REVIEW

Arch. Immunol. Ther. Exp., 2006, **54**, 85–101
PL ISSN 0004-069X

DOI 10.1007/s00005-006-0011-5

Monoclonal and bispecific antibodies as novel therapeutics

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Received: 2005.09.06, **Accepted:** 2005.11.30, **Published online first:** 2006.03.24

Abstract

Gene amplification, over-expression, and mutation of growth factors, or the receptors themselves, causes increased signaling through receptor kinases, which has been implicated in many human cancers and is associated with poor prognosis. Tumor growth has been shown to be decreased by interrupting this process of extensive growth factor-mediated signaling by directly targeting either the surface receptor or the ligand and thereby preventing cell survival and promoting apoptosis. Monoclonal antibodies have long been eyed as a potential new class of therapeutics targeting cancer and other diseases. Antibody-based therapy initially entered clinical practice when trastuzumab/Herceptin became the first clinically approved drug against an oncogene product as a well-established blocking reagent for tumors with hyperactivity of epidermal growth factor signaling pathways. In the first part of this review we explain basic terms related to the development of antibody-based drugs, give a brief historic perspective of the field, and also touch on topics such as the "humanization of antibodies" or creation of hybrid antibodies. The second part of the review gives an overview of the clinical usage of bispecific antibodies and antibodies "armed" with cytotoxic agents or enzymes. Further within this section, cancer-specific, site-specific, or signaling pathway-specific therapies are discussed in detail. Among other antibody-based therapeutic products, we discuss: Avastin (bevacizumab), CG76030, Theragyn (pemtumomab), daclizumab (Zenapax), TriAb, MDX-210, Herceptin (trastuzumab), panitumumab (ABX-EGF), mastuzimab (EMD-72000), Erbitux (certuximab, IMC225), Panorex (edrecolomab), STI571, CeaVac, Campath (alemtuizumab), Mylotarg (gemtuzumab, ozogamicin), and many others. The end of the review deliberates upon potential problems associated with cancer immunotherapy.

Key words: Bexxar, Gleevec, Imantib, Mitumomab, Tositumomab, Trastuzumab.

Abbreviations: ADCC – antibody-dependent cellular cytotoxicity, bsAbs – bispecific antibodies, CDC – complement-mediated cytotoxicity, CML – chronic myeloid leukemia, EGFR – epideremal growth factor receptor, ELISA – enzyme-linked immumosorbent assay, HGFR – hepatocyte growth factor receptor, HAMA – human anti-mouse antibodies, IFN – interferon, mAbs – monoclonal antibodies, NHL – non-Hodgkin's lymphoma, NSCLC – non-small cell lung cancer, PI3-K – phosphatidyloinositol 3-kinase, PDGF – platelet-derived growth factor, PSMA – prostate-specific membrane antigen, SRC – Rous sarcoma virus transforming oncogene, TAA – tumor-associated antigen, TNF – tumor necrosis factor, TK – tyrosine kinase, NRTK – non-receptor TK, TKI – TK inhibitors, VEGF – vascular endothelial growth factor.

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INTRODUCTION

In multicellular organisms, normal cells differentiate, proliferate, migrate, and survive or die at certain time points in a precise and controlled manner. A well-coordinated interaction between extracellular signals and intracellular response forms the basis of these events. Growth factors play a crucial role in these interactions. The lack of an appropriate survival or growth-promoting signal will by default lead to the activation of apoptotic process [92, 120, 130, 145]. This so-called "death by default" can be activated both through the

self-activation of death receptors (autocrine suicide) or by the activation of the mitochondrial/apoptosome--dependent/intrinsic death pathway [32, 57, 88]. Several strategies have been developed to explore the inhibition of signaling through cell growth or cell survival pathways. For example, peptides derived from "contact sites" between critical molecules within death-signaling and other pathways competitively inhibit these signals [22, 58, 77, 100].

Growth factors bind to cell surface receptors and regulate by activation of a chain of reactions inside of the cell. These signals then influence cell proliferation, differentiation, and survival. Thus, cell surface receptors can be called signal transducers, which convert an extracellular ligand-binding event into an intracellular signal that leads to an alteration of the cell's behavior. Withdrawal of growth factors often causes cells to undergo apoptosis. In contrast, inappropriate growth factor expression leads to loss of regulation of cell growth and prevention of apoptosis, which contributes to the development of cancer and other diseases [80, 83]. Uncontrolled regulation of proteins involved in the diverse signaling pathways activated by growth factors or alterations in the receptor kinases themselves are frequently found in various cancers. Furthermore, nonspecific functioning of growth factor receptors contributes to drug resistance by blocking apoptotic signaling pathways. Therefore, growth factors, their receptors, and all components of the involved signaling cascades are frequent targets for cancer therapeutics [11].

MECHANISMS OF ANTIBODIES USED AS THERAPEUTICS

There are a variety of potential mechanisms for the use of antibodies as therapeutic agents against cancer and other diseases. For example, antibodies may target specific components of tumor development, such as angiogenesis, growth factor receptors (e.g. EGFR), or ligand-receptor interactions, or via directly killing tumor cells by activating death receptor pathways [91, 152]. Additionally, antibodies can invoke an immune response by inducing complement-mediated cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). They can also inhibit tumor progression by directly inducing apoptosis [152] or by preventing the expression of proteins that are necessary for tumor development [154]. For example, the CD4 antigen has been used to block the function of specific molecules without killing the cells. This co-receptor, along with the T cell receptor complex, can bind MHC class II molecules, which initiate the immune response. Antibodies against CD4 can promote the tolerant state in T cells to suppress immune aggression and control T cells during the post-transplantation period [53]. A study by Taetle et al. [147] found that antibodies against interleukin (IL)-6 and transferrin receptors were also able to inhibit growth in factor-dependent myeloma cells.

Trauth et al. [152] and Yonehara et al. [165] originally reported using monoclonal antibodies (mAbs) to a human cell surface antigen (anti-FAS and anti-APO-1 mAbs) that gave a cytotoxic (apoptotic) effect similar to that of tumor necrosis factor (TNF)-α. The cell killing was independent of complement, and relied on the activation of the Fas/CD95/APO-1 death receptor. The Fas antigen is part of the TNF receptor family [71].

Shan et al. [140] demonstrated that proliferation of B cells could be inhibited, and apoptosis could be induced, when CD20 was extensively cross-linked with murine anti-CD20 mAb in the presence of cells expressing the Fc receptor. This strategy is being tested in an experimental therapy against CD20-expressing B cell lymphomas (see below).

Attempts to use antibodies against cancer began in the 1950's and relied on polyclonal antibody preparations, but the technology gained feasibility after 1975, when Kohler and Milstein developed techniques for the production of mAbs (identical antibodies directed against specific antigens). Initially, murine, rabbit and rat antibodies were studied; however, they had several associated problems. The host would often generate antibody response against these antibodies because of their recognition as foreign antigens. The antibody complexes that formed were then quickly cleared from the organism. Also, the immune response sometimes caused adverse effects such as "serum-sickness" and anaphylaxis. In addition, these antibodies were sometimes unable to stimulate cytotoxic humoral or cellular immune responses such as CDC and ADCC, which are necessary to destroy malignant cells. In order to overcome this problem, chimeric or "hybrid antibodies" were constructed by linking human antibody backbone regions with murine or primate variable regions. A more advanced version of this are "humanized antibodies", which consist of a human framework immunoglobulin (Ig) containing only rodent sequences encoding the three complementarity determining regions. These antibodies activated immune response (both CDC and ADCC) and displayed better performance in clinical trials [161].

Attempts have been made to further improve the cytotoxic activity of therapeutic mAbs. For example, conjugation of therapeutic mAbs with β-glucan markedly enhances the recruitment of complement and thus improves their CDC or ADCC activity. For example Herceptin, Rituxan, and Erbitux (Table 1) promote tumor regression by enhancing leukocyte-mediated killing of tumor cells coated with the iC3b-receptor. β-glucan functions here via the iC3b-receptor (found on tumors) and complement receptor 3 (found on leukocytes). Combinations of mAb and β-glucan have been shown to significantly increase tumor regression in models involving breast and liver tumors [64].

Dimeric and trimeric antibodies

Single-chain Fv antibody fragments (scFvs) can be engineered to form dimers and trimers by varying the

Table 1. Examples of mAb-based therapeutics, and "small molecule" inhibitors targeting the same pathways

Target	Name	Mode of action	Company
Abl, Src	BMS354825	Inhibitor of Bcr-Abl tyrosine kinase	Bristol-Myers
Abl, Src	AZD0530	Orally active Src kinase inhibitor	Astra Zeneca
Abl, Src	AP23464	ATP-analogue-based protein kinase inhibitor	Ariad
Abl, Src	SKI-606	Inhibits the tyrosine kinases Abl and Src causing a decrease in proliferation and onset of apoptosis in CML cells	Wyeth-Ayerst
Anti-idiotypic mAb, GD3 ganglioside mimetic	BEC2 (mitumomab)	Vaccine mimicking GD3 glycopeptide	ImClone Systems, Merck KGaA
Anti-iodiotypic mAb, CEA mimic	CeaVac	Stimulates immune response to CEA	Titan Pharmaceuticals
CA 125	Ovarex	mAb with high specificity to CA 125, an over-expressed TAA in a majority of ovarian cancers. Induces an immune response against CA 125	Altarex
CD20	Rituxan (rituximab)	Lysis of B lymphocytes through activation of CDC and ADCC	IDEC Pharmaceuticals, Genentech
CD20	Zevalin (ibritumomab tituxetan)	Radioimmunotherapy delivered by binding CD20	IDEC Pharmaceuticals
CD20	Tositumomab (Bexxar)	^{131}I -radiolabeled mAb capable of binding CD20 and delivering a specific dose of radiation and initiating immune response, targets B cells	Corixa; Titan Pharmaceuticals. GlaxoSmith-Kline
CD22	Epratuzumab (LymphoCide)	Binds to the extracellular domain of CD22 and induces internationalization and phosphorylation	Immunomedics
CD33	Mylotarg (gemtuzumab ozogamicin)	Chemoimmunotherapeutic agent targeting CD33 on leukemic cells	Wyeth Laboratories/AHP
CD33	Zamyl	Binds CD33 to induce immune response	Protein Design Laboratories
CD52	Campath (alemtuizumab)	mAb that specifically targets malignant lymphocytes. Binds CD52 and triggers antibody-mediated lysis of B cells	Millennium, BTG; ILEX Oncology; Hoffman--LaRoche
c-kit	Gleevec (STI 571, imantib)	Receptor-tyrosine kinase inhibitor	Novartis
EpCam	Panorex (edrecolomab)	Murine mAb targeting the epithelial cell adhesion molecule	GlaxoSmith-Kline, Centocor
Erb1/EGFR	Erbitux (certuximab, IMC225)	mAb that blocks the action of epidermal growth factor receptors (attaches to and blocks EGFR)	ImClone Systems, Merck KgaA
ErbB1/EGFR	EMD 72000 (mastuzimab)	mAb that blocks the action of epidermal growth factor receptors	Merck KgaA
ErbB1/EGFR	ABX-EGF (panitumumab)	mAb that blocks the action of epidermal growth factor receptors	Abenix
ERBB2/HER2/neu	Herceptin (trastuzumab)	Blocks EGF by attaching to HER2	Genentech
ERBB2/HER2/neu X CD64 (FcγRI)	MDX-210	Bispecific Ab that directs immune response against cells over-expressing ERBB2	Medarex, Immuno Designed Molecules
HMFG	TriAb	Anti-idiotype antibody targeting ovarian cancer. Mimics the HMFG antigen triggering immune response	Titan Pharmaceuticals
IL-2 receptor, CD25	Daclizumab (Zenapax)	mAb that blocks the action of IL-2 receptor	Protein Design Labs; Hoffman-LaRoche
PEM	Theragyn (pemtumomab)	Activates ADCC by targeting PEM (MUC1)	Antisoma
Src	CG76030	Inhibits the activity of Src and Abl kinase	Pfizer
VEGF	Avastin (bevacizumab)	Angiogeneis inhibitor	Genentech BioOncology

length of their polypeptide linkers. An scFv fragment with a linker length of 3–12 residues cannot fold into a functional Fv domain and therefore associates with another scFv molecule to form a bivalent dimer. Trimers and tetramers can be formed by further reducing the linker length. These molecules have the advantage of increased tumor penetration and faster clearance rates than the parental Ig due to their smaller size. Designing therapeutic antibodies to include the Fc domain prolongs serum half-life and complement-mediated effects. In addition, antibody fragments can be fused with a wide variety of molecules to alter functionality or introduce a secondary activity, such as radioisotopes for cancer imaging, enzymes for prodrug therapy, or lipids for improved systemic delivery [82].

Bispecific diabodies (also called bispecific antibodies) are formed through the association of two different scFv molecules, each containing a VH and VL domain (variable regions from both heavy and light chain) from a different parent Ig. The bispecific antibodies (bsAbs) are able to cross-link different target antigens either on the same cell or on two different cells, thus allowing more efficient recruitment of cytotoxic T cells to their target cancer cells or more effective activation of CDC. For example, Holliger et al. [63] designed bispecific diabodies that could cross-link colon cancer cells to serum Ig which then induced the complement cascade, including phagocytosis, while directing synergistic T cell cytotoxicity. The "classic" bsAbs antibodies are discussed below.

Arming antibodies for stronger cytotoxic effect

Direct arming of antibodies is a strategy to enhance the effectiveness of anti-tumor antibodies (Table 1). This is accomplished by covalently linking antibodies to molecules or proteins that are used to destroy tumor cells, such as radionuclides, toxins, or cytokines. Antibodies linked to cytokines are able to stimulate the anti-tumor immune response without the toxicity associated with systemic cytokine delivery. Antibodies can also be armed indirectly by attaching engineered antibody fragments to the surface of liposomes loaded with drugs or toxins for tumor-specific delivery [20]. For example, in preclinical studies, anti-HER2 scFv immunoliposomes containing doxorubicin showed increased retention in the circulation and improved efficiency compared with free doxorubicin, non-antibody conjugated liposomal doxorubicin, and the anti-HER2 mAb trastuzumab [115]. Another example is gemtuzumab ozogamicin, a humanized anti-CD33 mAb covalently linked to the cytotoxin calicheamicin. CD33 is expressed on early myeloid cells as well as leukemic blast cells, but is rarely expressed outside the hematopoietic system. This makes it an attractive target for use in therapy. Gemtuzumab ozogamicin binding to CD33 leads to endocytosis followed by cleavage of the covalent linkage between the antibody and calicheamicin inside the lysosomes. The calicheamicin is released and it induces sequence-specific cleavage of double-stranded DNA [46]. Gemtuzumab ozogamicin is also discussed in a paragraph below that is focused on the treatment of hematological malignancies. Calicheamicin is also being studied in conjugation with the murine mAb CTM01 for targeting the MUC1 antigen found on a variety of solid tumors of epithelial origin, such as breast, lung, ovarian, and colon cancers [55].

Radioimmunotherapy employs radiolabeled antibodies. The antibodies serve here as a vector targeting tumor antigens. For example, radioactive anti-carcinoembryonic antigen antibodies are used in the treatment of colorectal cancer. This strategy works through the accumulation of high energy β-particles within the tumor which are emitted from a radionuclide (^{131}I, ^{90}Yt, ^{111}In). Using this method in conjunction with a gamma-detector also makes it possible to locate and stage tumors [166]. Recently, the first radioimmunoconjugate was approved for the treatment of non-Hodgkin's lymphoma (NHL). Zevalin is a ^{90}Yt-labeled anti-CD20 mAb that incorporates β-emitting radioisotopes [122].

As another example serves antibody-directed enzyme prodrug therapy, a strategy that employs enzyme immunoconjugates to locally activate prodrugs. This method uses antigens that are present on the tumor cells to target enzymes to the tumor site. After the enzyme-antibody conjugate has been delivered and has had sufficient time to bind target cells and be cleared from the system, a prodrug is administered and activated extracellularly at the tumor site [124]. An example of this is the rituximab-alliinase->alliin->allicin therapeutic system. Rituximab is a chimeric mouse/human mAb designed to treat NHL. It recognizes the CD20 antigen, which is expressed on malignant and normal B cells but is not found on other tissues [50]. The effectiveness of rituximab can be enhanced by covalently linking (arming) this antibody with the enzyme alliinase. The alliinase-rituximab complex specifically binds B cells and lymphoma cells. This is followed by injection of the chemical alliin (found in plants such as garlic and onion), which is converted by the alliinase to allicin. Allicin then penetrates the tumor and kill cells. More examples of "armed antibodies" can be found below in paragraphs describing tumor site-specific treatment.

Bispecific antibodies

Because bsAbs recognize two distinct antigens, they can stimulate effector cells to direct their cytotoxic effects against tumor cells. To accomplish this, a bsAb must directly bind a target cell to a triggering molecule on the immune effector cell, such as a cytotoxic T cell. A wide variety of effector responses can be directed against tumor cells by changing the specificities of the anti-effector and anti-target components of the bsAb. Two examples of cytotoxic triggering receptors are the TCR and the Fc receptors [138]. For example, CD3×CD19 bsAbs have been used in combination with CD28 monospecific antibodies for the activation of

autologous T cells in the lymph nodes of patients with low-grade NHL [96].

Another example of a therapeutic bsAb is MDX-210, a chemically cross-linked, partly humanized antibody that binds to FcγR1 and the HER2/neu oncogene product [158]. MDX-210 will recognize FcγR1 on monocytes and macrophages as well as the HER2/neu oncogene product on breast, ovarian, and other malignancies [125]. HER2/neu oncogene targeting is discussed to a greater extent in the following paragraphs, and death-receptor-directed bi-specific antibodies are discussed in detail in the paragraph below that focuses on death receptors.

Antibody-based targeting of tumor progression

This approach aims to achieve the control of tumor progression (tumor stasis) rather than complete elimination of tumor cells (cure). Three treatment targets discussed are angiogenesis, invasion (tumor cell adhesion), and cell signaling pathways.

Matrix metalloproteinases (MMPs) are a family of enzymes required for angiogenesis and metastasis, as they mediate turnover and remodeling of the extracellular matrix. A group of anti-angiogenic agents has been designed to target MMPs in an attempt to stop angiogenesis. One strategy to inhibit invasion centers on targeting proteins that mediate adhesion, for example integrin [7]. Integrin promotes cell adhesion, which is required for normal cells to grow. When this cellular attachment is lost, apoptosis is generally initiated. Cancer cells are able to circumvent apoptosis in the absence of integrin by increasing levels of integrin-linked kinase. Integrin-linked kinase inhibitors have been found to stop tumor growth and metastasis in mouse studies, which may indicate clinical applications for this approach.

TUMOR-RELATED ANTIGENS

Oncogenic transformation can occur by several means, including mutations within the components of stimulatory pathways, alteration of DNA-damage control machinery, cell-cycle progression, or by changes within genes that control programmed cell death [11, 114, 119]. The acquisition of the malignant phenotype is usually accompanied by a change in antigenicity due to the expression of "tumor-specific antigens" (TSA) [160]. Furthermore, genetic instability, which frequently occurs in cancers that have acquired mutations within the myc proto-oncogene, or within proteins that guard cell-cycle check-points, contribute to the creation of new fusion proteins with antigenic properties [114]. These are the changes that form a basis for modern mAb-based therapies. More commonly, tumors express tumor-associated antigens (TAA), which are present on tumor cells and on normal cells during fetal development [160]. Also, the etiology of some cancers may be related to viral infections. For example "hairy" T cell leukemia is caused by human T cell leukemia virus [90], certain non-Hodgkin's B cell lymphomas are caused by Epstein-Barr virus, and cervical cancer may be caused by certain strains of papillomavirus. Most likely, the viral infection only predisposes to, rather than solely causes, the cancer to occur. These malignancies may occasionally express "viral antigens" (proteins completely foreign to our cells, encoded by viral genetic material) [24].

Solid tumors

The human epidermal growth factor receptor-2 (EGFR2)/HER2 gene is over-expressed in approximately 25–30% of breast cancers [142, 143] (Table 1). The HER2 gene (also known as neu or c-erbB-2) encodes a transmembrane glycoprotein receptor ($p185^{HER2}$) that has partial homology with the EGFR and contains intrinsic tyrosine kinase (TK) activity with this receptor [2, 29, 151]. Interestingly, when compared with normal breast epithelial cells, the HER2 gene may have up to 100-fold greater expression [142]. Trastuzumab (Herceptin) is a mAb that targets the antigen p185Her2, the extracellular domain of EGFR2 [21]. Similarly, EGFR (also known as HER1 or c-erbB-1) displays greater expression in many forms of cancer and appears to be involved in disease progression [61, 164]. Cetuximab (Erbitux), a chimeric mAb that binds HER1, prevents signal transduction and induces apoptosis and inhibition of cell growth [16]. Therapeutic targeting of EGF-dependent signal transduction pathways is discussed in detail in one of the following paragraphs, which is solely dedicated to this aspect.

A murine IgG2A mAb termed edrecolomab (Panorex), is being used in the treatment of colorectal cancer [1, 54, 137]. Edrecolomab is a mAb that targets the human epithelial cell adhesion molecule (Ep-CAM or CO17-1A) and is the first mAb approved for cancer treatment in Germany [1]. In addition to edrecolomab, a new recombinant IgG1 antibody, MT201 shows promise as an additional therapy targeted against the Ep-CAM antigen [108].

Solid tumors can be efficiently, although not curatively, targeted by interfering with their blood supply. Bevacizumab (Avastatin), a recombinant humanized mAb anti-vascular endothelial growth factor (VEGF), prevents vascularization of tumors by binding to and inhibiting the VEGF-receptor [157]. In this manner, bevacizumab inhibits growth of the microvasculature supplying the tumor, thereby indirectly terminating tumor growth. Bevacizumab has been validated in a phase III trial for the first time as an anti-angiogenic strategy in patients with previously untreated metastatic colorectal cancer [69]. Targeting of VEGF is discussed in greater detail in a paragraph solely dedicated to VEGF.

CeaVac is an anti-id antibody to the carcinoembryonic antigen [39] that specifically recognizes an epitope on CEA, present on malignant cells in 95% of all cases

of colorectal cancer, 70% of lung adenocarcinomas, and 50% of breast cancers [70] (Table 1). In a study of 32 patients, CeaVac displayed humoral and cellular immune response in all patients through measurement of IgG titers and T cell proliferation [39].

Prostate-specific membrane antigen (PSMA) is a cell surface peptidase that is expressed by malignant prostate epithelial cells and vascular endothelial cells but not normal endothelium in benign tissues or non-prostate malignancies [107]. PSMA expression is greatly increased in primary and metastatic prostate cancer [125]. A mAb for the external domain of PSMA, huJ591, which is radiolabeled with various radionuclides, is in preclinical and phase I trials [3, 87, 101].

Hematological malignancies

B and T cell surface antigens found on leukemia or lymphoma cells are eyed as targets for therapeutic mAbs. Two radiolabeled mAbs licensed for use are ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar) [23]. Ibritumomab tiuxetan is a murine antibody that is conjugated to ^{90}Y and tositumomab is a murine antibody conjugated to ^{131}I[84]. Both of these antibodies target the CD20 antigen on the cell surface and are used in the treatment of NHL [84]. The CD20 surface antigen is also targeted by a chimeric antibody, containing murine light- and heavy-chain variable region sequences and human constant region sequences, known as rituximab (Rituxan, Mabthera®), which is not conjugated [42, 49, 125].

The CD33 antigen was originally identified on human myeloid cells and consists of a 67-kDa glycoprotein [117], the expression of which appears to be related to a potential role in the regulation of differentiation of myeloid cells [40]. Nevertheless, anti-tumor compounds such as gemtuzumab ozogamicin (Mylotarg®) recognize CD33 on the surface of cells of the hematopoietic system [40]. Gemtuzumab ozogamicin is a conjugated mAb that is linked to the DNA-cleaving antibiotic ozogamicin-γ^{I} [73, 167]. Gemtuzumab ozogamicin was approved for treatment of relapsed myeloid leukemia in 2000 by the Food and Drug Administration (FDA) [10].

Alemtuzumab (Campath) is a humanized mAb designed for the treatment of B cell chronic lymphocytic leukemia in patients unresponsive to fludarabine therapy [36, 113]. Alemtuzumab targets CD52, a cell surface glycoprotein that is highly expressed on the surface of normal and malignant B and T lymphocytes [47, 125].

The IL-2 Tac receptor is expressed in adult T cell leukemia and hairy T cell leukemia [125]. This receptor can be targeted by dacluzumab (Zenapax), a chimeric mAb that is predominantly used for the prevention of transplant rejection and other chronic inflammatory conditions [125].

The CD22 antigen is a B cell restricted transmembrane sialoglycoprotein [27]. This antigen is targeted by epratuzumab (LymphoCide), a chimeric mAb that is in clinical trials for the treatment of NHL [125].

Virtually all B cells express CD19, the function of which is Ig-based activation and proliferation [118]. Two anti-CD19 antibodies, HU37 and BU12, were tested and radiolabeled with ^{90}Y in nude mice [153]. The results support the use of ^{90}Y-labeled anti-CD19 for treatment of B cell malignancies [153]. Quite interestingly, an α-helical amphipathic peptide termed D-$(KLAKLAK)_2$, when conjugated to an anti-CD19 mAb, effectively killed B lymphoid cell lines [97].

As shown by the above examples, TAA-directed antibodies are increasingly being used in the clinic. It can be predicted that as researchers identify more TAAs, the mAb-based treatment of cancer and also autoimmune diseases will become more common.

THERAPEUTIC CELL-SELECTIVE TARGETING OF DEATH RECEPTORS WITH BISPECIFIC ANTIBODIES

In most cases, mAbs directed against certain cell surface receptors imitate the physiological function of the natural ligand instead of blocking it. These so-called "bio-mimetic" antibodies can exert powerful biological effects by modifying a targeted biological response. Biomimetic antibodies are used as effector agents in the form of bsAbs in order to induce a target cell-restricted activation of the desired immune receptors. In a plethora of clinical trials using these reagents, apparent therapeutic effects were observed, in particular if the bsAbs were administered locally [18, 75, 110]. After systemic administration, toxic side effects were apparent such as the cytokine release syndrome, which is induced in particular by bsAbs containing an anti-CD3 specificity [72, 104, 150]. Recently there has been a shift towards recruitment of immune system elements and the subsequent induction of apoptosis in consequence of inflammatory processes directed against tumor-associated and TSA contemporarily with death receptors in high amounts [8, 74, 79]. Theoretically, there is a robust potential to use the death receptors such as Apo--1/Fas/CD95, CD40, CD30, TNF receptors, and TRAIL receptors, not only in cancer, but also in other diseases [43, 89]. Since there is compelling evidence available for CD95 in this aspect, we will converge more towards this death receptor.

Biomimetic anti-CD95 antibodies are capable of prompting apoptosis in CD95-positive and -sensitive target cells; however, the administration of such antibodies in mice vastly killed the animals by instigating massive liver cell death [112]. In light of these results, target cell restriction is necessary if augmentative anti--CD95 antibodies are used for tumor cell killing *in vivo*. Since the agonistic activity of anti-CD95 antibodies requires antibody multimerization, the employment of bsAbs would be indicative. Jung et al. [76] constructed bispecific $F(ab')_2$ fragments directed to CD95 and different TAAs expressed on cell lines of the B cell lineage (such as CD19, CD20 and CD40) and found that these antibodies are able to induce conspicuous apop-

tosis in the SKW6.4 cell line carrying these target antigens. CD95-sensitive Jurkat cells that do not express the above-mentioned tumor antigens were left unaffected; however, Jurkat cells were killed by apoptosis as onlooker cells if SKW6.4 cells were present during the lytic assay [76]. By using solid tumor-derived cell lines with reduced expression of CD95, selective cell killing could still be observed. The same group also found that under these more austere conditions, some tumor antigens, such as melanoma-associated proteoglycan, are better than other partners (such as the EGFR) for CD95 antibody-based bispecific constructs. They also found that the efficacy of such a construct depends on properties of the target antigen other than its expression level on the cell surface, for instance its spatial configuration within the membrane. Thus the activity of a given bsAb is difficult to predict. More importantly, it was ascertained that the killing of solid tumor cells was generally less imposing and significantly depended on reagents that sensitize targets for apoptosis, such as cycloheximide. This is not surprising, since tumor cells are often resistant to Fas-mediated cell death either because the molecule *per se* or the components of the intracellular Fas signaling cascade are mutated or down-regulated during the process of tumorigenesis [15, 119, 162]. The application of the above-portrayed principle to death receptors other than CD95, e.g. receptors for TNF (TNF-R I and TNF-R II) or for its immediate relative member, namely TRAIL (DR4 and DR5), would be remunerative. Wajant et al. [155] reported that a recombinant fusion protein composed of a targeting antibody (directed against the tumor-associated FAP protein) and TRAIL induced apoptosis preponderantly in cells expressing TRAIL-R2 (DR4). TRAIL-R1-expressing cells could not be killed selectively since this receptor is triggered by soluble TRAIL protein. Once again, the problem of target cell-restricted selective stimulation arises. More recently, the same group engendered a fusion protein which consisted of the anti-Fas-associated phosphatase (FAP)-1 antibody and FasL. Interestingly, they opposed the growth of human tumor cells in a nude mouse model. Notably, the fusion proteins did not induce toxicity in mice unless they were cross-linked with an anti-FLAG antibody, indicating that this protein may wield that type of selectivity required for successful *in vivo* application [131]. Although this abstraction can be painlessly drawn out to other target cells and other death receptors, it is conceived that selective Fas stimulation on tumor cells is particularly indulging. In addition to the selective induction of apoptosis, it may touch off the origination of an inflammatory and anti-angiogenic response that would make the immune system aware of the menace. If these effects could be induced *in vivo* in a tumor cell-restricted manner, an auspicious new class of bispecific reagents would become available for experimental or even clinical tumor therapy based on apoptosis.

MONOCLONAL ANTIBODIES AS BLOCKERS OF SIGNALING THROUGH GROWTH-FACTOR RECEPTORS

Many studies have shown that mAbs can be used for therapeutic purposes to block growth factor receptors that are over-expressed on cancer cells, including EGFR, TGFαR, VEGFR, IGF-1R, HGFR, Brc-Ab1 kinase, PDGF-R, and c-kit [19, 25, 86, 103, 146]. Antibodies are also being tested for the targeting of tumor angiogenesis. Thus, besides VEGF, targets for mAb-based anti-angiogenic therapies include the basic fibroblast growth factor, several other growth factor receptors, and a number of cell adhesion molecules [144]. To date, 17 antibodies have been approved by the FDA, and 8 of them are used to combat malignant diseases (Table 1) [144].

Receptor TKs

Receptor TKs are transmembrane enzyme-linked cell surface receptors with an extracellular ligand binding domain, an intracellular TK domain, which phosphorylates specific tyrosines on a small set of signaling proteins, and a carboxyl terminal segment with multiple tyrosine residues for autophosphorylation. All 16 structural subfamilies, each dedicated to its complementary family of protein ligands, function in the same way. The binding of a signaling protein to the ligand binding domain on the outside of the cell triggers receptor dimerization or oligomerization of the receptor, which causes an intracellular rearrangement of the kinase domain and therefore its activation. The neighboring activated kinase domains first cross-phosphorylate each other on multiple tyrosines (autophosphorylation) and then activate other proteins by transferring a phosphate group from ATP. Most of the proteins activated by the receptor TKs are also kinases. Their activation leads to an expanding cascade of phosphorylations within the cytosol, which leads either directly to phosphorylation of transcription factors, thereby activating cell growth, proliferation, differentiation and cell survival, or indirectly to the activation of a second messenger, which mediates cell behavior.

One of the most frequently deregulated receptor TKs in human cancers are members of the epidermal growth factor ErbB receptor family (ErbB1/EGFR, ErbB2/HER2/neu, ErbB3/HER3 and ErbB-4/HER4), which regulate cell proliferation through the Ras-mitogen-activated protein kinase pathway and cell survival and transformation through the phosphatidylinositol 3-kinase (PI3-K)/AKT pathway [136]. Of the four HER family receptors, most attention has been given to ErbB1/EGFR and ErbB2/HER 2/neu [11, 100].

Targeting ErbB2/HER2/neu. Several laboratories have developed mAbs (mumAb4D5) recognizing the extracellular domain of the HER2/neu proteins generated by a HER2 over-expressing NIH 3T3 line. These

antibodies were able to inhibit growth of breast cancer cell lines via ADCC [85]. In 1992, Carter et al. [21] created a recombinant humanized version of mumAb4D5, called rhumAb4D5, to eliminate the risk of immunological consequences of mouse antibodies administered to humans. RhumAb4D5 achieves a 3-fold higher affinity for ErbB2/HER2/neu and is able to successfully mediate ADCC in breast tumor lines. RhumAb4D5, named trastuzumab or herceptin, became the first clinically approved drug against an oncogene product as a well--established blocking reagent for EGF signaling, activating pro-apoptotic pathways and therefore causing anti--tumor activity and increasing sensitivity to chemotherapy and radiotherapy. In addition, trastuzumab increases the amount of hypoxia-induced cell death in breast cancer cells. Experimental evidence further indicated that trastuzumab enhances the responsiveness of ERBB2--overexpressing breast cancer cells to taxanes, anthracyclines, and platinum compounds (cisplatin, doxorubicin, paclitaxel) [106]. Moreover, a combined treatment with cyclooxygenase (COX) 2 inhibitors might be promising [95] due to the correlation between ErbB2/HER2/neu and prostaglandins [52]. Treatment with trastuzumab is well tolerated. Low-grade fever, chills, and fatigue have been observed frequently, mostly with the first administration. Unfortunately, a combinatorial treatment of trastuzumab with doxorubicin or paclitaxel causes increased cardiotoxicity by an unknown mechanism.

Targeting ErbB1/EGFR. Certuximab (IMC225, Erbitux™) is one of the first mAbs, which binds competitively, and with high affinity to ErbB1/EGFR to have entered clinical trial. It is a chimeric humanized mAb and is applied in different kinds of cancers, such as head and neck, colorectal, non-small cell lung cancer (NSCLC), and pancreatic cancer. In all cancers so far tested, certuximab successfully increased the average survival rate of patients and stabilized the progression of the disease when combined with other drugs. In head and neck cancer, certuximab has been tested in combination with chemotherapy and radiotherapy [17]. Furthermore, a combination of certuximab with cis/carboplatin and 5-fluorouracil showed an increased disease control rate. In colorectal cancer the combination of certuximab and irinotecan was proven to significantly increase the response rate and time to progression of the disease [30]. In NSCLC a combined treatment of certuximab with paclitaxel and carboplatin or gemcitabine and carboplatin lead to an increased time-to--progression and survival rate. The same results could be obtained by combining certuximab and doxetaxel, achieving a 66% rate of stable disease. A phase II trial of certuximab and gemcitabine has recently been published for treatment of pancreatic cancer [163] and demonstrated the same increased rates of time-to-progression, one-year progression-free survival, and overall survival rate. Mucosotis, which is the most common radiation-related side effect, was not worsened by concomitant cetuximab.

Phase II studies focusing on ABX-EGF (panitumumab), which binds the EGFR with high affinity, revealed promising results, mainly stabilization of disease progression, with dose-dependent skin toxicity as the main side effect [127]. Another humanized antibody against the EGFR is EMD 72000 (mastuzumab). A phase I study of combinatorial treatment with EMD 7200 and gemcitabine in advanced pancreatic cancer has shown a stabilization of disease progression in 11 of 17 patients.

A new strategy in targeting the EGFR pathway is a combined treatment of mAbs and TK inhibitors (TKIs). An increased inhibition of EGFR-dependent signaling and, therefore, an induction of apoptosis could be achieved by a combinatorial therapy with gefitinib [98] or erlotinib [66] and certuximab. In addition, the concomitant use of two mAbs (two-antibody therapy) might be a promising strategy of inhibiting tumor growth and progression of disease. Monoclonal antibodies either target different parts of the same receptor or different ErbB receptors with the goal of interfering in the formation (dimerization) of active signaling complexes.

Targeting the vascular VEGF. VEGF is a member of the platelet-derived growth factor (PDGF) family. It stimulates angiogenesis and lymphangiogenesis and increases the permeability of vascular endothelium. VEGF receptors are highly expressed in vascular endothelial cells and play an important role in the regulation processes of physiological and pathological growth, development, and maintenance of blood and lymphatic vessels. Over-expression of VEGF was found in various tumor samples and correlates with a high expression of VEGF receptors, which leads to an increase in tumor proliferation rate and poor survival. Bevacizumab (Avastin™) is a humanized mAb against VEGF which inhibits VEGF-induced uncontrolled angiogenesis and lymphangiogenesis and, therefore, the formation and growth of tumors. Hurwitz et al. [68] showed an increase in median survival, progression-free survival, response rate, and duration of response in a phase III trial with colorectal cancer patients. Toxicity, mainly in from of hypertension, was low. Furthermore, renal cancer represents a suitable target for bevacizumab, because its main cause is an upregulation of VEGF, induced by a biallelic loss of the von Hippel-Lindau tumor suppressor gene. In contrast, no significant differences in a phase III study with metastatic breast cancer patients could be seen. The use of bevacizumab in combination with gemcitabine for pancreatic cancer is currently under investigation. In head and neck carcinoma, angiogenesis has been linked with tumor progression. That is why a combinatorial treatment with mAb and TKI might be a successful approach in this case. Current studies evaluate a combined treatment with bevacizumab and erlotinib [99] and could demonstrate an increase in disease stabilization. Bevacizumab and erlotinib are also being tested in NSCLC, metastatic renal cancer, and breast cancer.

Targeting the c-kit receptor TK. The mAb imanitib

(STI 571, Gleevec™), primarily designed to treat chronic myeloid leukemia (CML) by targeting the non-receptor TK Abl, is also an inhibitor of a receptor TK, the c-kit kinase. C-kit TK receptor is a member of the type III receptor TK family that also includes, amongst others, the PDGF receptors. The c-kit receptor is a mast and stem cell growth factor receptor, but is expressed in other tissues as well. In gastrointestinal stromal tumors, a frequently expressed constitutively activated form of the c-kit receptor has been identified. Therefore, imanitib is currently the drug of choice in gastrointestinal stromal tumors therapy. Other tumors with c-kit-activating mutations are currently being evaluated for treatment efficacy by Gleevec.

NON-RECEPTOR TKIS – "SMALL MOLECULES" IN ACTION

Beside the receptor TKs discussed in the last paragraph, non-receptor TKs (NRTKl; e.g. SRC, ABL, FAC, JAC 2 and SYK) [81] have become increasingly eyed as targets for anticancer therapy. NRTKs are mostly cytoplasmic proteins and thus cannot be easily reached by antibodies; therefore, cell-permeable chemical compounds, so called "small molecules", are instead being applied as inhibitors. NRTKs are generally composed of at least two regions. In addition to the kinase domain, NRTKs have ATPase activity and several signaling and protein-protein interaction domains such as SH2, SH3, and the PH domain [38, 93, 135]. The TK kinase domain is approximately 300 residues comprising the N-terminal region and is composed of a 5-stranded β-sheet and one α-helix. The C-terminal domain is a large cytoplasmic domain composed mainly of α-helices [60]. For activation, ATP binds in the cleft between the two regions and then the tyrosine-containing sequence of the substrate protein interacts with the residues of the C-terminal region. The activation process also involves heterologous protein-protein interaction which enables trans-phosphorylation [149]. Increased understanding of the molecular pathophysiology of cancer identified NRTKs as prominent oncoproteins. Since then, the development of therapeutic agents for a variety of cancers was directly linked to control abnormal activation of TKs, whether the deregulation was due to enhanced expression, mutation, or auto-activation stimulations leading to abnormal downstream oncogenic signaling [116].

Rous sarcoma virus-transforming oncogene (SRC), a NRTK, is either over-expressed or constitutively active in a large percentage of colon and breast cancer patients [109]. The increased expression or activity correlates with the stage and metastatic potential of some neoplasia. In spite of the fact that details of several steps in SRC's oncogenic pathways still remain elusive, the knowledge of SRC activation facilitates the development of strategies to potentially inhibit SRC's pathways [133]. The rational is to design drugs that interfere with binding of either the ATP molecule or the substrate proteins: Two main approaches were established: 1) the development of SRC homologues incorporating non-hydrolyzable phosphotyrosine mimics and exhibiting molecular recognition and targeting properties. This approach targets the tyrosine-binding SH2 domain in SRC, where the non-peptide ligands illustrate the systematic replacement of the phosphate group by multiple nonhydrolyzable, mono- or di-anionic functional groups. Specifically, these are phenylalanine (Phe) analogs composed of 4' and 3' substituents. Several compounds were synthesized and incorporated into a bicyclic benzamide template including 4',3'-diphosphono-Phe, 4',3'-dicarboxymethyloxy-Phe, and 4'-phosphono-3'-carboxymethyloxy-Phe [14]. 2) The second approach focuses on the development of ATP-analogue-based SRC kinase inhibitors, a strategy that is also widely used to develop non-targeted TK inhibitors. ATP-based inhibitors display antiproliferative activity by blocking cell-cycle progression and eventually promote apoptosis. This strategy targets the ATP binding site in the kinase domain, where the adenine region contains two hydrogen bonds formed by the interaction of N-1 and N-6 amino group of the adenine ring. The two hydrogen bonds are frequently targeted in ATP-based inhibitors. However, the inhibitors designed according to the above principle lack the specificity. In contrast, ATP-inhibitors may be designed to target the phosphate region in the kinase to favor improving the selectivity of the inhibitor towards a specific kinase [28]. Examples of such approaches are being carried out by ARIAD Pharmaceuticals, which led to the development of the two *in vivo*-active compounds AP-22408 and AP-23236.

Another example for NRTKs is the oncoprotein c--Abl. Several decades ago, the Philadelphia chromosome was identified and is characterized by a reciprocal translocation between chromosomes 9 and 22 [37, 128, 132]. This translocation links the *c-abl* TK oncogene on chromosome 9 to the 5' half of the *bcr* gene on chromosome 22 and creates the fusion gene *bcr-c-abl* [59, 129]. The fusion gene produces a chimeric 8.5-kb transcript that codes for the p210bcr-abl protein. The product of the p210bcr-abl gene is a constitutively active TK leading to continuous activation of both growth and anti-apoptotic pathways [51]. The Philadelphia chromosome and *bcr-c-abl* translocation are found in >95% of CML and some acute lymphoblastic leukemias [129].

For decades, the modes of treatment for CML involved interferon (IFN)-based therapy, chemotherapy, and stem cell transplantation, which was the only proved curative therapy. During the past decade the anticancer drug imatinib mesylate was developed (STI571, Gleevec; Novartis, Basel, Switzerland). Imatinib is a small molecule inhibitor for the BCR-ABL TK [11]. It is a phenylaminopyrimidine-based compound that prominently inhibits the TK ABL and also PDGF receptor TKs [51]. This inhibition is relatively selective, but also extends to the c-kit protein, another TK [34]. In contrast to other CML therapeutic

approaches, in the phase I trial for imatinib, CML patients who were resistant or intolerant to IFN-α treatment produced complete hematological responses. 98% of chronic-phase patients and 55% of those in blast phase displayed significant improvement [35]. Further clinical testing has confirmed these striking results [78, 111, 134, 148]. Furthermore, a possible survival benefit was anticipated from using imatinib due to the high rate of hematological responses and marked reduction in patients who had already progressed to accelerated or blast phases of the disease [33]. On the molecular level, the clinical benefits of the drug were confirmed by classical cytogenetics approaches such as fluorescence *in situ* hybridizations and reverse transcription PCR for the detection of BCR-ABL transcript. Cytogenetics tests are also used as an early indicator of patient responses to imatinib mesylate upon the onset of treatment [148]. The inhibitory effects of imatinib mesylate on CML paved the way to elucidate other downstream biomarkers that are also affected by the drug, such as PI3-K, AKT, and Ras [31].

However, the emergence of resistance to imatinib highlighted the need to focus on strategies that can identify the cause and overcome this resistance phenomenon. Resistance was often characterized with the reactivation of kinase activity within the leukemic clone; either BCR--ABL itself or BCR-ABL triggered downstream signaling pathways. Further studies revealed that resistance occurred due to several reasons, including rapid amplification of the fused *bcr-c-abl* gene, failure of imatinib to bind the BCR-ABL protein in its active conformation, and the selection of mutant clones [114]. The mutant clones exhibited a number of point mutations in the kinase domain of the *abl* oncogene in regions that are critical for the binding of the inhibitor [9, 62, 123, 126]. Due to the variability in patients' resistance towards imatinib, two strategies were employed: 1) escalation of imatinib dosage, which in some cases was enough to overcome the resistance, and 2) alternative drugs that target more than one pathway, which opened the gate for dual treatment involving both targets, ABL and SRC.

BCR-ABL activates multiple signaling pathways, including members of the SRC kinase family, such as Lyn and Hck [65]. Furthermore, studies have demonstrated that multiple domains of BCR-ABL interact with and activate SRC kinases independently of BCR-ABL kinase activity [41]. This fact suggested the possibility that molecules with "dual" kinase inhibitory activity against either ABL or Src might be of benefit to CML patients exhibiting imatinib resistance.

Several compounds are being tested for dual activity in CML, including pyrido[2,3-d]pyrimidine compounds [67]. Those compounds are described as being equally inhibitory to Abl- and Src-family kinases *in vitro*. The dual inhibitory effect towards multiple targets in the same oncogenic pathway is a favorable approach, as it may effectively allow "combination" therapy using a single drug. Moreover, the smaller size of the pyrido[2,3--d]pyrimidines compared with imatinib may resolve the paradox of drug binding exhibited by some BCR-ABL variants. However, the main reason for their increased potency is probably due to their ability to inhibit BCR--ABL irrespective of its activation status, in contrast to imatinib, which can only bind BCR-ABL with its activation loop only in the active conformation [56, 105]. Other compounds that are being tested as inhibitors for BCR-ABL/SRC-NRTK include SKI-606 and 4-anilino--3-quinolinecarbonitrile and the SRC kinase inhibitor BMS-354825, which is an orally bioavailable ABL kinase inhibitor that showed two-log greater potency than imatinib. BMS-354825 retained activity against 14 of 15 imatinib-resistant BCR-ABL mutants in pre-clinical studies. BMS-354825 is currently in phase I of clinical trials [48, 139]. Additional compounds that are in different stages of clinical trials are listed in Table 1.

POTENTIAL DIFFICULTIES OF ANTIBODY-BASED THERAPIES

Although mAbs, which are considered to be potential "magic bullets" for cancer and other disease treatment, are currently being used to a broad degree, there are certain problems that still need to be solved. Various factors are responsible for the low efficacy of antibodies. First, incomplete antibodies, such as those discussed in the first chapters of this review, have short *in vivo* half--lives and do not kill cells with great efficiency, as they do not always fix human complement or elicit ADCC with human mononuclear cells. Also, in many cases they are not directed against growth receptors that are essential for cell survival and proliferation[156]. Conjugated antibodies in which drugs, toxins, or radionuclides are attached to naked mAbs have been constructed, but only a few of them are currently used for therapy due to many side effects. The side effects typically are a result of the type of substance that is attached to the bsAb.

Above all, unless so called "humanized antibodies" are developed, there is an immunogenity problem, irrespective of whether the antibody is naked or conjugated. Monoclonal antibodies are produced in mice and they trigger an immune response when injected into humans producing human anti-mouse antibodies (HAMA). This results in the elimination of therapeutic antibodies from the host and also causes the formation of immune complexes that can result in damage to the kidneys. Monoclonal antibodies raised in humans would lessen the problem, but most attempts that have been made have been unsuccessful. This problem has been reduced to some extent by the use of genetic engineering to produce mouse-human hybrid antibodies (e.g. infliximab, rituximab, vitaxin, etc.)

Hybrid antibodies include both chimeric (human constant region plus mouse variable region) and humanized antibodies (human framework Ig containing only rodent sequences encoding the three complementarity-determining regions). Although these antibodies diminish HAMA response and produce ADCC, it is thought that

they will be immunogenic in immunocompetent humans because of non-self variable regions [13] and, moreover, they have low cytotoxicity to target cells. Human IgG_1 is the most widely used chimeric mAb for tumor therapy as it effectively triggers ADDC by mononuclear effector cells, activates human complement [12], and has an extended plasma life [45]. However, human IgG_1 also binds to Fc receptors on non-cytotoxic cells, such as FcγRII on platelets and B cells, FcγRIIIb on polymorphonuclear leukocytes, and to Fc receptors, which even inhibit effector cell activation (such as FcγRIIb on monocytes/macrophages). This interaction with the inhibitory FcγRII isoform was demonstrated to diminish Herceptins activity in animal models [26]. To overcome the difficulties associated with mAbs, bsAbs have been proposed. *In vitro* studies have shown that chemically linked bsAbs directed against the Fcγ receptors FcγRIII (CD16) and FcγRI (CD64) and the Fcα receptor FcαRI (CD89) were more effective than conventional Ig antibodies. Animal studies have also confirmed the efficacy of the molecules, but results from clinical trials have been less promising because of their short plasma half-lives when compared with conventional antibodies.

Furthermore, antibody structure also has a profound effect on tumor targeting. IgG, being a large protein of approximately 150 kDa mass, has slower kinetics of distribution and severely limits tissue penetration compared with small molecules. Therefore, alteration of antibody structure can improve quantitative and selective tumor targeting [159]. In spite of the above-mentioned potential difficulties, at least 17 mAbs have been approved by the FDA, while many are still in clinical trials. Some antibodies are also used effectively in combination with chemotherapy.

CONCLUDING REMARKS

Combining cytotoxic drugs with novel agents that specifically interfere with key pathways controlling cancer cell survival, proliferation, invasion, and/or metastatic spreading, mitogenic signaling, DNA integrity, and/or cell-cycle progression can render cancer cells more susceptible to irreparable damage. In this context, the use of mAbs is a useful therapeutic approach for several reasons: 1) because the cellular targets for these agents and their mechanism(s) of action are different from those of other cytotoxic drugs, it is possible that using them in combination with chemotherapy can prevent cross-resistance, and 2) alterations in the activity of signaling pathways that regulate mitogenic signals cannot only directly cause perturbation of cell growth, but may also increase the sensitivity of cancer cells to conventional chemotherapy and radiation therapy [6, 25].

Besides the direct role in cancer therapy, antibodies are being used on a daily basis to detect and to positively identify tumor antigens, thus being invaluable in every clinical pathology lab. Furthermore, the enzyme-linked immunosorbent assay (ELISA), a versatile method for the detection of antigens in body fluids, on the cell surface, and in cell lysates, plays a key role not only for diagnosis, but also for the assessment of the prognosis of the patient. Thus, ELISAs are mostly being used to detect the level of TAA. For example, a high level of these antigens, after a round of chemotherapy or after surgery, is a negative prognostic factor, indicating the presence of still significant tumor mass in the body. Recent studies indicate that ELISAs could be applied for the quantitative assessment of *in vivo* programmed cell death, for example in the course of cancer chemotherapy [4]. Cytochrome c, which normally resides within the intermembrane space of mitochondria, becomes released from mitochondria upon apoptotic stimuli. We have previously observed that a significant portion of cytochrome c leaves the cell and can be detected in the extracellular fluids, thus serving as an early and sensitive marker *in vivo* [121]. Interestingly, besides cancer therapy, large quantities of cytochrome c could also be found in the plasma and serum of patients with the fulminant hepatitis [5, 43].

The high (selective) affinity of antibodies to their targets makes them a desirable "weapon" against cancer and other diseases. Still, for a majority of diseases, including cancer, concurrent selective approaches exist. For example, viral proteins, e4orf4 and apoptin, have recently been identified that show selective toxicity towards cancer cells [94, 102]. It appears that these proteins target the same pathways that are normally active in aberrantly proliferating cells and redirect their activity towards promotion of apoptotic processes. In addition to antibodies, small peptides and natural products derived from the components of the immune system are also gaining popularity as anticancer agents [44, 58, 100, 141].

In the above review we have focused on the therapeutic applications of antibodies. We discussed both the unique advantages of antibodies arising from their specificity towards targeted antigens, as well as potential shortcomings, determined mainly by their antigenicity and their large size (150 kDa or more), which makes them somewhat inferior in comparison to "small molecules" to penetrate tumors. Despite these shortcomings, antibodies are becoming more and more important therapeutic tools. It is expected that, as we learn how to improve the pharmacokinetic properties of antibodies and how to diminish their adverse interactions with the immune system, mAbs will gain importance as therapeutic molecules due to their targeting specificity.

Acknowledgment: M. Los thankfully acknowledges the support by the CFI-Canada Research Chair program and the programs financed by the PCRFC, CCMF, and the HSC Foundation (Winnipeg). S. Louis, S. Loewen, and I. Gehrke thankfully acknowledge the support from CIHR training program. E. P. Booy has been supported by the CIHR CGS Master's Award.

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