

## Anticancer Therapeutics: Targeting Macromolecules and Nanocarriers to Hyaluronan or CD44, a Hyaluronan Receptor

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**Abstract:** The complex system involved in the synthesis, degradation and binding of the high molecular weight glycosaminoglycan hyaluronic acid (hyaluronan or HA) provides a variety of structures that can be exploited for targeted cancer therapy. In many cancers of epithelial origin there is an upregulation of CD44, a receptor that binds HA. In other cancers, HA in the tumor matrix is overexpressed. Both CD44 on cancer cells and HA in the matrix have been targets for anticancer therapy. Even though CD44 is expressed in normal epithelial cells and HA is part of the matrix of normal tissues, selective targeting to cancer is possible. This is because macromolecular carriers predominantly extravasate into the tumor and not normal tissue; thus CD44–HA targeted carriers administered intravenously localize preferentially into tumors. Anti-CD44 antibodies have been used in patients to deliver radioisotopes or mertansine for treatment of CD44 expressing tumors. In early phase clinical trials, patients with breast or head and neck tumors treated with anti-CD44 conjugates experienced stabilized disease. A dose-limiting toxicity was associated with distribution of the antibody–drug conjugate to the skin, a site in the body with a high level of CD44. HA has been used as a drug carrier and a ligand on liposomes or nanoparticles to target drugs to CD44 overexpressing cells. Drugs can be attached to HA via the carboxylate on the glucuronic acid residue, the hydroxyl on the *N*-acetylglucosamine or the reducing end which are located on a repeating disaccharide. Drugs delivered in HA-modified liposomes exhibited excellent antitumor activity both *in vitro* and in murine tumor models. The HA matrix is also a potential target for anticancer therapies. By manipulating the interaction of HA with cell surface receptors, either by degrading it with hyaluronidase or by interfering with CD44–HA interactions using soluble CD44 proteins, tumor progression was blocked. Finally, cytotoxic drugs or prodrug converting enzymes can be attached to the HA matrix to generate a cytotoxic fence around the tumor. This review describes how the complex interplay among cancer biology, the CD44–HA interaction, drug carriers and drug targeting has been used to improve anticancer therapies. As these approaches evolve, they hold forth the prospect of significantly improved targeted anticancer treatments.

**Keywords:** Antibody; biodistribution; cancer; chemotherapy; drug conjugate; EPR effect; liposome; polymer; prodrug; recombinant protein

### 1. The Biology of the CD44–Hyaluronan Interaction

Hyaluronan (HA) (Figure 1) is a high molecular weight glycosaminoglycan extracellular matrix component essential for proper cell growth, organ structural stability and tissue

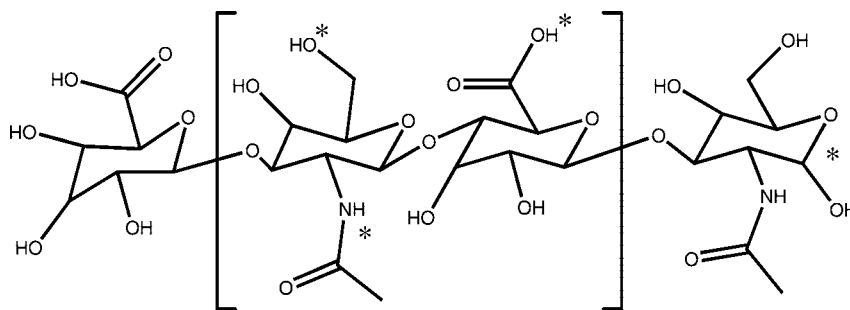
organization. The amount of HA in a tissue depends upon a complex interplay among HA synthesis by HA synthases,<sup>1</sup> HA internalization by cell surface receptors<sup>2</sup> and extracellular degradation by hyaluronidases.<sup>3</sup> HA turnover is due to local

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**Figure 1.** HA structure: polymeric repeat of D-glucuronic acid and N-acetylglucosamine. The asterisk (\*) represents potential sites of chemical conjugation.

cellular catabolism, removal by an HA endocytosing receptor (LYVE-1) on cells located in the lymphatics<sup>4</sup> and systemic clearance from the blood by the HARE receptor on liver sinusoidal endothelial cells.<sup>5</sup> In skin, HA has a half-life of over a day.<sup>6</sup> In contrast, circulating high molecular weight HA (HMW-HA) has a half-life of two to five minutes.<sup>7</sup> The net outcome of the various HA clearance processes results in a total turnover of about five grams of HA per day in humans.<sup>8</sup>

HA acts through CD44, its principal receptor, and RHAMM (receptor for HA mediated motility)<sup>9</sup> to regulate cell proliferation and movement. Many of the downstream pathways following CD44 activation become deregulated in cancer, leading to tumor growth, progression and metastasis.<sup>10–12</sup> Changes in both CD44 and HA expression have

been widely observed in tumors from cancer patients and occur in animal models of tumor growth.<sup>13</sup> During carcinogenesis, expression of the standard form of CD44 is upregulated in certain cancers. Non-native CD44 variants may also arise from alternate splicing of cytoplasmic regions of CD44 during translation.<sup>13</sup>

Interference with the CD44–HA interaction by either targeting drugs to CD44,<sup>14</sup> targeting drugs to the HA matrix<sup>15</sup> or interfering with HA matrix–CD44 interactions<sup>16</sup> are viable strategies for cancer treatments. The CD44–HA system is illustrated in Figure 2. In this figure we indicate options for anticancer therapeutics that specifically interfere with or exploit aspects of the CD44–HA pathway. This review discusses these options.

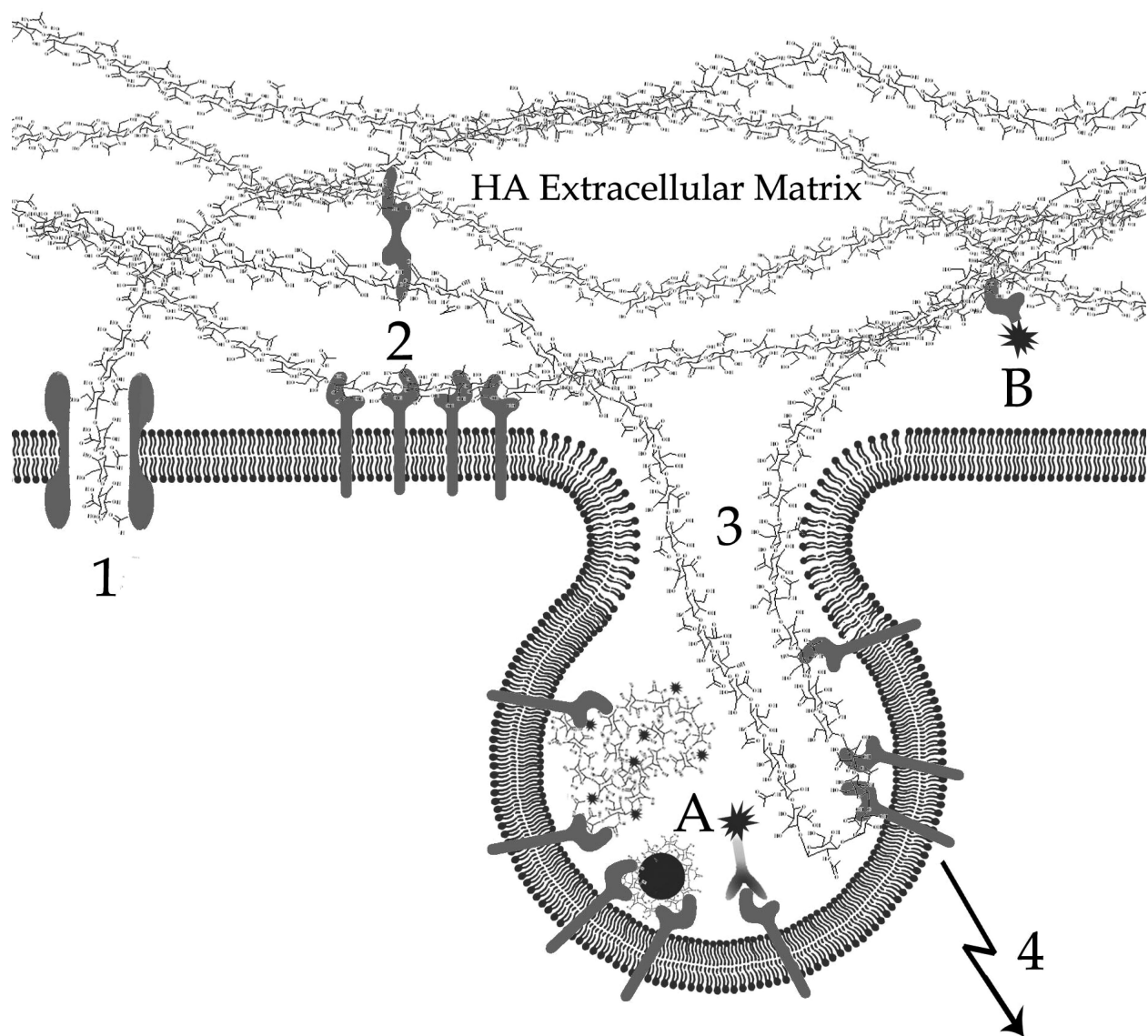
## 2. Targeting to CD44 with Anti-CD44 Antibodies or Hyaluronan

Although the ideal anticancer drug would have high specificity and activity against cancers, most drugs distribute throughout the body and are toxic to healthy as well as neoplastic cells. To minimize adverse effects, drugs can be formulated to increase the concentration at the target site and decrease drug concentrations elsewhere in the body. This goal has been achieved to some extent with anti-CD44 antibody–drug conjugates, which target drugs to CD44 that is overexpressed on various tumors.<sup>13</sup> Anti-CD44 antibody–drug conjugates have shown promise during clinical development (Table 2). However, CD44 is endogenously expressed at low levels in healthy tissues;<sup>17</sup> because of this expression, side effects still occur.<sup>18,19</sup>

Antibody targeted therapies are effective in cancer treatments for three principal reasons: (1) The epitope recognized by the antibody is overexpressed on the tumor cell. (2) The

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**Figure 2.** HA regulation: (1) HA is synthesized by HA synthases on the cell surface, (2) interacts with extracellular binding proteins and cell surface receptors which (3) internalize HA and lead to (4) degradation by hyaluronidases. The CD44–HA system can be utilized for (A) intracellular delivery of antibody- or HA-conjugated drugs and drug carriers or for (B) extracellular localization of HA binding proteins or protein conjugates.

**Table 1.** Hallmarks of the Enhanced Permeability and Retention (EPR) Effect

healthy tissue	cancerous tissue
Small molecules diffuse across capillary walls.	Small molecules diffuse across capillary walls and enter through large gaps in the capillary walls.
Large molecules cannot diffuse due to tight capillary cell junctions.	Large molecules enter through large gaps in the capillary walls.
Small molecules are cleared by a well-formed lymphatic system.	No lymphatic clearance is present.

a long enough period that a high fraction of the injected dose passes through the tumor. The therapeutic advantages of mechanisms one and three are self-evident. Mechanism two arises because tumors often exhibit a phenomenon known as the enhanced permeability and retention effect (EPR).<sup>20</sup> The properties of the EPR effect are summarized in Table 1. This phenomenon is thought to occur because solid tumors have a much leakier blood supply than healthy capillary beds; particles of up to approximately 0.5  $\mu\text{m}$  can extravasate into

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antibody has better access to the tumor than to normal tissues that also express the epitope. (3) The antibody circulates for

**Table 2.** Clinical Trials of Antibody Anti-CD44 Conjugates

antibody	drug	injection method	cancer type	effect	ref
U36	Re-186	single intravenous	head and neck squamous cell carcinoma	stable disease in 5 of 9 patients, mild myelotoxicity	51
BIWA 4	Tc-99m	single intravenous	head and neck squamous cell carcinoma	tumor targeting	53
BIWA 4	Re-186	dose escalation	head and neck squamous cell carcinoma	stable disease in 3 of 6 patients, limiting myelotoxicity	52
BIWA 4	Re-186	single intravenous	early stage breast cancer	moderate tumor identification, no correlation with CD44v6 expression	54
BIWA 4	mertansine	dose escalation	head and neck squamous cell carcinoma	moderate disease stabilization, skin toxicity	18
BIWA 4	mertansine	dose escalation	head and neck squamous cell carcinoma	low interpatient pharmacokinetic variability, skin toxicity	19
BIWA 4	mertansine	dose escalation	CD44v6 positive metastatic breast cancer	stable disease in 12 of 24 patients, dose limiting toxicity	55

the tumor from the blood.<sup>20</sup> Tumors also lack a well-developed lymphatic system. This combination allows mac-

romolecules or nanoparticles to passively accumulate within tumors due to leakage from the improperly formed tumor vasculature and remain within the tumor due to limited lymphatic clearance.<sup>20</sup>

Administering a drug in a carrier alters the drug's distribution profile by directing the drug away from sites of toxicity and, by exploiting the EPR effect, into the tumor. Successfully targeted anticancer therapies utilize a specific drug target and also exploit the altered biology of cancerous tissue to achieve a therapeutic effect. Drug carriers may also improve drug circulation time, stability or solubility depending on the carrier's properties.<sup>21</sup>

HA exhibits a number of properties of a successful drug carrier. This water soluble, nonimmunogenic polysaccharide has multiple functional groups available for chemical conjugation<sup>22–24</sup> (Figure 1). Furthermore, since it is the major CD44 ligand, HA can be used to target cells on which CD44 is expressed<sup>24,25</sup> (Table 3). Several reviews have previously discussed the advantages of HA as a drug carrier and a targeting ligand for cancer, as well as other pathologies.<sup>22–27</sup>

HA targeting increases drug accumulation on CD44 expressing cells and HA-attached drug can enter the cell via endocytosis.<sup>28,29</sup> Interestingly, CD44 binding and endocytotic activity, and therefore the cellular localization of HA targeted drugs, are greatly affected by CD44 posttranslational modifications. Such modifications are not constant across all CD44 expressing cells. Glycosylation is required for CD44 to bind HA on certain cell types, while glycosylations rich in sialic acid decrease HA binding.<sup>30–32</sup> Circulating lymphocytes

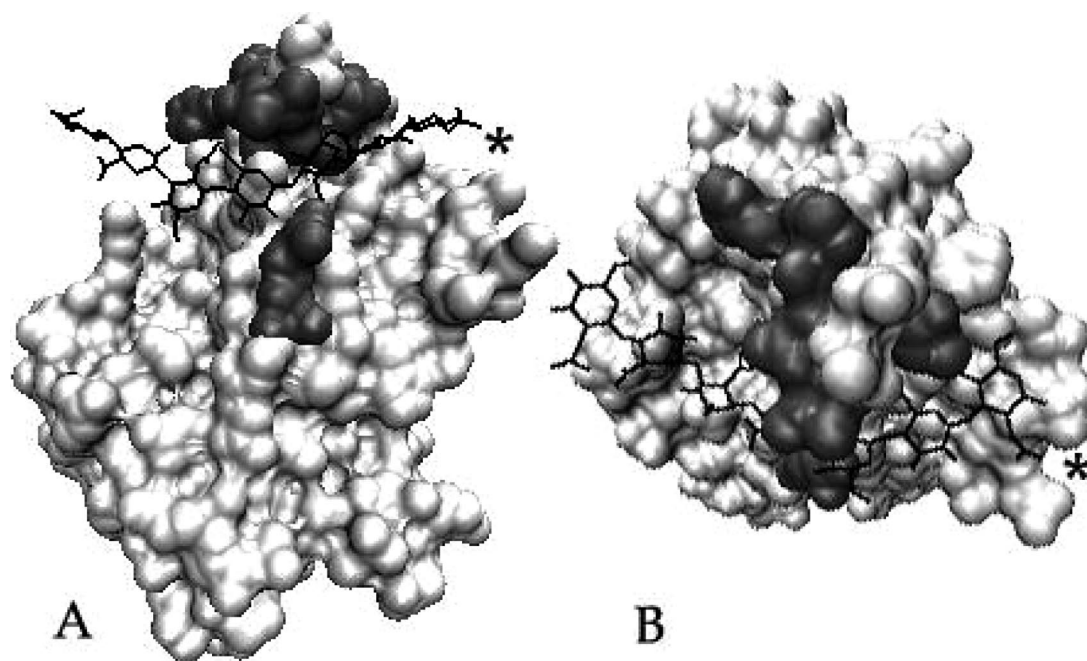
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Table 3. Targeted Drugs and Drug Carriers in Vitro

carrier	drug	linkage	cell lines	effect	ref
LMW-HA	paclitaxel	hydrazide to glucuronic acid carboxylate	HBL-100 (human mammary carcinoma) HCT-116 (human colon carcinoma) SK-OV-3 (human ovarian carcinoma)	cytotoxicity, CD44 specific uptake	28, 29
HMW-HA	butyrate	ester to <i>N</i> -acetylglucosamine hydroxyl	MCF-7 (human mammary adenocarcinoma) HepB3 (human hepatocellular carcinoma) NCI-H460 (human nonsmall cell lung carcinoma) NCI-H460 M (human nonsmall cell lung carcinoma)	inhibited proliferation, CD44 specific uptake	57, 59, 64
HMW-HA	paclitaxel	ester to glucuronic acid carboxylate	RT-4 (human bladder carcinoma) RT-112/84 (human transitional cell carcinoma)	cytotoxicity	63
HMW-HA	paclitaxel	hydrazide to glucuronic acid carboxylate	NMP-1 (human ovarian carcinoma) SK-OV-3ip (human ovarian carcinoma)	cytotoxicity	62
HMW-HA	carborane	ester to glucuronic acid carboxylate	HT-29 (human colorectal adenocarcinoma) MCF-7 (human mammary adenocarcinoma) OVCAR-3 (human ovarian adenocarcinoma) RT-112/84 (human transitional cell carcinoma) HCT-116 (human colon carcinoma)	CD44 specific uptake	58
LMW-HA	siRNA	disulfide cross-linked via the glucuronic acid carboxylate	A549 (human alveolar squamous cell carcinoma)	CD44 specific uptake, gene silencing	72
HMW-HA	peptide	hydrazide cross-linked via the glucuronic acid carboxylate	HBL-100 (human mammary carcinoma) HCT-116 (human colon carcinoma) SK-OV-3 (human ovarian carcinoma) B16F10 (murine melanoma)	peptide internalization	73
HPMA polymer LMW-HA	doxorubicin	hydrazide to glucuronic acid carboxylate		cytotoxicity, CD44 specific uptake	60
liposome	doxorubicin	reductive amination to terminal end		cytotoxicity, CD44 specific uptake	37, 38, 41
HA oligos	doxorubicin	amide to glucuronic acid carboxylate	MDA-MB-231 (human breast adenocarcinoma)	cytotoxicity, CD44 specific uptake	76



**Figure 3.** HA–CD44 binding interaction: The structure of the extracellular portion of CD44 is shown with important binding residues shown in gray (A front view, B top view). Saccharides 2–8 of a cocrystallized HA8 are shown in the link domain binding groove (\* reducing end). (Reproduced from Protein Data Bank structure reported in Banerji 2007.<sup>43</sup>)

express CD44 but do not bind HA until the CD44 is deglycosylated upon lymphocyte activation.<sup>30,33,34</sup> For CD44 to internalize via endocytosis it must be acylated.<sup>35</sup> Thus posttranslational modifications of CD44 greatly influence its ability to bind and internalize HA and must be considered during development of an HA-ligand targeted carrier.

Carriers, such as HMW-HA, can cross-link multiple receptors, potentially inducing binding and endocytosis. Drug potency is improved by increased cellular uptake of the drug–carrier complex and, for certain drugs, by circumvention of multidrug resistance efflux pumps.<sup>36</sup> Potency may also be improved by altering the drug’s location inside the cell so that the intrinsic activity of the drug in the carrier is higher than the intrinsic activity of the free drug.<sup>37,38</sup> A confounding difficulty for HMW-HA as a targeting carrier is that HA is cleared from circulation by the liver.<sup>39</sup> For HMW-HA to be a useful intravenous targeting carrier, strategies must be devised to reduce HA clearance from the blood.

Drug carrier–target cell interactions can be tuned to be effective only at sites of high receptor density by taking advantage of multiple ligands with moderate affinity but high specificity.<sup>40</sup> In the case of CD44, this can be achieved by employing short HA oligosaccharides as targeting moieties on a larger carrier.<sup>37,41</sup> CD44 is reported to interact with a minimum HA length of 6 to 8 saccharides.<sup>42</sup> The extracellular portion of CD44 with an octasaccharide HA positioned in the binding groove is illustrated in Figure 3.<sup>43</sup> Selecting HA oligosaccharides long enough to bind to CD44 but too short to bind to the HARE receptor may permit an HA-targeted carrier to avoid elimination by the liver while maintaining targeting to cells that overexpress CD44. This

is because short oligosaccharides maintain a high enough affinity with individual CD44 that binding to multiple CD44 by different HA on the same carrier creates an avidity strong enough for effective targeting (Table 4).

**2A. Anti-CD44 Antibody Conjugates.** The most effective strategy for CD44 targeted anticancer therapeutics currently under investigation is anti-CD44 antibodies that “actively” target drugs to CD44, disrupt CD44 matrix interactions and, by occupying CD44, induce CD44 signaling, which can cause apoptosis.<sup>44</sup> Anti-CD44 antibodies as targeting ligands for either radiolabels or anticancer chemotherapeutics in squamous cell carcinoma (SCC) of the head and neck (Table 2) stabilized disease progression in several patients.<sup>18,19</sup>

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Table 4. Targeted Drugs and Drug Carriers *in Vivo*

carrier	drug	injection method	tumor model	effect	ref
anti-CD44 antibodies	Re-186	intravenous	HNX-OE (human HNSC carcinoma) in HSD nude mice via subcutaneous implantation	delayed tumor growth	50
HMW-HA	epirubicin, mitomycin C	intraperitoneal, subcutaneous	MH-134 (murine ascites hepatoma) in C3H/He mice via subcutaneous foot pad or back injection LLC (murine lung carcinoma) in C57BL/6 mice via subcutaneous abdominal injection	decreased tumor size, inhibited lymph node and lung metastasis, decreased systemic toxicity	14
HMW-HA	butyrate	intraperitoneal, subcutaneous	LL3 (murine lung carcinoma) or B16F10 (murine melanoma) in BD2F1 mice via intrasplenic injection	reduced liver metastasis, increased survival time	57
HMW-HA	butyrate	intratumoral	LL3 (murine lung carcinoma) in BD2F1 mice via subcutaneous injection	inhibited tumor growth, reduced lung metastasis	64
HMW-HA	paclitaxel	intraperitoneal	RT-112/84 (human transitional cell carcinoma) in SCID mice via subcutaneous flank injection	inhibited tumor growth, decrease local toxicity	63
HMW-HA	paclitaxel	intraperitoneal	NMP-1 (human ovarian carcinoma) or SK-OV-3ip (human ovarian carcinoma) in HSD mice via intraperitoneal injection	reduced tumor burden, decreased systemic toxicity, increased survival time	62
HMW-HA	DNA	intramuscular	rat hind limb	transfection	74
liposome HMW-HA	mitomycin C, doxorubicin	intravenous	C26 (murine colon carcinoma) in BALB/c mice via footpad injection B16F10 (murine melanoma) or D122 (murine metastatic lung carcinoma) in C57BL/6 mice via intravenous tail vein injection	prolonged drug circulation time, increased drug accumulation in tumors, decreased metastasis, inhibited tumor growth, prolonged survival	69, 70
liposome HMW-HA	mitomycin C, doxorubicin	intravenous	P388/ADR (murine leukemia) in BDF <sub>1</sub> mice via intraperitoneal injection PANC-1 (human pancreatic carcinoma) in CD1-Nu mice via subcutaneous injection	prolonged drug circulation time, increased drug accumulation in tumors, decreased metastasis, inhibited tumor growth, prolonged survival	69, 70
PLGA particle HMW-HA	doxorubicin	intravenous	EAT (ehrlich ascites) in BALB/c mice via intraperitoneal injection	moderately increased tumor accumulation, weak tumor growth inhibition	71

A number of monoclonal antibodies capable of targeting SCC were identified.<sup>45</sup> The most specific antibody was developed as a clinical radioisotope carrier by converting it to a human/mouse chimera that had a slightly lower half-life in circulation but a much higher capacity for cell lysis in a mouse xenograft tumor model.<sup>46</sup> Later improvements led to antibodies specific for CD44v6, a variant overexpressed in SCC. The antibody VFF18 was shown to target SCC in spontaneous epidermoid carcinoma bearing nude mice.<sup>47</sup> This mouse monoclonal antibody (renamed BIWA 1) and a similar chimeric antibody, U36, were evaluated for targeting, pharmacokinetic parameters and safety in hu-

mans.<sup>48</sup> The monoclonal antibody BIWA 1 targeted SCC cells *in vivo*, was safe and localized to tumors; however, antimouse antibodies were found in patients.<sup>48</sup> The chimeric U36 antibody was also well-tolerated and resulted in the highest *in vivo* tumor targeting compared to several other monoclonal or humanized antibodies.<sup>49,50</sup> Treatment of refractory head and neck SCC patients with U36 conjugated to the radioisotope rhenium-186 (<sup>186</sup>Re) resulted in partially stabilized disease at the maximum tolerated dose.<sup>51</sup> To reduce immunogenicity, a humanized monoclonal antibody, bivatuzumab (BIWA 4), was developed. Bivatuzumab radiolabeled with either technetium-99m (<sup>99m</sup>Tc) or <sup>186</sup>Re was safely administered to patients. There was minimal toxicity and a minimal anti-antibody response.<sup>52,53</sup> Treatment of patients with <sup>186</sup>Re-BIWA 4 resulted in stable disease in three of the six patients at the maximum tolerated dose, but the tumor did not completely regress in any patient.<sup>52</sup> The <sup>186</sup>Re-BIWA 4 antibody conjugate was also well tolerated in patients with early stage breast cancer but did not target the tumor as effectively as in the patients with head and neck tumors. In the breast cancer patients, tumor localization appeared unrelated to CD44v6 expression and patients experienced unfavorable accumulation of the conjugate in nontarget organs.<sup>54</sup>

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Conjugation of BIWA 4 to a cytotoxic drug, mertansine, resulted in dose-limiting toxicities associated with skin-related disorders, including one fatal event of toxic epidermal necrolysis, which manifested by detachment of the epidermis from the dermis. This toxicity was most likely due to the presence of CD44v6 in the skin.<sup>17</sup> BIWA 4 antibodies (with or without conjugated mertansine) had half-lives of over 3 days, with low interpatient pharmacokinetic variability.<sup>18,19</sup> This slow elimination allowed the antibody to reach the skin as well as the targeted tumor. There was limited anti-antibody response in the patients which did not seem to influence the pharmacokinetics of the BIWA 4–mertansine. Stable disease was achieved in 50% of patients with CD44v6 positive metastatic breast cancer, who previously received anthracycline and taxanes, regardless which of eight dose levels they received.<sup>55</sup>

Although the clinical trials for BIWA 4 were stopped due to skin-related toxicities, the antibody–drug conjugate showed some clinical success (disease stabilization or tumor regression) in both SCC and metastatic breast cancer. Using this antibody as a targeting moiety on a drug carrier (which would have less access to the skin), thus altering its distribution via physical mechanisms, may allow for an alternative use of these antibodies as targeting ligands in a clinical setting.

**2B. Hyaluronan Conjugates as Drug Carriers.** HA has several chemical groups to which drugs can be conjugated.<sup>22</sup> The structure of HA, with possible chemical modification sites, is shown in Figure 1. The carboxylate on the glucuronic acid, the *N*-acetylglucosamine hydroxyl, and the reducing end have all been successfully utilized in conjugation reactions with drugs. The acetyl group may be enzymatically removed from the *N*-acetylglucosamine and so is also a potential site for drug conjugation.<sup>56</sup> Drug conjugation creates a macromolecular prodrug; the conjugated drug becomes active upon release from the HA. A summary of various HA conjugates with specific conjugation methods used, as well as the cell lines and details of the conjugated drugs or drug carriers, is listed in Table 3.

In many of the studies described below, uptake of HA conjugates was measured using radiolabeled conjugates in

which binding at 4 °C was compared to uptake at 37 °C,<sup>57,58</sup> fluorescent HA conjugates (such as FITC or BODIPY)<sup>14,28,29,59</sup> or the drug as a visualizing agent itself (doxorubicin).<sup>60</sup> These measurements provided direct evidence that HA conjugates were specifically and efficiently internalized into cells that expressed CD44.

The earliest reports of an HA–drug conjugate designed to specifically target overexpressed CD44 were performed by Akima *et al.*, who showed uptake of a fluorescent HA conjugate in Lewis lung carcinoma cells.<sup>14</sup> More importantly, this HA–drug conjugate decreased cancer progression in several models of cancer. HA-conjugated mitomycin c reduced systemic toxicity and allowed a higher relative dose to be administered in mice bearing tumors in the foot pad, although it did not decrease metastasis more than unconjugated mitomycin c. When treating primary ascites tumors implanted into the back of mice, HA–mitomycin c decreased tumor burden more than free mitomycin. An epirubicin conjugate showed no improvement. In a metastatic lung carcinoma model, HA–mitomycin c decreased the number of metastatic lung nodules at a much lower dose than free mitomycin c.<sup>14</sup>

Drug–HA conjugates have also been used to deliver drugs with physical properties that limit dosing. For instance, the antimitotic chemotherapeutic agent paclitaxel has low aqueous solubility. Conjugation of paclitaxel to HA increases drug solubility and, when incubated with cells that express CD44, a LMW-HA–paclitaxel conjugate increased cellular uptake and cytotoxicity *in vitro*.<sup>28</sup> CD44 negative control fibroblasts tolerated a much higher dose of conjugate drug than CD44 positive cells.<sup>28</sup> Uptake of HA-conjugated paclitaxel was dose-dependent and CD44-specific, as it could be blocked by both excess HA and anti-CD44 antibodies. Notably, uptake could not be blocked by chondroitin sulfate, a sulfated polysaccharide of glucuronic acid and *N*-acetylglucosamine similar to HA. Luo *et al.* found that HA–drug conjugates are internalized via CD44 and drug is released mainly by intracellular enzymatic hydrolysis.<sup>28,29</sup> Others have devised improved synthetic methods for HA–paclitaxel conjugates.<sup>61</sup>

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Auzenne *et al.* observed CD44-specific paclitaxel cytotoxicity *in vitro* in two human ovarian carcinoma cell lines, NMP-1 and SK-OV-3. *In vivo*, the HA–paclitaxel conjugate decreased tumor burden in mice bearing abdominal tumors of either cell type. HA–paclitaxel conjugate delivered intraperitoneally in the NMP-1 tumor model also moderately increased survival time compared to multiple doses of free drug. Preliminary *in vivo* toxicity studies showed that the HA conjugate was well tolerated.<sup>62</sup>

Conjugation of drug to HMW-HA can also serve to physically localize the drug. *In vitro* treatment of transitional cell carcinoma (superficial bladder cancer) cells showed a higher antiproliferative effect than free paclitaxel. Injection of the conjugate within the bladder did not improve therapeutic efficacy and only slightly decreased the amount of paclitaxel reaching the systemic circulation. However, the HA–paclitaxel conjugate had significantly decreased local toxicity.<sup>63</sup>

To improve the uptake of carboranes for boron neutron capture therapy the carborane was attached to HA. This conjugate was taken up specifically by CD44 positive cells, but the amount of conjugate internalized in each cell line was not directly proportional to the receptor density. Cells that expressed 10-fold fewer CD44 receptors internalized similar amounts of HA conjugate. Meo *et al.* suggested that cell lines with lower expression of CD44 may recycle CD44, increasing the uptake of the drug conjugate.<sup>58</sup> In this case, healthy cells that express low endogenous levels of CD44 would also take up the conjugate; however, activation of the carborane occurs only in regions irradiated by the neutron beam so that therapy can be concentrated in the tumor.

Attaching butyric acid, a histone deacetylase inhibitor, to HMW-HA targets the drug conjugate to CD44 expressing cells, leads to internalization and improves *in vivo* delivery by altering the release profile of the drug. Coradini *et al.* showed, in several human tumor models (breast, lung, melanoma and hepatocellular), that HA-conjugated butyric acid increased apoptosis, inhibited cell growth *in vitro* and decreased tumor burden *in vivo*. Initial studies of HA-conjugated butyric acid showed rapid cellular uptake in a human breast carcinoma cell line *in vitro*. This interaction was blocked by an anti-CD44 antibody.<sup>59</sup> Further *in vitro*

studies in both primary and metastatic nonsmall cell lung cancer cell lines also showed increased uptake of the HA–butyric acid conjugate. The most effective HA–butyric acid conjugate resulted in a 10-fold decrease in IC<sub>50</sub> compared to free butyric acid, suggesting that intracellular delivery of butyric acid, via the HA internalization pathway, increases cytotoxicity. Daily *in vivo* intratumoral injections both significantly decreased primary tumor mass and reduced metastases. Intraperitoneal and subcutaneous injection of butyric acid conjugates in intrasplenic hepatocellular carcinoma or melanoma mouse models resulted in fewer liver metastases.<sup>57</sup> Intratumoral treatment of nonsmall cell lung carcinoma bearing mice showed a decrease in the number of metastatic colonies in the lung.<sup>64</sup> However, intravenous injection resulted in rapid accumulation in clearance organs.<sup>57</sup>

HA–drug conjugates have effective cytotoxicity at a cellular level, but studies performed *in vivo* often did not employ intravenous dosing, opting for intratumoral, subcutaneous or intraperitoneal injection, and avoided many HA clearance mechanisms. The short half-life of HMW-HA conjugates<sup>57,65,66</sup> is most likely due to HA clearance from the blood by the HARE receptor present in the liver and spleen.<sup>5</sup>

Improvement in the distribution of HA conjugated drugs *in vivo* may be achieved by reducing endogenous HA blood clearance mechanisms. Luo *et al.* suggested that systemic HA clearance can be reduced by pretreatment with a high molecular weight saccharide that competes with HA for binding during clearance but not during target cell drug internalization.<sup>29</sup> HARE binds chondroitin sulfate and HA; CD44 binds only to HA.<sup>39</sup>

In rats with hepatic metastases of colon adenocarcinoma cells, pretreatment by intravenous injection of either chondroitin sulfate or HA resulted in higher tumor accumulation of a subsequent intravenous HA dose.<sup>67</sup> In perfused liver, preblocking with an anti-HARE antibody or immediate predosing with free HA resulted in decreased HA clearance of the second dose.<sup>68</sup> These results parallel the *in vitro*

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observations of Luo *et al.*, strongly suggesting that cancer treatments utilizing HA targeting to CD44 overexpressing tumor types may benefit from pretreatment with a HARE blocking moiety such as free chondroitin sulfate, unconjugated HA or antibodies.<sup>29,67,68</sup>

## 2C. Hyaluronan in a Targeted Nanocarrier System.

When attached to a nanocarrier, HA can act as a protective structural component, a small (oligosaccharide) targeting moiety and a targeting coating. Pharmacokinetic properties, such as circulation time and biodistribution, are influenced by incorporating the targeting and cell-specific uptake properties of HA onto large carriers. Cargos delivered to CD44 overexpressing cells include anticancer drugs: epirubicin,<sup>14</sup> doxorubicin,<sup>37,38,41,69–71</sup> paclitaxel,<sup>28,29,62,63</sup> and mitomycin c,<sup>14,70</sup> as well as agents such as siRNA,<sup>72</sup> iron oxide magnetic particles<sup>73</sup> and DNA.<sup>74</sup> These studies are summarized in Tables 3 and 4.

Several different methods have been used to improve the distribution of doxorubicin, which has significant cardiotoxicity as a free drug. Liposomes (nanosized phospholipid vesicles) bearing oligosaccharide HA conjugated to a phosphatidylethanolamine lipid have been used as a targeted drug carrier for doxorubicin.<sup>37,38,41,69,70</sup> Two different approaches to attach HA to liposomes were developed.<sup>41,75</sup> In the earliest report, HMW-HA was coupled via the glucuronic carboxylate to phosphatidylethanolamine in preformed liposomes.<sup>75</sup> This coupling method results in multipoint attachment of the HA to a liposome. The number and distribution of the resulting attachment points was difficult to characterize. The HMW-HA liposomes were originally proposed as a bioadhesive formulation<sup>75</sup> and then subsequently used to target cytotoxic drugs to tumors.<sup>69,70</sup> Peer and co-workers encapsulated doxorubicin or mitomycin c within the aqueous core of the HMW-HA liposome. Mitomycin c encapsulated in HMW-

HA liposomes increased cytotoxicity compared to nontargeted liposomes (but not free drug) in CD44 overexpressing cells.<sup>70</sup>

HMW-HA liposomes were very successful at decreasing tumor burden in several different tumor models. HA coated liposomes carrying either doxorubicin or mitomycin c also showed an increased circulation time over traditional, nontargeted liposomes<sup>69,70</sup> with a half-life slightly less than that of PEGylated “stealth” liposomes. This method of conjugation, in which a HMW-HA was attached to the liposome surface at multiple sites, avoided immediate clearance by the liver HA receptor. Both drug-loaded HA-coated liposomes increased drug accumulation in the CD44 expressing tumor, decreased systemic toxicity and increased survival time in multiple cancer models.<sup>69,70</sup> Disease regression included significantly decreased solid tumor growth and a reduced metastatic lung tumor burden in colon cancer models for mitomycin c<sup>70</sup> and doxorubicin containing targeted liposomes, increased survival in models of intraperitoneal ascites tumors and reduced tumor burden in a solid pancreatic tumor model.<sup>69</sup>

The second conjugation method attached oligosaccharide HA via the reducing end of a phosphatidylethanolamine by reductive amination.<sup>41</sup> A mixture of oligosaccharides of increasing disaccharide number was coupled to phosphatidylethanolamine.<sup>41</sup> The precise composition of the oligosaccharide mixture was not completely specified. This lipid-HA was incorporated at a defined surface density in the liposome and the binding of radiolabeled and fluorescent liposomes to cells that expressed different levels of CD44 examined. Liposome uptake was dependent on the expression of CD44. The uptake was CD44 specific and could be blocked by both free HA and anti-CD44 antibodies.<sup>41</sup> Liposome uptake was also dependent on the density of the HA oligosaccharide targeting ligand,<sup>37</sup> suggesting that receptor clustering might occur because of multivalent ligand presentation on the liposomal surface. Targeting was observed with as little as 0.1 mol % HA-ligand on the liposome. Encapsulated doxorubicin was significantly more cytotoxic to CD44 overexpressing cell types in culture than was free drug. Uptake of targeted liposomes was modeled and provided a quantitative confirmation of the hypothesis that the increased cytotoxicity of the HA-targeted liposome stemmed specifically from the internalization of the doxorubicin containing liposomes.<sup>37</sup>

Doxorubicin-containing PLGA (polylactide-co-glycolide) nanoparticles showed improved tumor accumulation when targeted with HA. In two separate studies, HA was conjugated to PLGA via a polyethylene glycol (PEG) spacer prior to particle formation<sup>71</sup> or directly onto the surface of a preformed particle.<sup>76</sup> Both types of coated nanoparticles showed improved sustained release compared to untargeted

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nanoparticles. Preformed particles also showed higher specific uptake and cytotoxicity in CD44 overexpressing cell types than in cells with low CD44 expression.<sup>76</sup> *In vivo* studies using particles with HA conjugated via a PEG spacer showed significantly decreased hemolytic and subacute toxicity profiles and modestly increased doxorubicin levels within the tumor.<sup>71</sup> However, there was no significant difference between the uptake of targeted and nontargeted particles in the liver. Most importantly there was no difference in tumor progression among the drug treated control, the targeted or nontargeted PLGA nanoparticle.<sup>71</sup> In this case, incorporating a targeting ligand onto the particle did not improve drug therapy.

Cross-linked HA particles have also been used for the protected delivery of DNA<sup>74</sup> and siRNA.<sup>72</sup> DNA incorporated prior to cross-linking of the HA carboxylate side chains was released from the particle upon exposure to hyaluronidase but not buffer. Release was sustained for several weeks. However, HA mediated CD44 specificity of transfection was not investigated. Rather, targeting was achieved by antibodies to specific selectins.<sup>74</sup> Thiosulfated HA nanogels cross-linked with a glutathione labile disulfide bond were developed for siRNA delivery. These nanogels had CD44 specific uptake. Release of siRNA by glutathione, an intracellular reductive agent, induced silencing of a fluorescent reporter protein gene in a colon carcinoma cell line *in vitro*.<sup>72</sup>

Results from the above studies using HA targeted nanocarriers are consistent with many of the studies performed on HA–drug conjugates and validate the potential of targeting CD44 to improve anticancer drug delivery.

**2D. Alternate CD44 Targeting Ligands.** Alternatives to HA for highly specific targeting ligands are also being investigated. Peptide mimetics of both HA itself<sup>77</sup> and a collagen triple helix peptide that interacts specifically with a chondroitin sulfate proteoglycan modification found on CD44 variants overexpressed in metastatic melanoma<sup>78</sup> were designed to interact with the HA receptors RHAMM and CD44 respectively. The CD44 specific peptide amphiphile was incorporated into liposomes via a C<sub>16</sub> tail and engendered specificity for CD44 positive cells, with liposome uptake correlating with the amount of CD44 on the surface of a human metastatic melanoma line studied *in vitro*.<sup>78</sup>

### 3. Targeting to the Hyaluronan Matrix

Based on the intimate relationship between tumor survival and the extracellular matrix, regulated in part by the CD44–HA interaction, HA itself is also a viable target for anticancer treatments. Manipulating the interaction between cancer cells and HA by disrupting HA binding to cell surface

receptors can sometimes lead to disease regression. HA within the matrix, acting as a reservoir for nascent HA binding proteins,<sup>79</sup> can be used as a conjugate–drug target site and provides a further opportunity for improved targeted chemotherapeutic delivery.

Degradation of the HA matrix by exogenously added hyaluronidase decreases tumor growth.<sup>80</sup> Intravenous injection of hyaluronidase into mice bearing human breast carcinoma tumors resulted in substantial tumor regression. Although the tumors were grown initially in the presence of an HA gel, and grew more slowly without an HA matrix,<sup>80</sup> this study illustrated the combinatorial effect of targeted treatment (hyaluronidase disruption of the HA matrix) and utilization of cancer biology (access of the hyaluronidase to the tumor, potentially via the EPR phenomenon). A conceptually simple method to increase the passive targeting of nanocarriers to tumors is to inject hyaluronidase into the tumor. The disruption of the HA matrix permitted increased delivery of intravenously injected untargeted liposomes containing doxorubicin in a human osteosarcoma solid tumor model.<sup>81</sup>

Disrupting binding of HA to the cell surface with soluble HA binding proteins can also decrease tumor progression. The soluble portion of HA binding receptors (either as individual molecules or as immunoglobulin fusion constructs) decreased tumor growth in several cell lines and animal tumor models. Administration of CD44–immunoglobulin fusion protein significantly decreased *in vitro* tumor cell growth in models of human melanoma<sup>82</sup> and lymphoma.<sup>16</sup> Soluble CD44–immunoglobulin constructs, injected intravenously<sup>16</sup> or delivered by slow release infusion pump implanted subcutaneously near the tumor,<sup>82</sup> decreased tumor growth and inhibited invasion.

Mammary carcinoma and malignant melanoma transfected to overexpress soluble CD44 had significantly decreased HA binding *in vitro* and slower tumor progression *in vivo*.<sup>83–85</sup>

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Transfected mammary carcinoma cells showed decreased monolayer invasion, HA binding and HA internalization.<sup>83</sup> When injected intraperitoneally, cells expressing soluble CD44 grew poorly and did not invade or form tumors on the peritoneal wall. When injected intravenously, the cells could both adhere and invade pulmonary stroma but underwent apoptosis before forming tumors.<sup>84</sup> Melanoma cells expressing soluble CD44 bound less HA *in vitro* and formed fewer tumors *in vivo*.<sup>85</sup> Decreased proliferation was not observed in all tumor cell lines since overexpression of soluble CD44 in human colon carcinoma cells increased proliferation *in vitro*.<sup>86</sup>

Addition of exogenous soluble RHAMM<sup>87,88</sup> and a peptide mimetic of RHAMM consisting of three repeats of an HA binding motif<sup>89</sup> inhibited tumor growth. *In vivo*, pretreatment of fibrosarcoma cells with soluble RHAMM prior to either subcutaneous or intravenous injection decreased primary tumor formation, limited metastasis, and decreased lung nodule formation.<sup>87</sup> *In vitro* studies using the RHAMM peptide mimetic suggested that soluble RHAMM may cause apoptosis.<sup>83</sup>

Soluble CD44 and RHAMM mutated to eliminate HA binding ability did not exhibit any antitumor effects, suggesting that interference with HA binding between the cell and the tumor HA matrix may also contribute to decreased tumor growth.<sup>84,85</sup> The above studies showed that decreased tumor growth by overexpression of soluble HA binding proteins is due to both increased apoptosis and physical mechanisms of CD44–matrix disruption.<sup>83–85,87,88</sup> These results suggest that either mechanism may be a potential avenue for improved anticancer therapeutics.

The tumor matrix can be a target for drug delivery. It is a multivalent ligand accessible from the blood due to the EPR effect. The concept of matrix attachment therapy is to

target the tumor matrix with a drug carrier or prodrug converting enzyme. This concept has a number of advantages over targeting the tumor itself: (1) The number of binding sites is very large so that a high concentration of a matrix targeted system can accumulate at the site. (2) The tumor cannot shed the matrix as it can shed a surface antigen. (3) The tumor cannot directly internalize the delivery system, as it could a system targeted to the cell surface. (4) Targeting the matrix may kill matrix-associated fibroblasts that supply growth factors that promote tumor growth. (5) Targeting the matrix may also kill local endothelial cells that provide a blood supply to the growing tumor. The combination of these mechanisms could have a synergistic effect to stop tumor progression.

To implement matrix attachment therapy, Park and co-workers<sup>15</sup> prepared a recombinant fusion protein consisting of a soluble-HA binding domain fused to a yeast cytosine deaminase. The fusion protein could convert the prodrug 5-fluorocytosine into cytotoxic 5-fluorouracil. When the fusion protein was injected intratumorally into a C26 colon carcinoma tumor model and the animals were supplied with 5-fluorocytosine in the drinking water, tumor progression was slowed and long-term survivors ensued. The presence of the drug, active enzyme and an HA binding component were all required to observe an antitumor effect.<sup>15</sup> This strategy of targeting the matrix may also be possible for other carriers, such as liposomes, polymeric micelles or nanocapsules.

#### 4. Conclusion

The importance of the CD44–HA interaction in tumor growth and progression provides multiple opportunities for intervention: targeting therapeutics to the CD44 receptor, interfering with the CD44–HA signaling pathway, removing the HA matrix with hyaluronidase to enable passive carrier uptake or targeting the HA tumor matrix to provide a sustained drug source within the tumor. Anti-CD44 antibodies targeting either radioisotopes or cytotoxic drugs have shown promise in human clinical trials. The targeting of drug-loaded liposomes with surface attached HA has shown promise in various animal tumor models. Alternative approaches, such as targeting the tumor matrix to disrupt CD44–HA signaling pathways or targeting matrix HA to provide a sustained source of drug, are promising research avenues in the quest for effective approaches for the control of tumor progression and metastasis.

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