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Guided Molecular Missiles for Tumor-Targeting Chemotherapy—Case Studies Using the Second-Generation Taxoids as Warheads

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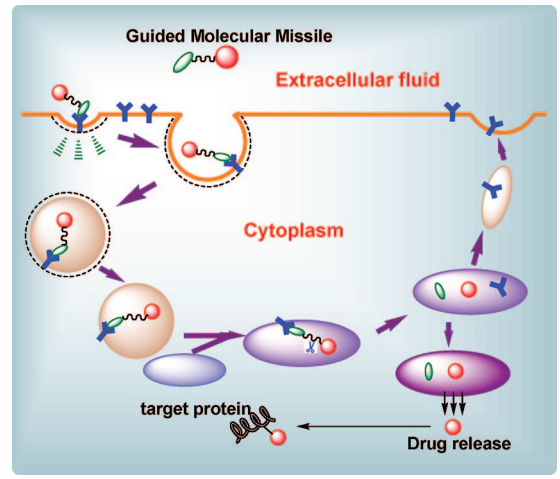

A long-standing problem in cancer chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects. Therefore, the development of innovative and efficacious tumor-specific drug delivery protocols or systems is urgently needed. A rapidly growing tumor requires various nutrients and vitamins. Thus, tumor cells overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors.

This Account presents our research program on the discovery and development of novel and efficient drug delivery systems, possessing tumor-targeting ability and efficacy against various cancer types, especially multidrug-resistant tumors. In general, a tumor-targeting drug delivery system consists of a tumor recognition moiety and a cytotoxic warhead connected directly or through a suitable linker to form a conjugate. The conjugate, which can be regarded as a "guided molecular missile", should be systemically nontoxic, that is, the linker must be stable in blood circulation, but upon internalization into the cancer cell, the conjugate should be readily cleaved to regenerate the active cytotoxic warhead.

These novel "guided molecular missiles" are conjugates of the highly potent second-generation taxoid anticancer agents with tumor-targeting molecules through mechanism-based cleavable linkers. These conjugates are specifically delivered to tumors and internalized into tumor cells, and the potent taxoid anticancer agents are released from the linker into the cytoplasm. We have successfully used omega-3 polyunsaturated fatty acids, in particular DHA, and monoclonal antibodies (for EGFR) as tumor-targeting molecules for the conjugates, which exhibited remarkable efficacy against human tumor xenografts in animal models.

We have developed self-immolative disulfide linkers wherein the glutathione-triggered cascade drug release takes place to generate the original anticancer agent. The use of disulfide linkers is attractive beacuse it takes into account the fact that the concentration of glutathione is much higher (>1000 times) in tumor cells than in blood plasma. In order to monitor and elucidate the mechanism of tumor-targeting, internalization, and drug release, several fluorescent and fluorogenic probes using biotin as the tumor-targeting module were developed and used. Then, the progressive occurrence of the designed receptor-mediated endocytosis, drug release, and drug binding to the target protein (microtubules) has been successfully observed and confirmed by means of confocal fluorescence microscopy.

These "guided molecular missiles" provide bright prospects for the development of highly efficacious new generation drugs for cancer chemotherapy.

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Introduction

Cancer is the second major cause of death (the number 1 cause of death for the age 85 or younger population) in the U.S. Despite the significant progress in the development of cancer detection, prevention, surgery, and therapy, there is still no common cure for patients with malignant diseases. In addition, the long-standing problem of chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects, such as hair loss and damage to the liver, kidney, and bone marrow. Therefore, various drug-delivery protocols and systems have been explored in the last 3 decades.[1]

In general, a tumor-targeting drug-delivery system consists of a tumor recognition moiety and a cytotoxic warhead connected directly or through a suitable linker to form a conjugate. The conjugate, which can be regarded as a "guided molecular missile", should be systemically nontoxic. This means that the linker must be stable in blood circulation. Upon internalization into the cancer cell, the conjugate should be readily cleaved to regenerate the active cytotoxic warhead.

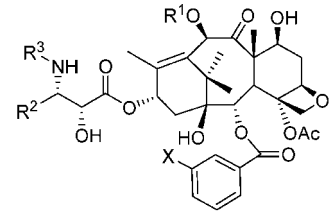
A rapidly growing tumor requires various nutrients and vitamins. Therefore, tumor cells overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors.[1] For example, monoclonal antibodies,[2–6] polyunsaturated fatty acids,[7,8] folic acid,[9,10] aptamers,[11] oligopeptides,[12] and hyaluronic acid[13] have been applied as tumor-specific moieties to construct "guided molecular missiles".

This Account describes the progress in the novel molecular approaches to the design and discovery of "guided molecular missiles" for tumor-targeting chemotherapy in our laboratory.

Use of Second-Generation Taxoids as "Warheads"

Paclitaxel and docetaxel have brought about significant impact on the current cancer chemotherapy, mainly because of their unique mechanism of action,[14] but seriously suffer from the lack of tumor specificity and multidrug resistance (MDR). Paclitaxel and docetaxel are effective against breast, ovary, and lung cancers but do not show efficacy against colon, pancreatic, melanoma, and renal cancers. For example, human colon carcinoma is inherently multidrug-resistant because of the overexpression of P-glycoprotein (Pgp), which is an effective ATP-binding cassette (ABC) transporter, effluxing out hydrophobic anticancer agents, including paclitaxel and docetaxel.[15]

TABLE 1. Cytotoxicity (IC_{50}, nM) of Selected Second-Generation Taxoids against Human Cancer Cell Lines



Taxane	R^1	R^2	R^3	X
Paclitaxel	Ac	Ph	PhCO	H
Docetaxel	H	Ph	*t*-Boc	H
SB-T-1103	EtCO	*i*-Bu	*t*-Boc	H
SB-T-1104	*c*-PrCO	*i*-butenyl	*t*-Boc	H
SB-T-1213	EtCO	*i*-butenyl	*t*-Boc	H
SB-T-1214	*c*-PrCO	*i*-butenyl	*t*-Boc	H
SB-T-1216	Me_2NCO	*i*-butenyl	*t*-Boc	H
SB-T-1217	MeOCO	*i*-butenyl	*t*-Boc	H
SB-T-11033	EtCO	*i*-Bu	*t*-Boc	MeO
SB-T-121303	EtCO	*i*-butenyl	*t*-Boc	MeO

taxane	MCF7[a]	NCI/ADR[b]	R/S ratio[c]
paclitaxel	1.7	550	324
docetaxel	1.0	723	432
SB-T-1103	0.35	5.1	15
SB-T-1104	0.51	7.9	15
SB-T-1213	0.18	4.0	22
SB-T-1214	0.20	3.9	20
SB-T-1216	0.13	7.4	57
SB-T-1217	0.14	9.7	69
SB-T-11033	0.36	0.61	1.7
SB-T-121303	0.36	0.79	2.2

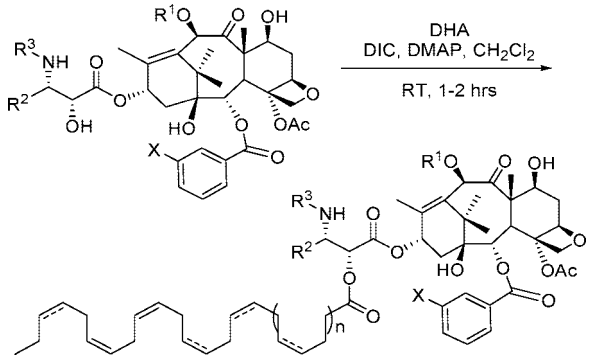
[a] Human mammary tumor cell line (Pgp−). [b] Human ovarian tumor cell line (Pgp+). [c] IC_{50} (NCI/ADR)/IC_{50} (MCF7); IC_{50} = the half-maximal inhibitory concentration for tumor growth.

On the basis of our structure–activity relationship (SAR) study of taxoids, we have developed a series of highly potent second-generation taxoids.[16–20] Most of these taxoids exhibited 2–3 orders of magnitude higher potency than those of paclitaxel and docetaxel against drug-resistant cell lines expressing MDR phenotypes. Accordingly, these highly potent taxoids have been used as the warhead of our "guided molecular missiles". Selected second-generation taxoids are listed in Table 1.

Use of Polyunsaturated Fatty Acids (PUFAs) as a Tumor-Targeting Module

PUFAs are ideal candidates for tumor-specific guiding molecules. Representative naturally occurring PUFAs possess 18, 20, and 22 carbons and 2–6 unconjugated *cis* double bonds separated by one methylene, such as linolenic acid (LNA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These PUFAs are included in vegetable oils, cold-water fish, and meat. DHA is classified as a nutritional additive by the Food and Drug Administration (FDA) in the U.S.

SCHEME 1



Thus, DHA and its metabolites are considered to be safe to humans.[21,22] PUFAs have exhibited anticancer activity against CFPAC, PANC-1, and Mia-Pa-Ca-2 pancreatic and HL-60 leukemia cell lines, and their antitumor activities have been evaluated in preclinical and clinical studies.[23,24] Perfusion studies demonstrated that some PUFAs are taken up more rapidly by tumor cells than normal cells.[25,26] In addition, PUFAs are readily incorporated into the lipid bilayer of tumor cells, which results in disruption of the membrane structure and fluidity.[27] This has been suggested to influence the chemosensitivity of tumor cells.[28] These findings strongly suggest the benefit in the use of PUFAs for tumor-targeting drug delivery.

Bradley et al.[7] prepared the DHA conjugate of paclitaxel (Taxoprexin) by linking DHA to the C-2′ position of paclitaxel. The conjugate exhibited substantially increased antitumor activity and reduced systemic toxicity as compared to paclitaxel. Furthermore, the conjugate is stable in blood plasma, and high concentrations in tumor cells are maintained for a long period of time. Taxoprexin was selected as a first-track development drug candidate by the FDA and has advanced to human phase III clinical trials.[29]

Although Taxoprexin exhibits an impressive antitumor activity against drug-sensitive tumors, this conjugate would not be effective against multidrug-resistant tumors because the released paclitaxel would be caught by the Pgp efflux pump and eliminated from the cancer cells. As mentioned above, many of the second-generation taxoids developed in our laboratory showed 2−3 orders of magnitude higher activity against drug-resistant cancer cells and tumor xenografts in mice.[16−19] Thus, we hypothesized that the PUFA conjugates of the second-generation taxoids would be efficacious against drug-resistant tumors, for which DHA-paclitaxel is ineffective. To prove this hypothesis, the conjugates of DHA, LNA, and LA with the second-generation taxoids were synthesized and their efficacy was assayed *in vivo* against human tumor xenografts.

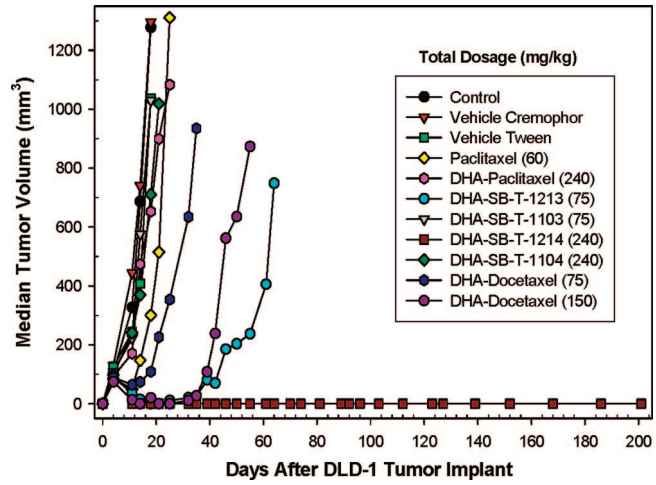


FIGURE 1. Effect of DHA-taxoid conjugates on human colon tumor xenograft DLD-1.

The synthesis of PUFA conjugates of the second-generation taxoids is straightforward. A free taxoid is coupled to a PUFA at the C-2′ hydroxyl group (Scheme 1) in the presence of diisopropylcarbodiimide (DIC) and 4-(dimethylamino)pyridine (DMAP), to afford the corresponding conjugates.

The PUFA-taxoid conjugates thus obtained were assayed for their efficacy against a drug-resistant human colon tumor xenograft DLD-1 and a drug-sensitive human ovarian tumor xenograft A121 in severe combined immunodeficiency (SCID) mice. As we anticipated, paclitaxel and DHA paclitaxel were totally ineffective against the drug-resistant DLD-1 tumor xenograft (Figure 1). In contrast, DHA-SB-T-1214 achieved complete regression of the DLD-1 tumor in 5 of 5 mice at 80 mg/kg dose administered on days 5, 8, and 11 (tumor growth delay > 187 days). Systemic toxicity was monitored by the weight loss of the animals throughout the *in vivo* experiments. A minor weight loss (<10%) was observed on days 12−22 but was all tolerated by the animals. [Note: no systemic toxicity was observed when the q7d × 3 (i.e., drug was given every 7 days) schedule was used with the same antitumor efficacy. SB-T-1214 (free drug) at the same dose was found to be toxic to the animals.] This is a very promising result that identifies this compound as the leading candidate for further preclinical studies and drug development.

In the case of the drug-sensitive tumor A121 xenograft, the efficacy of DHA paclitaxel reported by Bradley et al.[7] was confirmed by our results. However, two of the new DHA taxoids exhibited even better activity; i.e., DHA-SB-T-1213 and DHA-SB-T-1216 delayed the tumor growth for more than 186 days and caused complete regression of the tumor in all surviving mice even at the nonoptimized dose (Figure 2).

The impressive results obtained with DHA taxoids prompted us to investigate the use of different PUFAs and

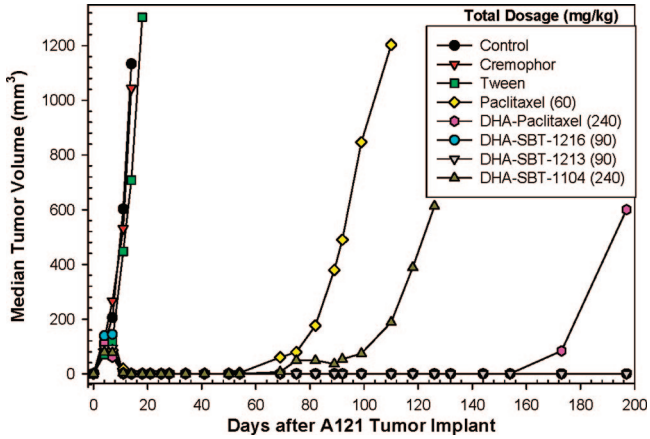


FIGURE 2. Effect of DHA-taxoid conjugates on human ovarian tumor xenograft A121.

their efficacy. We synthesized the conjugates of SB-T-1213 with DHA, LNA, and LA and examined their efficacy against DLD-1 colon tumor xenograft (Pgp+). LNA-SB-T-1213 exhibited strong antitumor activity (tumor growth delay > 109 days), while LA-SB-T-1213 did not show meaningful efficacy in the same assay, which revealed the marked difference between omega-3 PUFA (DHA and LNA) and omega-6 PUFA (LA).

The remarkable efficacy of PUFA-taxoid conjugates against drug-resistant and drug-sensitive human tumor xenografts provides bright prospects for further investigations for the applications of those conjugates in cancer chemotherapy.

Use of Monoclonal Antibodies as a Tumor-Targeting Module

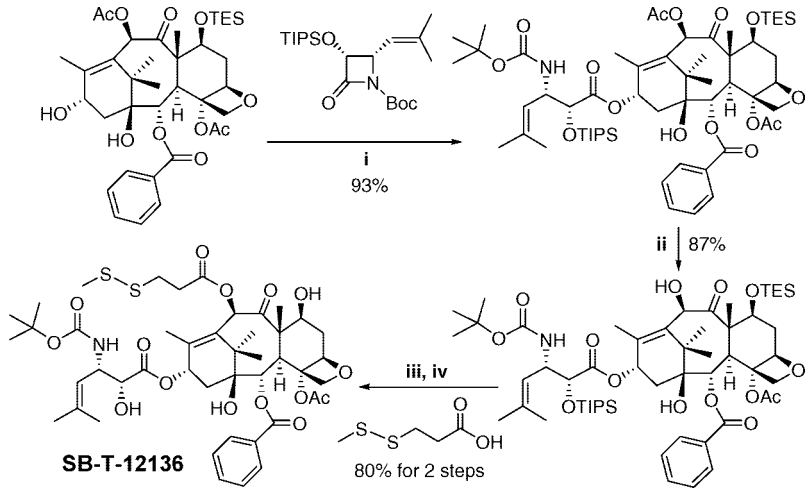
Monoclonal antibodies (mAbs), which have shown high binding specificity for tumor-specific antigens, are ideal to deliver cytotoxic drugs selectively to tumor cells.[6] A mAb-drug immunoconjugate would target the tumor cells and was internalized to release the original cytotoxic agent in its active form.[30,31] The most desirable mAb-drug immunoconjugate should be stable during circulation and should not bind to normal tissue cells. A mAb-calcheamicin conjugate "Mylotarg" has been approved for clinical use.[5] Several other mAb-drug conjugates have advanced to human clinical trials.[32,33]

The practical efficacy of such immunoconjugates heavily depends upon the nature of the cytotoxic agents as well as the tumor specificity of mAbs. Two research groups investigated paclitaxel-mAb conjugates[34,35] as potential tumor-specific anticancer agents. However, the results were disappointing. As mentioned above, we have developed a series of highly potent second-generation taxoids.[16–20] Accordingly, in principle, we should be able to develop novel chemotherapeutic agents with high potency and exceptional tumor specificity by linking these second-generation taxoids with mAbs.[4]

Design of mAb-Taxoid Conjugates.[4] Use of an appropriate linker between a taxoid and an mAb is crucial for the efficacy of the resulting immunoconjugate. It is required that the linker is stable for an extended period of time upon storage and also in circulation *in vivo*, while it is readily cleavable inside of cancer cells. Among possible linker units reported, we chose to employ a disulfide linker unit because of its favorable characteristics.[6,32,36] The use of disulfide linkers is attractive by taking into account the fact that the concentration of glutathione is much higher (>1000 times) in tumor cells than that in blood plasma.[37] It is expected that the mAb module of the conjugate binds to the specific antigens on tumor surfaces, and the whole conjugate is internalized via endocytosis. The disulfide bond is then cleaved by an intracellular thiol, such as glutathione, to release taxoid in its active form.

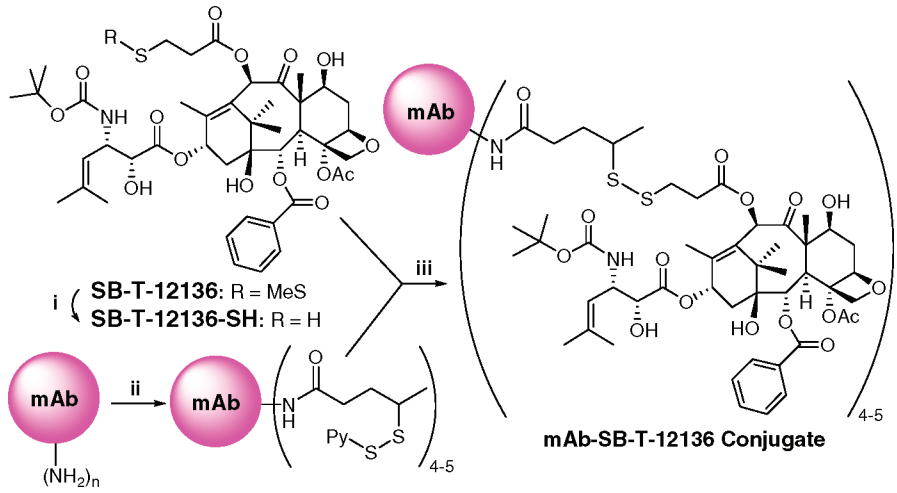
To synthesize a mAb-taxoid conjugate, both a taxoid and a mAb need to be modified to form a disulfide linkage by the disulfide–thiol exchange reaction. Because the necessary modification of mAb had been worked out prior to this project, the critical issue was to find highly potent second-generation taxoids modified with a sulfhydrylalkanoyl group, which would be the actual cytotoxic agent in the target cancer cells. As the logical precursor (or synthon) for the sulfhydrylalkanoyl group is the methyldisulfanyl (MDS)-alkanoyl group, we decided to synthesize MDS-alkanoyltaxoids. It has been shown that the number of tumor-associated antigens on the cancer cell surface is limited (estimated to be 10^5 molecules/cell). Thus, the cytotoxic agents that can be effectively used in these conjugates must have an IC_{50} value of 10^{-10}–10^{-11} M against target cancer cells.[6] At that time, a couple of the second-generation taxoids were shown to possess cytotoxicity in the required range.[16] Thus, those taxoids were chosen for modification with a MDS-alkanoyl group. Because incorporation of a MDS-alkanoyl group into these taxoids may well affect the cytotoxicity of the resulting taxoids, a SAR study was necessary to determine the optimal position for the introduction of a MDS-alkanoyl group. Thus, we synthesized a series of novel taxoids bearing a MDS-alkanoyl group at the C-10, C-7, C-2′, and C-2 positions, and their cytotoxicity was assayed. The SAR study of these MDS taxoids indicated that cytotoxicity could be retained (although it caused an 8 times loss in activity) when a MDS-propanoyl group was attached to the C-10 position of a taxoid. Modifications at all other positions were found to be detrimental to the potency. This was

SCHEME 2[a]



[a] (i) LiHMDS (1.5 equiv), β-lactam (1.5 equiv), tetrahydrofuran (THF), −40 °C, 40 min; (ii) N_2H_4–H_2O, EtOH, 3 h; (iii) $MeSS(CH_2)_2CO_2H$ (10 equiv), DIC (11 equiv), DMAP, CH_2Cl_2, overnight; (iv) HF–pyridine, pyridine, CH_3CN, overnight.

SCHEME 3[a]



[a] (i) DTT; (ii) SPP (10 equiv in ethanol), 50 mM potassium phosphate buffer, pH 6.5, NaCl (50 mM), EDTA (2 mM), 90 min; (iii) 50 mM potassium phosphate buffer, pH 6.5, NaCl (50 mM), EDTA (2 mM), SB-T-12136-SH (1.7 equiv per dithiopyridyl group, in EtOH), 24 h.

an important finding, which was totally unexpected. Accordingly, a 10-MDS-propanoyl taxoid, SB-T-12136, was selected as the warhead precursor.

Syntheses of MDS Taxoid SB-T-12136.[4] We originally planned the synthesis of SB-T-12136 using the β-lactam ring-opening coupling protocol[19,38] with appropriately modified baccatins. However, we found that the introduction of the 3-MDS-propanoyl group at the C-10 position of a taxoid was not a trivial matter, because of the occurrence of retro-Michael addition during the acylation. Accordingly, it became obvious that this group should be introduced at a later stage of the synthesis. After several tries and errors, we were able to synthesize this taxoid as shown in Scheme 2 in high yield.

Conjugation of Taxoid with mAbs Targeting Epidermal Growth Factor Receptor (EGFR).[4] The EGFR is known to be overexpressed in several human squamous cancers, such as head and neck, lung, and breast cancers. Murine monoclonal antibodies directed against the human EGFR were used as the tumor-targeting moieties in immunoconjugates. Three such immunoglobulin G class monoclonal antibodies, KS61 (IgG2a), KS77 (IgG1), and KS78 (IgG2a), were linked to SB-T-12136 via disulfide bonds. The preparation of mAb-taxoid conjugates is illustrated in Scheme 3. SB-T-12136 was treated with dithiothreitol (DTT) to generate SB-T-12136-SH bearing a free thiol functionality. The anti-EGFR mAb was modified with *N*-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP) to attach 4-pyridyldithio (PDT)-pentanoyl groups. Recov-

ery of the antibody was about 90%, with ~4−5 PDT-propanoyl groups linked per antibody molecule on average. Then, the modified mAb was conjugated with SB-T-12136-SH (2 equiv), which proceeded with virtually complete conversion. The mAb-taxoid conjugates were purified by gel filtration, which separated aggregates from monomeric species, and only the fractions corresponding to the monomeric conjugates were collected. Recovery of the conjugate was 65−70%. Three immunoconjugates, KS61, KS77, and KS78 taxoid, were thus prepared. Preliminary matrix-assisted laser desorption ionization time-of-flight (MALDI−TOF) analyses of the KS77-taxoid conjugate in comparison with KS77 strongly support that ~4−5 taxoids, on average, are attached to the mAb. The final formulation of the conjugate was in phosphate-buffer saline (PBS), containing 20% propylene glycol and 0.1% Tween 80 (v/v). A conjugate of SB-T-12136 with monoclonal antibody mN901 that does not bind to EGFR was also prepared in a similar manner for comparison.

***In Vitro* Cytotoxicity Assay.**[4] *In vitro* cytotoxicity was determined in a clonogenic assay after a continuous exposure of the cells to the conjugates. It is expected that antigen-expressing cancer cells could only be targeted by an immunoconjugate bearing a mAb specific to the antigen. In fact, mN901 taxoid exhibited no cytotoxicity against the A431 cell line, expressing EGFR. In sharp contrast, KS78 taxoid showed high potency (IC_{50} = 1.5 nM) against the same A431 cell line. It should be noted that the addition of an excess of unconjugated anti-EGFR antibody, e.g., KS61 at 3×10^{-8} M to the KS61-taxoid conjugate, abolished its cytotoxicity against A431 cells, indicating that cytotoxicity depended upon the specific binding of the conjugate to the antigen on cells. These results demonstrate that the binding of anti-EGFR mAb-taxoid conjugate to EGFR is highly specific. Moreover, it is strongly indicated that the immunoconjugate KS78 taxoid generates a highly cytotoxic agent SB-T-12136-SH upon binding to EGFR, followed by internalization and the subsequent cleavage of the disulfide linkage.

***In Vivo* Tumor Growth Inhibition Assay.**[4] The antitumor activities of two anti-EGFR-mAb-taxoid conjugates, KS61 taxoid and KS77 taxoid, were evaluated against human tumor xenografts in SCID mice (Figure 3). Each mouse was inoculated with 1.5×10^6 A431 human squamous cancer cells, and the tumors were allowed to grow for 11 days to an average size of 100 mm^3. The mice were then randomly divided into four groups. The first group received KS61-taxoid conjugate (10 mg/kg, qd × 5, administered *in vivo*). The second group received KS77-taxoid conjugate in the same manner. The third group received free taxoid (0.24 mg/kg, qd × 5, administered *in vivo*) at the same dose as that present in the conjugate. A control group of mice received PBS using the same treatment schedule as in groups 1−3. The weights of the mice and tumor sizes were measured twice weekly. The results are shown in Figure 3. The tumors in the control group of mice grew to a size of nearly 1000 mm^3 in 31 days. Treatment with free taxoid (same dose as that in the mAb-taxoid conjugates) showed no therapeutic effect. Also, treatment with mAb KS77 (2× dose of the mAb-taxoid conjugate) exhibited only a moderate delay in tumor growth, i.e., the tumor size reached 800 mm^3 within 25 days after the treatment (data not shown). In contrast, both anti-EGFR-mAb-taxoid conjugates, especially KS61 SB-T-12136, showed remarkable antitumor activity, resulting in complete inhibition of tumor growth and elimination of tumor cells in all of the treated animals for the duration of the experiment. Necropsy on day 75, followed by histopathological examination, showed residual calcified material at the tumor site but no evidence of tumor cells. These data also indicate that targeted delivery of the taxoid using a tumor-specific mAb is essential for the activity because an equivalent dose of unconjugated taxoid shows no antitumor activity. Notably, the doses of mAb-taxoid conjugates used are nontoxic to the mice as demonstrated by the absence of any weight loss. The results clearly indicate that the "guided molecular missiles" combining the second-generation taxoids with mAbs highly specific to the antigen on tumor cell surfaces are very promising as potential chemotherapeutic agents with few side effects.

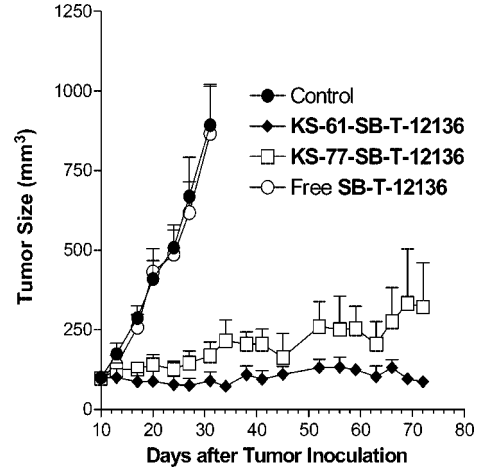


FIGURE 3. Antitumor activity of anti-EGFR mAb-taxoid conjugates against A431 xenografts in SCID mice.

Second-Generation Self-Immolative Disulfide Linkers

For the development of efficacious tumor-targeting drug conjugates, efficient mechanism-based linkers are essential

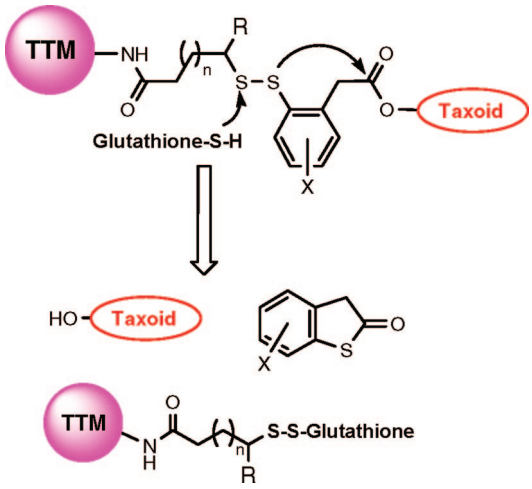


FIGURE 4. Second-generation self-immolative disulfide linkers (TTM = tumor-targeting module).

because the conjugates should be stable during circulation in the blood but readily cleavable in the tumor. Also, these linkers should be bifunctional so that this linker module can be connected to the warhead at one end and the tumor-targeting module at the other end. As described above, we invented novel mAb-taxoid conjugates as tumor-targeting anticancer agents, which exhibited extremely promising results in human cancer xenografts in SCID mice. The results clearly demonstrate the tumor-specific delivery of a taxoid anticancer agent, curing all animals tested, without any noticeable toxicity to the animals.[4] As the linker for these mAb-taxoid conjugates, we used a disulfide linker, which was stable in blood circulation but efficiently cleaved by glutathione or other thiols in the tumor. However, in these first-generation mAb-taxoid conjugates, the orginal taxoid molecule was not released because of the compromised modification of the taxoid molecule to attach the disulfide linker. Accordingly, the cytotoxicity of the taxoid released in these conjugates (SB-T-12136-SH) was 8 times weaker than the parent taxoid (SB-T-1213).[4]

To solve this problem, we have been developing the second-generation mechanism-based bifunctional disulfide linkers, which can be connected to various warheads as well as tumor-targeting modules. One of our approaches is to use self-immolative disulfide linkers, wherein the glutathione-triggered cascade drug release takes place to generate the original anticancer agent via thiolactone formation and ester bond cleavage (Figure 4). This mechanism-based drug release concept was nicely proven in a model system by monitoring the reaction with ^{19}F NMR using fluorine-labeled compounds (Figure 5).[39] The strategic design of placing a phenyl group attached to the disulfide linkage directs the cleavage of this linkage by a thiol to generate the desirable thiophenolate or sulfhydrylphenyl species for thiolactonization. This type of self-immolative disulfide linker is readily applicable to a range of tumor-targeting drug conjugates.

Use of Biotin as Tumor-Targeting Module of the Fluorescent and Fluorogenic Molecular Missiles

All living cells require vitamins for survival, but the rapidly dividing cancer cells require certain vitamins to sustain their rapid growth. Accordingly, the receptors involved in the uptake of the vitamins are overexpressed on the cancer cell surface. Thus, those vitamin receptors serve as useful biomarkers for the imaging and identification of tumor cells as well as tumor-targeting drug delivery. Vitamin B_{12}, folic acid, biotin, and riboflavin are essential vitamins for the division of all cells but particularly for the growth of tumor cells. The folate receptors were recognized as potentially excellent biomarkers for targeted drug delivery, and significant advancement has been

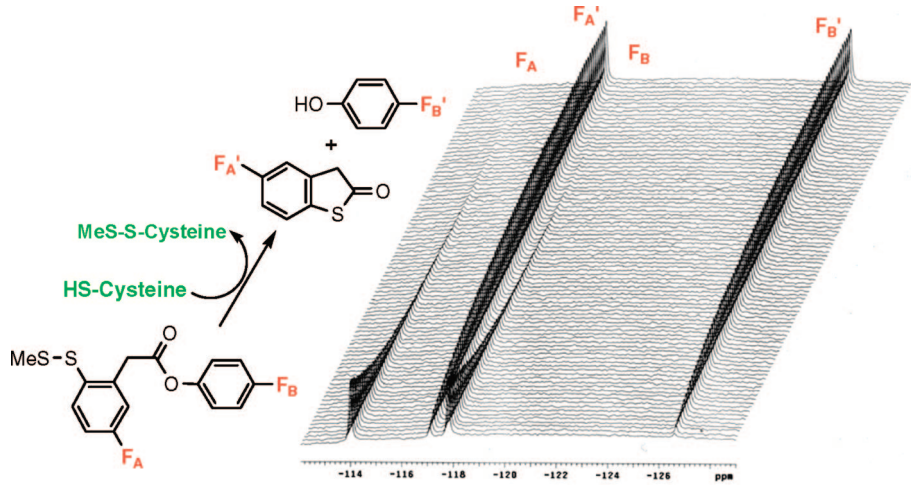


FIGURE 5. Proof-of-concept model for the mechanism-based drug release.

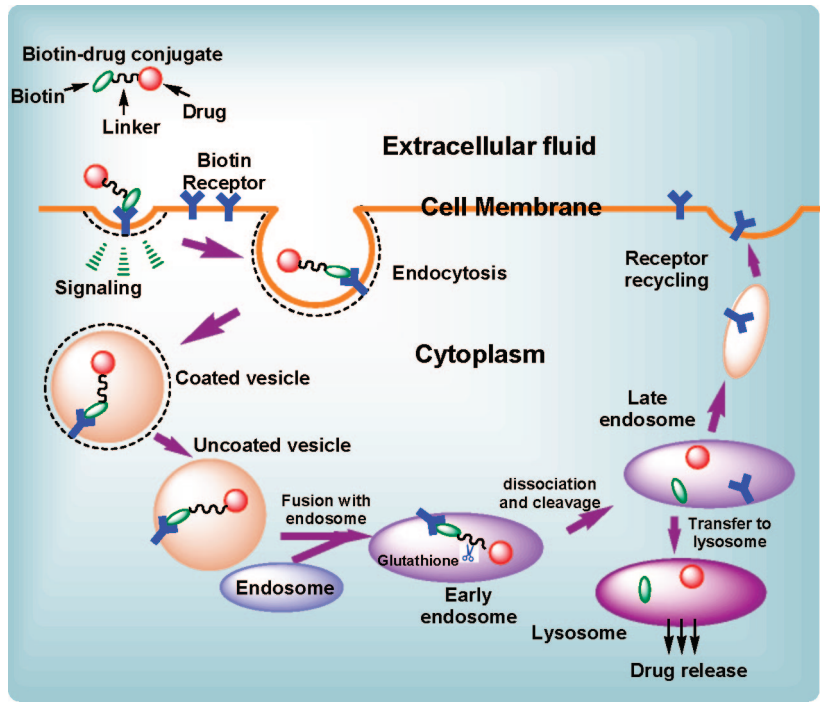
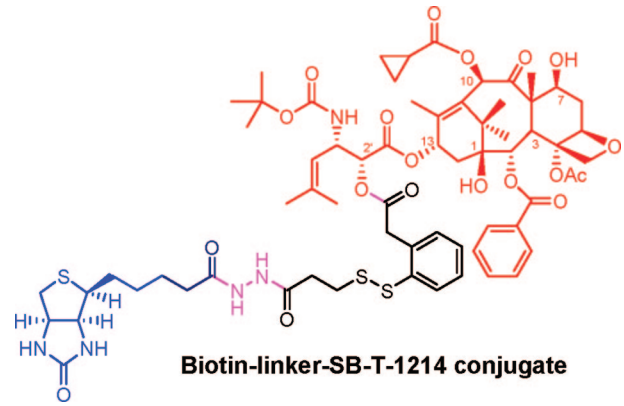


FIGURE 6. Receptor-mediated endocytosis of biotin–drug conjugates.

made to date.[9,10,40,41] However, the biotin receptors were not studied for this purpose until recently. Biotin (vitamin H or vitamin B_7) is a growth promoter at the cellular level, and its content in tumors is substantially higher than that in normal tissues. Recently, it has been shown that the biotin receptors are even more overexpressed than the folate and/or vitamin B_{12} receptors in many cancer cells, e.g., leukemia (L1210FR), ovarian (Ov 2008, ID8), Colon (Colo-26), mastocytoma (P815), lung (M109), renal (RENCA, RD0995), and breast (4T1, JC, MMT06056) cancer cell lines.[42]

Accordingly, we chose biotin as the tumor-targeting molecule for our molecular missile bearing the second-generation disulfide linker with SB-T-1214 as the warhead. We also launched a mechanistic study on the validation of tumor-targeting drug delivery by monitoring the internalization through receptor-mediated endocytosis (RME) (Figure 6) and drug release, using fluorescent and fluorogenic molecular probes for the biotin-taxoid conjugate.

We designed and synthesized three fluorescence-labeled biotin conjugates, i.e., (i) biotin–flurorescein (A), (ii) biotin–linker–coumarin (B) (fluorogenic probe), and (iii) biotin–linker–taxoid–fluorescein (C) (Figure 7). The conjugate A was designed to observe RME (Figure 6), while the conjugate B was designed for confirming the internalization via RME of the biotin-linker unit and the release of coumarin, which becomes fluorescent only when it is released as a free molecule (it is conjugated to the linker via an ester bond) via disulfide cleavage by endogenous thiol, glutathione in particular. The conjugate C was designed to validate the whole internalization by RME and drug-release processes, in which the freed fluorescent taxoid should bind to the target protein, microtubules, in the cancer cells.



For the syntheses of these fluorescent and fluorogenic probes, we used the hydrazide of biotin to couple with fluorescein isothiocyanate (FITC) as well as the self-immolative disulfide linker unit. The synthesis of biotin–linker–SB-T-1214–fluorescein is illustrated in Scheme 4. First, fluorescein was connected to the C7 position of SB-T-1214 via a 4-hydroxybutanoic acid tether. Then, mono-TIPS-ester of the self-immolative disulfide linker carboxylic acid was coupled to SB-T-1214–fluorescein using DIC/DMAP. TIPS was removed by HF/Py, followed by esterification with *N*-hydroxysuccinim-

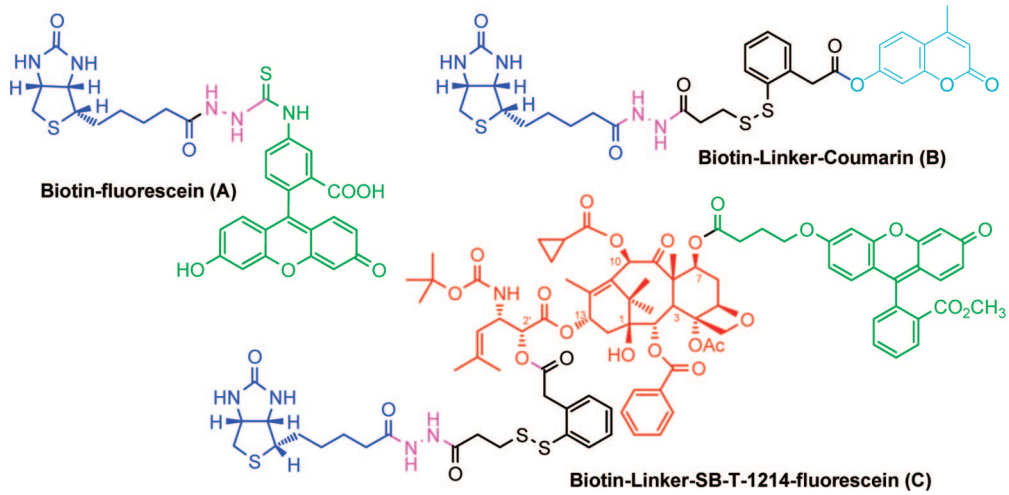
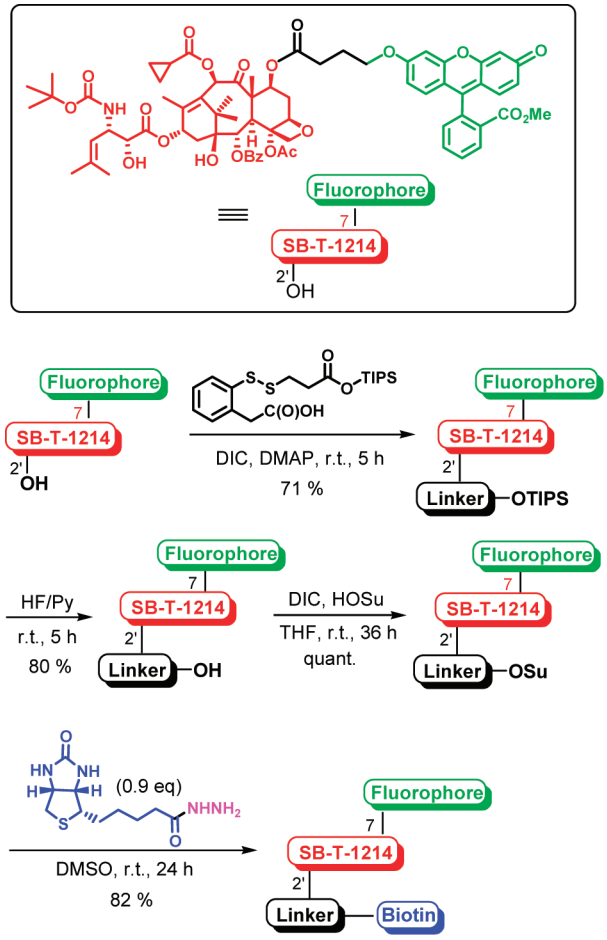


FIGURE 7. Fluorescent and fluorogenic probes for the internalization and drug release.

SCHEME 4



ide (HO-Su) using DIC to form the corresponding activated ester of the linker–SB-T-1214–flurorescein component. Finally, the biotin unit was introduced simply by reacting biotin–$NHNH_2$ with the linker–SB-T-1214–flurorescein component to give the conjugate C in good overall yield.

Cellular uptake of these three fluorescent and fluorogenic probes was monitored by confocal fluorescence microscopy (CFM). Figure 8a shows the observation of intense fluorescence when the L1210FR cell was incubated with biotin–fluorescein (probe A) (100 nM) at 37 °C for 3 h, followed by thorough washing of the cells by PBS and analysis. The result confirms the internalization of this fluorescent probe A into the leukemia cells. It has been shown that endocytosis is an energy-dependent process. Thus, endocytosis should be inhibited at low temperature (4 °C). The CFM image of the probe A, incubated at 4 °C (100 nM, 3 h), showed greatly diminished fluorescence in the cells, which clearly indicates that the probe A was internalized through endocytosis. To further confirm that this was a receptor-mediated endocytosis process, excess free biotin molecules (2 mM) were preincubated for 1 h to saturate the biotin receptors on the cancer cell surface, followed by the addition of the probe A (100 nM, 37 °C, 3 h). The CFM image revealed the virtually total absence of fluorescence, which confirms that this is indeed the receptor-mediated endocytosis.

Next, the fluorogenic probe B, biotin–linker–coumarin, (1 μM), was incubated with L1210FR at 37 °C for 3 h. After washing thoroughly with PBS, glutathione ethyl ester (2 mM) was added to the medium and cells were incubated for another 2 h at 37 °C. The addition of glutathione ethyl ester was to ensure the cleavage of the self-immolative disulfide linker to release free coumarin (fluorescent), as designed. As Figure 8b shows, fluorescent coumarin molecules (blue) are indeed released in the leukemia cells. The result confirms that the intracellular drug release via cleavage of the disulfide linkage by glutathione and the subsequent thiolactonization took place as designed. In the absence of additional glutathione, the observed blue fluorescence was substantially weaker. This

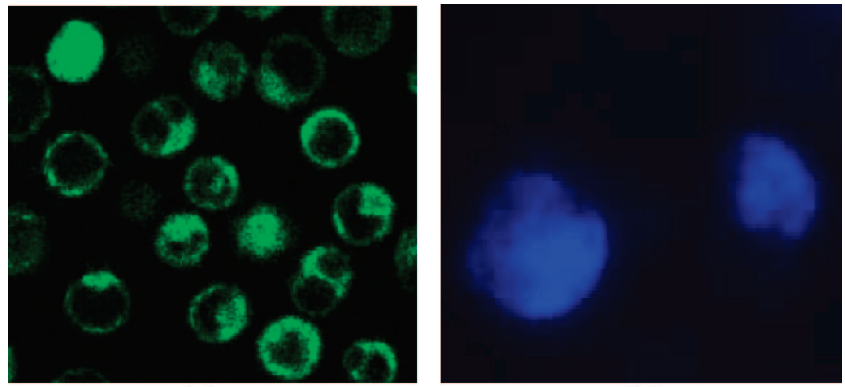


FIGURE 8. CFM images of L1210FR cells incorporating (a) biotin–fluorescein (probe A) and (b) biotin–linker–coumarin (probe B) after the addition of glutathione Et ester.

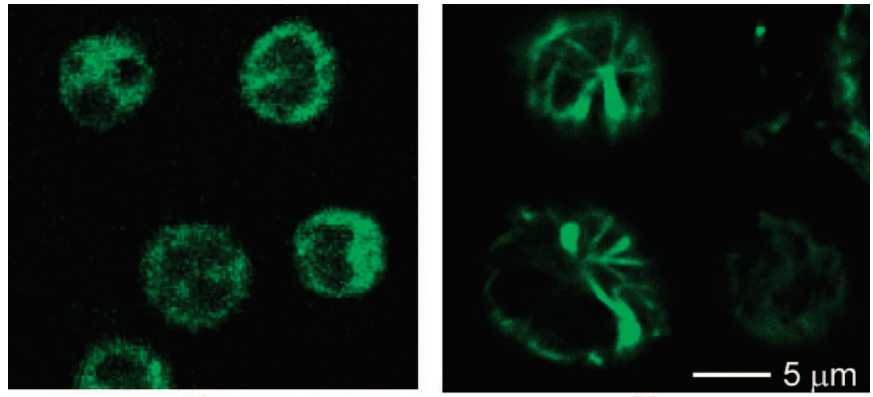


FIGURE 9. CFM images of L1210FR cells incorporating biotin–linker–SB-T-1214–fluorescein (probe C) (a) prior to the addition of glutathione Et ester and (b) after the addition of glutathione Et ester.

means that the concentration of intracellular glutathione in this leukemia L1210FR cell line is small under the *in vitro* experimental conditions, which are significantly different from *in vivo* conditions, where the glutathione supply in tumor tissues is more than adequate. On the other hand, this experiment using additional glutathione (ester) obviously demonstrates that the cleavage of the self-immolative disulfide linkage and drug release was caused by glutathione. Thus, this result confirms that the designed drug release using the fluorogenic molecule in place of the taxoid warhead has worked well.

Finally, the internalization and drug release of the biotin–linker–SB-T-1214–fluorescein conjugate (probe C) was investigated. First, the probe C (20 μM) was incubated with L1210FR cells at 37 °C for 3 h and analyzed. As Figure 9a shows, the whole conjugate was internalized in the same manner as that described above for the probe A (see Figure 8a). Next, glutathione ethyl ester (2 mM) was added to the medium, and the cells were incubated for another 1 h to ensure the drug release. Figure 9b shows the CFM image of this system, which is dramatically different from Figure 9a. This CFM image indicates that the released fluorescent taxoid binds to the microtubules, which are the drug target of the taxoid, highlighting the fluorescence-labeled microtubule bundles. Accordingly, the release of the taxoid warhead through the designed mechanism is shown to have taken place in the same manner as that observed for the fluorogenic probe B. In addition, the binding of the released fluorescent taxoid to microtubules is observed. Thus, it is concluded that the "guided molecular missile" successfully delivered the active warhead to the drug target, as designed, through receptor-mediated endocytosis and glutathione-triggered intracellular drug release via cleavage of the self-immolative disulfide linker and thiolactonization.

Closing Remarks

As described above, we have successfully designed and constructed novel "guided molecular missiles" for potential use in cancer chemotherapy, using the second-generation taxoids as the warheads, omega-3 PUFAs, mAbs, and biotin as the tumor-targeting modules, and mechanism-based disulfide linkers. Remarkable antitumor efficacies with minimum systemic tox-

icity have been demonstrated by DHA-taxoid conjugates as well as mAb-taxoid conjugates in animal models. The designed receptor-mediated endocytosis and drug-release processes by intracellular glutathione have been confirmed using fluorescent and fluorogenic probes bearing biotin as the tumor-targeting module by means of confocal fluorescence microscopy. The *in vivo* efficacy evaluation of the biotin-taxoid conjugate will begin shortly. We have also studied the use of functionalized single-wall carbon nanotubes (SWCNTs) as a template for multiple-warhead drug conjugates and obtained exciting preliminary results, which will be reported elsewhere. We have also been exploring the use of folate and aptamers as tumor-targeting modules as well as the construction of new generation "guided molecular missiles" bearing different multiple warheads in a molecule. Results will be reported in due course.

The author thanks his current and past graduate students and postdoctoral associates who have worked on this project for their enthusiasm, dedication, and persistent pursuit of their challenging goals. He is also grateful to Dr. Ralph J. Bernacki and his laboratory staff at the Roswell Park Cancer Institute for productive collaboration. This work was supported by the grants from National Institutes of Health (NIGMS and NCI). Generous support from Indena, SpA for the supply of 10-deacetylbaccatin III, as well as Immunogen, Inc. for collaborative work on the mAb-taxoid conjugates, is gratefully acknowledged.

BIOGRAPHICAL INFORMATION

Iwao Ojima received his B.S., M.S., and Ph.D. (1973) degrees from the University of Tokyo, Japan. He joined the Sagami Institute of Chemical Research and held a position of Senior Research Fellow until 1983. He joined the faculty at the Department of Chemistry, State University of New York at Stony Brook, first as an Associate Professor (1983) and was promoted to Professor (1984), Leading Professor (1991), and then Distinguished Professor (1995). He served as the Department Chairman from 1997 to 2003. He has been serving as the founding Director for the Institute of Chemical Biology and Drug Discovery (ICB&DD) from 2003. He has a wide range of research interests in synthetic, organic, and medicinal chemistry as well as chemical biology, including the discovery and development of anticancer agents and antimicrobials, targeted drug delivery, catalytic methodologies, and asymmetric synthesis. His awards and honors include Arthur C. Cope Scholar Award (1994), E. B. Hershberg Award for Important Discoveries of Medicinally Active Substances (2001) from the American Chemical Society, The Chemical Society of Japan Award (1999), Outstanding Inventor Award (2002) from the Research Foundation of the State University of New York, and Fellows of J. S. Guggenheim Memorial Foundation, the American Association for the Advancement of Science, and The New York Academy of Sciences.

FOOTNOTES

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