot reach, thereby avoiding undesired renal toxicity.

In contrast to folic acid-containing macromolecule-drug conjugates, only a few reports have been published on the folic acid-drug conjugates of low molecular weight for tumor-targeting, wherein paclitaxel, maytansine, and mitomycin C (Fig. 2) are employed as cytotoxic agents. For example, Lee et al.[74] attached paclitaxel to folic acid through an oligoethyleneglycol linker. Several paclitaxel conjugates bearing folic acid esters and carbamates at the C-7 or C-2′ position have been synthesized. All these conjugates showed better cytotoxicity than paclitaxel alone, that is, the ED_{50} values against A-549 (non-small cell lung), MCF-7 (breast), and HT-29 (colon) cancer cell lines in vitro were up to 10-fold, 30-fold, and 80-fold, respectively, better than those of paclitaxel. However, it was found that the FR-binding affinity of a representative conjugate, C-7-(PEG-3)-folylpaclitaxel, to the FR-positive KB cells was only 1/4 of the free folic acid. Moreover, unfortunately these conjugates all failed to demonstrate selective cytotoxicity to FR-expressing KB tumor cells in vitro and in vivo.[74]

Highly cytotoxic maytansinoid derivative DM1 was used by Ladino et al.[75] to form a disulfide-linked folic acid conjugate. This conjugate retained high specific affinity to FR and possessed remarkable potency against a panel of KB, SKOV-3, LoVo, HeLa, and SW620 cancer cell lines with IC_{50} values ranging from 10^{-11} to 10^{-10} M. The conjugate was about 100-fold less toxic to the FR-negative cell lines than the FR-positive cell lines, suggesting that attachment of the cytotoxic drug to folic acid markedly decreased non-specific delivery of the drug to primary cells.

Leamon and Reddy[76] (Endocyte Inc.) recently reported in vivo evaluation of two proprietary folic acid-drug conjugates, EC70 (the structure of the warhead was not disclosed) and EC72, having mitomycin C as the warhead. EC72 increased lifespan of nu/nu mice with human KB xenografts by 124%. There was no sign of non-specific toxicity, whereas mitomycin C at an equivalent regimen proved to be lethal. EC70 suppressed tumor growth by 5-fold after 52 days and increased lifespan by 333%. It should be noted that the parent drug alone was less active against tumor and more toxic against other tissues as observed by substantial weight loss (>20%).

Since a liposome vesicle can carry a drug cargo usually in the order of 10^3–10^4 molecules, liposomal formulations offer opportunities to develop efficacious tumor-targeting drug delivery systems. Goren et al.[77] attached folic acid to Doxil®, which is a sterically stabilized liposomal formulation of doxorubicin consisting of hydrogenated soy phosphatidylcholine, cholesterol, and polyethylene glycol(PEG)–distearoyl-phosphatidyl-ethanolamine.[78] The folic acid moiety is attached to a certain number of PEG tether terminals of Doxil® through an amide bond. Confocal fluorescence microscopy of rhodamine-labeled doxorubicin encapsulated inside the liposome showed rapid internalization into FR-positive cells, followed by drug-release in the cytoplasmic compartment, and doxorubicin was then detected in the nucleus. The entire process lasted only 1–2 h. FR-mediated uptake of the liposomes with entrapped doxorubicin into a multidrug-resistant subline of M109-HiFR cells was unaffected by P-glycoprotein efflux pump. The *in vitro* cytotoxicity of the liposome conjugated to folic acid was 10-fold stronger than a Doxil® but the cytotoxicity of the conjugate was the same as that of non-liposomal doxorubicin itself. The in vivo assay against M109-HiFR tumor in BALB/c mice for five weeks showed that the FR-targeting liposomal doxorubicin was considerably more efficacious than doxorubicin itself. The final tumor incidence for treated and untreated animals was 10% and 65%, respectively.

Stevens and Lee[79] reported FR-targeting emulsion formulation with entrapped paclitaxel. The liposome-like structure consisting of various unsaturated lipids, surfactants, phosphatidylcholine, 0.5% of folate–PEG–cholesterol, and paclitaxel (59 wt %) exhibited significant selectivity against FR-positive KB tumor cells in vitro. The IC_{50} value for the FR-positive KB cells was 18 nM, while that against FR-negative glioma cells was 1400 nM, that is, the efficacy of paclitaxel against the KB cells was 78 times higher than that against the glioma cells because of tumor-targeting drug delivery. In addition, the non-targeting emulsion showed a 200-fold higher IC_{50} value (i.e., IC_{50} = 3.6 μM) against the FR-positive KB cells.

5. Tumor-targeting with hyaluronic acid

Hyaluronic acid (or hyaluronan) (HA) is a linear, negatively charged polysaccharide, containing two alternating units of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc) with molecular weight of 10^5–10^7 (see Fig. 7).

HA is responsible for various functions within the extracellular matrix such as cell growth, differentiation, and

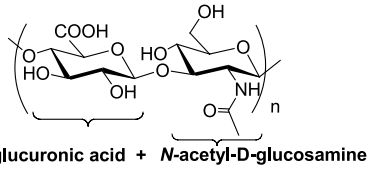

Figure 7. Hyaluronic acid.

migration.[80] A wide range of activities can be explained by a large number of HA-binding receptors such as cell surface glycoprotein CD44, receptor for hyaluronic acid-mediated motility (RHAMM), and several other receptors possessing HA-binding motifs, for example, transmembrane protein layilin, hyaluronic acid receptor for endocytosis (HARE), lymphatic vessel endocytic receptor (LYVE-1), and also intracellular HA-binding proteins including CDC37, RHAMM/IHABP, P-32, and IHABP4.[81,82]

It has been shown that the HA level is elevated in various cancer cells.[83] The higher concentration of HA in cancer cells is believed to form a less dense matrix, thus enhancing the cell's motility as well as invasive ability into other tissues[84] and also providing an immunoprotective coat to cancer cells.[85] It is well known that various tumors, for example, epithelial, ovarian, colon, stomach, and acute leukemia, overexpress HA-binding receptors CD44[86] and RHAMM.[87] Consequently, these tumor cells show enhanced binding and internalization of HA.[88]

CD44–HA interactions play various important physiological roles, including mediation or promotion of macrophage aggregation,[89] cell migration,[90] chondrocyte pericellular matrix assembly,[91] and leukocyte activation.[92] It has been shown that the overexpression of hyaluronic acid synthases increases the HA level, which leads to the acceleration of tumor growth and metastasis.[93–95] On the other hand, exogenous oligomeric HA inhibits tumor progression most likely by competing with endogenous polymeric HA.[96,97]

Although the mechanism of hyaluronan–CD44 binding is still not fully understood, it has been reported that the CD44 receptor contains the specific binding domain for HA, which consists of 160 amino acid residues.[82,86,98] The binding affinity of CD44 to HA was found to be dependent on the size of HA oligomers. For instance, hexamer (HA_6) and decamer (HA_{10}) are considered to be the minimum size oligosaccharides that bind to CD44. Larger oligomers ($\geqslant HA_{20}$) have higher binding affinity than smaller oligomers because of multiple interactions with more than one CD44 receptor simultaneously.[82,86,97] The dissociation constant (K_d) of CD44 with different HA oligomers varies according to the HA size. Shimada et al. reported a K_d value of 2.7×10^{-5} M for the 250 mer of HA with human CD44.[99] The high tumor specificity of the HA–CD44 interactions and high biocompatibility of HA were key factors for the design and synthesis of tumor-targeting bioconjugates bearing HA and cytotoxic agents.

HA can be coupled with an active cytotoxic agent directly to form a non-toxic prodrug. Alternatively, a suitable polymer with covalently attached HA and drug can be used as a carrier. Direct conjugations of a low molecular weight HA to cytotoxic drugs such as butyric acid,[100] paclitaxel,[101,102] and doxorubicin[103] (Fig. 2) have been reported. It has been shown that these bioconjugates are internalized into cancer cells through receptor-mediated endocytosis, followed by intracellular release of active drugs, thus restoring their original cytotoxicity.

The efficacy of bioconjugates depends on the level of cytotoxic agent loading to HA. The HA–butanoic acid conjugates were tested against MCF-7 cell line with different degrees of butanoic acid attachments, ranging from 0.1 to 2.24 per HA molecule. It was found that the IC_{50} values for 0.1, 0.14, 0.19, and 2.24 butanoic acid/HA attachments were 0.92, 0.54, 0.17, and 7.40 mM, respectively, as compared with that of 0.5 mM for sodium butyrate. It is worth mentioning that the cytotoxicity of highly loaded conjugate was found to be weaker than that of the drug alone.[100] The same tendency was observed for the HA–paclitaxel conjugates tested against HBL-100 (breast), SK-OV-3 (ovarian), and HCT-116 (colon) cancer cell lines.[101] This result can be attributed to the blocking of the receptor recognition sites of HA by paclitaxel moieties, which led to a decrease in the tumor-targeting selectivity as well as the cytotoxicity of the bioconjugate.

HA was also used as a tumor-targeting moiety in drug delivery system with *N*-(2-hydroxypropyl)methacrylamide (HPMA) polymer.[103] HMPA–HA–DOX bioconjugate with a lysosomally degradable peptide linkage clearly demonstrated better internalization and cytotoxicity as compared to non-targeting HMPA-DOX conjugate. The IC_{50} value of HPMA–HA–DOX against HBL-100 (breast) cell line was 0.52 μM for 36 wt % loading of HA, which is more than one order of magnitude better than that of non-targeting HMPA–DOX (18.7 μM). It was found that the systemic toxicity of HMPA–HA–DOX to the primary cells of murine fibroblast was very low (IC_{50} = 21.2 μM).

HA-containing liposomes with entrapped DOX have also been investigated as a tumor-targeting drug delivery system.[104] It has been confirmed that the binding affinity of liposomes is attributed to the specific CD44–HA interaction. It was observed that the degree of HA-liposome uptake by murine melanoma B16F10 tumor cell line was proportional to the loading of HA in the 0–3 mol % range. Even as low as 0.1 mol % HA-loading showed selective tumor-targeting. The HA-containing DOX liposomes showed almost one order of magnitude better cytotoxicity than the drug alone and more than two orders of magnitude better activity than non-targeting liposomes against B16F10 melanoma cells. In addition, the HA-containing DOX liposomes were not cytotoxic to CV-1 cells with a low level of CD44 expression.[104]

6. Tumor-targeting with peptides

Peptide-based targeting of tumor-associated receptors is an attractive approach in tumor-specific drug delivery because high-affinity sequences can be discovered through screening of combinatorial libraries. Recently, numbers of peptides and their conjugates with cytotoxic

agents that target different cancer cell receptors have emerged as potential tumor-specific chemotherapeutic agents.

Gastrointestinal (GI) peptides have many physiological functions as hormones, neurotransmitters, and growth factors. Each of these peptides usually targets more than one receptor. Thus, these peptides and their truncated analogs, possessing appropriate recognition properties, could serve as tumor-targeting molecules in combination with cytotoxic agents.

Somatostatin (SST) is a hormonal neuropeptide existing in two active forms, that is, SST-14 and SST-28 with 14 and 28 amino acid residues, respectively. SST-14 and SST-28 interact with cells through a minimum of five membrane receptor subtypes ($SSTR_{1-5}$) inhibiting the secretion of various hormones including the growth hormone (GH) also known as somatotropin.[105,106] The $SSTR_{1-5}$ membrane receptors are expressed at significantly elevated levels in tumor cells and possess high binding affinity to somatostatin.[107,108] Thus, somatostatin is a good candidate for delivery of cytotoxic agents specifically to GI tumor cells. However, SST-14 was found to have only a short life in plasma. Thus, more stable synthetic analogs of SST-14 have been developed. Among them, octapeptides RC-160 and RC-121 (Fig. 8) are 50 times more potent than somatostatin in inhibition of GH.[109] Although the binding affinities of somatostatin to different SST_{1-5} receptors ($SSTR_{1-5}$) are similar, the synthetic analogs show high binding affinity to $SSTR_2$ and $SSTR_5$, moderate affinity to $SSTR_3$ and poor affinity to $SSTR_1$ and $SSTR_4$.[108,110] Nagy et al.[111] have synthesized conjugates of somatostatin analogs, RC-160 and RC-121, with glutarate-linked doxorubicin and 2-pyrrolino-DOX (AN-201), which is 500–1000 times more cytotoxic than doxorubicin (Fig. 2).[112]

These conjugates were assayed for their in vitro cytotoxicity against MKN-45 gastric, MDA-MB-231 breast, PC-3 prostate, and MIA-PaCa-2 pancreatic cancers.

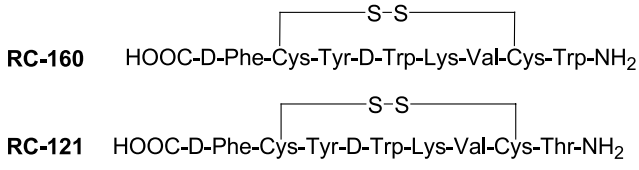

Figure 8. Somatostatin analogs.

The in vitro assay results showed the same or lower cytotoxicity of the conjugates than those of doxorubicin and AN-201 and lower binding affinity than their respective carriers, that is, RC-160 and RC-121. The affinity of these conjugates to SSTR was tested by competitive binding experiment using ^{125}I-labeled RC-160 on rat pituitary membranes. All conjugates demonstrated high binding affinity to the SSTRs ($IC_{50} < 80$ nM), although the RC-160 derivatives of doxorubicin and AN-201 were 4.5-fold and 46-fold weaker ligands than RC-160 alone, respectively.

For in vivo studies, the conjugate of AN-201 with RC-121 (AN-238) was selected on the basis of superior cytotoxicity to others in vitro. The antitumor activity assays against MXT murine mammary carcinoma in female BDF mice indicated that the conjugate AN-238 was tumor-specific since AN-201 alone at the same dose was highly toxic and did not show any antitumor activity.[111] Inhibition of tumor growth by AN-238 was dose dependent in the 150–300 nmol/kg range with no systemic toxicity.

Bombesin (BBN) and the bombesin-like peptide, gastrin-releasing peptide (GRP), consist of 14 and 27 amino acid residues, respectively, and have several physiological functions as gastrointestinal hormones and neurotransmitters.[113] Moreover, these peptides also function as growth factors and modulate tumor proliferation.[114] It was found that the bombesin-like peptides interact with four different receptors $BBNR_{1-4}$ and the receptor subtypes $BBNR_{1-3}$ were found in mammals.[115] The bombesin-like peptides and its receptors are produced in different cancer cells such as small cell lung, breast, prostatic, and pancreatic cancers.[114,116] The finding that bombesin-like peptides function as growth factor and possess a high binding affinity to the bombesin/GRP receptors has stimulated the development of bombesin/GRP antagonists as potential anticancer agents. Bombesin/GRP antagonists, such as RC-3095 and RC-3940-II (Fig. 9), showed highly potent cytotoxicity against CFPAC-1 and SW-1990 pancreatic,[117,118] H69 human small cell lung,[119] MKN45 and Hs746T gastric[120,121] cancer cell lines as well as nanomolar level binding affinity to bombesin/GRP receptors.[122–124]

The bombesin/GRP antagonists were further conjugated with doxorubicin and AN-201 for possible tumor-targeting drug delivery.[125] Cytotoxic agents were coupled to the bombesin/GRP antagonists at their amino termini via glutarate ester linker. Their affinity to bombesin/GRP receptors was examined on Swiss 3T3 cells using

Bombesin (BBN)	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
RC-3095	[D-Tpi^6, Leu$^{13}\Psi$(CH_2NH)Leu^{14}]-BBN(6-14)
RC-3940-II	[Hca^6, Leu$^{13}\Psi$(CH_2NH)Tac^{14}]-BBN(6-14)
B_4	[$^{13}\Psi^{14}$(CH_2NH)Leu^{14}]-BBN(7-14)

Hca = hydrocinnamic acid; Tpi = 2,3,4,9 tetrahydro-1H-pyrido-[3,4-b]indole-3-carboxylic acid
Tac = thiazolidine carboxylic acid; Ψ = pseudo peptide bond

Figure 9. Bombesin and its analogs.

[^{125}I-Tyr^4]bombesin as radioligand for competitive binding assay. The results show that the conjugates possess high affinity to bombesin/GRP receptors on the basis of their excellent inhibitory activity of the radioligand binding (IC_{50} = 0.6–1 nM).

Cytotoxicity of the conjugates against human cancer cell lines, CFPAC-1b (pancreatic), DMS-53 (small cell lung), PC-3 (prostate), and MKN-45 (gastric), was similar to that of non-conjugated doxorubicin or AN-201 itself.[125] Preliminary in vivo studies against the nitrosamine-induced pancreatic cancer model in golden hamsters showed that conjugate B_4-AN-201 exhibited significant antitumor activity and was less toxic at the same dose as AN-201.[125] B_4-AN-201 decreased the growth of AGS human gastric tumor xenograft in nude mice by 89%, while AN-201 showed only 50% inhibition.

Cytotoxicity of the conjugate of truncated bombesin with paclitaxel (Ptx), Ptx-PEG-BN(7–13) was assayed against human non-small cell lung cancer (NSCLC) cell line NCI-H1299.[126] The peptide moiety was connected to the cytotoxic drug through a polyethylene glycol spacer (MW 3400) and a succinate linker. The bombesin analogue, BN(7–13), was attached via amide bond, while paclitaxel was coupled through an ester linkage. It was found that Ptx-PEG-BN(7-13) (IC_{50} 14 nM after 24 h exposure) was more than twice as potent as paclitaxel (IC_{50} 35 nM after 24 h exposure). The results suggest that the receptor-mediated tumor-targeting by BN(7-13) enhanced the drug delivery into the cancer cells. Moreover, the binding affinity of the complex was retained as compared with the unconjugated peptide. Ptx-PEG-BN(7-13) exhibited 80–90% inhibition of [^{125}I-Tyr^3]bombesin binding to the receptors in BNR-11 (murine fibroblasts) and NCI-H1299 (NSCLC) cell lines.

7. Conclusion

Major progress in the recombinant gene technology has opened access to human-compatible mAbs, which provides significant advantages for the mAb-based tumor-targeting. The validity of this approach has been proven by the success of Mylotarg®, which was approved by the FDA as well as the fact that more than several mAb–drug conjugates are in various stages of human clinical trials. Major challenges for mAb-based drug delivery include the identification of target antigens that are overexpressed specifically on the tumor surface, the circumvention of potential immunogenicity of the conjugates, and the determination of the right size of the conjugates that can penetrate into tumors, yet not to be cleared by kidneys too fast.

Folic acid has very long history in cancer chemotherapy. It appears very popular in prodrug design because it is a small molecule and readily available, besides the fact that the distribution of the folic acid receptors in tumors is well described. Folic acid is ideally suited for the design of tumor-targeting macromolecules and formulations, such as water-soluble polymers, nanoparticles, nanotubes, liposomes, micelles, and emulsions. However, the applications of folic acid in small-size conjugates have had only limited success so far to date.

Tumor-targeting with hyaluronic acid (HA) has been showing great promise in the cancer therapy, especially those conjugates consisting of polymer, a HA oligomer, and a cytotoxic agent. The major advantage of HA is its hydrophilicity, which allows for easy formulation in aqueous media. Moreover, overexpression of CD44 receptors on tumor surface has been well studied. However, a possible disadvantage of HA is the lack of accurate structural characterization of the conjugates.

Polyunsaturated fatty acid (PUFA) conjugates of cytotoxic agents constitute a rather unexplored class of anticancer agents with high potential as efficacious chemotherapeutic agents. The efficacies demonstrated in the animal models show great promise in this approach. However, the detailed mechanism of tumor-targeting by PUFAs needs to be clarified. High availability as well as their known benefits as food additives and supplements are clear advantages of this approach.

Peptide-based targeting has a high potential for tumor-specific drug delivery of cytotoxic agents. A clear advantage of this approach is an excellent probability that highly tumor-specific peptide sequences for various cancers could be discovered by screening appropriate combinatorial libraries. Since most gastrointestinal cancers are difficult to treat due to their multidrug-resistance, this approach may shed a light on the development of efficacious chemotherapy. One of the inherent problems is the stability of these peptides in circulation, although this can be solved by appropriate design and modifications to prevent or slow down the amide hydrolysis.

As described above, tumor-targeting drug delivery in cancer therapy has been evolving from the traditional chemotherapy with the hope to minimize undesirable side effects and improve the quality of life of cancer patients. The authors sincerely hope that some breakthroughs come out of this line of approaches for new and efficacious cancer chemotherapy.

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