Mechanism-Based Tumor-Targeting Drug Delivery System. Validation of Efficient Vitamin Receptor-Mediated Endocytosis and Drug Release

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An efficient mechanism-based tumor-targeting drug delivery system, based on tumor-specific vitamin-receptor mediated endocytosis, has been developed. The tumor-targeting drug delivery system is a conjugate of a tumor-targeting molecule (biotin: vitamin H or vitamin B-7), a mechanism-based self-immolative linker and a second-generation taxoid (SB-T-1214) as the cytotoxic agent. This conjugate (**1**) is designed to be (i) specific to the vitamin receptors overexpressed on tumor cell surface and (ii) internalized efficiently through receptor-mediated endocytosis, followed by smooth drug release via glutathione-triggered self-immolation of the linker. In order to monitor and validate the sequence of events hypothesized, i.e., receptor-mediated endocytosis of the conjugate, drug release, and drug-binding to the target protein (microtubules), three fluorescent/fluorogenic molecular probes (**2**, **3**, and **4**) were designed and synthesized. The actual occurrence of these processes was unambiguously confirmed by means of confocal fluorescence microscopy (CFM) and flow cytometry using L1210FR leukemia cells, overexpressing biotin receptors. The molecular probe **4**, bearing the taxoid linked to fluorescein, was also used to examine the cell specificity (i.e., efficacy of receptor-based cell targeting) for three cell lines, L1210FR (biotin receptors overexpressed), L1210 (biotin receptors not overexpressed), and WI38 (normal human lung fibroblast, biotin receptor negative). As anticipated, the molecular probe **4** exhibited high specificity only to L1210FR. To confirm the direct correlation between the cell-specific drug delivery and anticancer activity of the probe **4**, its cytotoxicity against these three cell lines was also examined. The results clearly showed a good correlation between the two methods. In the same manner, excellent cell-specific cytotoxicity of the conjugate **1** (without fluorescein attachment to the taxoid) against the same three cell lines was confirmed. This mechanism-based tumor-targeting drug delivery system will find a range of applications.

INTRODUCTION

Cancer is the second leading cause of death (the number 1 cause of death under the age of 85) in the U.S. and remains one of the most challenging diseases to combat. Traditional cancer chemotherapy is based on the premise that tumor cells are more likely to be killed by anticancer drugs because of the faster proliferation of those cancer cells (*1*). However, in reality, the representative cytotoxic chemotherapeutic agents, such as paclitaxel, cisplatin, and doxorubicin, cannot distinguish cancer cells from normal cells. This lack of selectivity leads to undesirable side effects associated with these drugs (*2*), i.e., patients would be at risk of systemic cytotoxicity if exposed to the high doses of cytotoxic agents required to eradicate the tumor. Accordingly, the development of tumor-specific delivery systems for anticancer agents, recognizing the intrinsic differences between normal and tumor cells, is an urgent need for efficacious cancer chemotherapy, and has been receiving increasing attention in recent years (*3*).

In general, a tumor-targeting drug delivery system (DDS) consists of a tumor recognition moiety and a cytotoxic agent connected directly or through a suitable linker to form a conjugate (*4*). The tumor-targeting DDS should be systemically nontoxic. This means that the linker must be stable in blood circulation, but upon internalization into the tumor cells, the conjugate should be readily cleaved to regenerate the active cytotoxic agent.

Cancer cells overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors (*3*). For example, monoclonal antibodies (*1, 2, 5*−*7*), polyunsaturated fatty acids (*8, 9*), folic acid (*10*−*12*), aptamers (*13, 14*), transferrin (*15*), oligopeptides (*16*), and hyaluronic acid (*17*) have been employed as tumor-specific "guiding modules" to construct tumor-targeting drug conjugates.

We have constructed efficacious tumor-targeting DDS based on highly potent new-generation taxoids, which were discovered and developed in our laboratory, in combination with monoclonal antibodies (mAb's: targeting epidermal growth factor receptor) as well as omega-3 polyunsaturated fatty acids (*2, 4, 9*). These tumor-targeting drug conjugates exhibited remarkable efficacy against tumor xenografts in mouse models (*2, 4, 9*), and the mAb-taxoid conjugates demonstrated exceptional tumor-targeting specificity (*2, 4*). However, mAb's are very large (ca. 150 kD) and expensive, containing modification sites that are difficult to pinpoint, and are potentially immunogenic, while drug conjugates with omega-3 fatty acids provide only passive tumor-targeting delivery, although efficacious in vivo. Accordingly, we turned our attention to explore small molecules as tumor-targeting molecules and found that vitamins would serve as excellent guiding molecules (*4, 18*).

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Although all living cells require vitamins for their survival, rapidly dividing cancer cells, in particular, require certain vitamins to sustain their rapid growth (*19, 20*). Consequently, the receptors involved in uptake of the vitamins are overexpressed on the cancer cell surface. Therefore, those vitamin receptors serve as useful biomarkers for the imaging and identification of tumor cells as well as tumor-targeting drug delivery. Vitamin B12, folic acid, biotin, and riboflavin are essential vitamins for the division of all cells, but particularly for the growth of cancer cells. The folate receptors were recognized as potentially excellent biomarkers for targeted drug delivery, and significant advancement has been made to date (*10, 11, 21, 22*). In contrast, the biotin receptors were not investigated for this purpose until recently. Biotin (vitamin H or B-7) is a growth promoter at the cellular level, and its content in tumors is substantially higher than that in normal tissues (*19*). Recently, it has been shown that the biotin receptors are overexpressed more than the folate and/or vitamin B-12 receptors in many cancer cells, e.g., leukemia (L1210FR), ovarian (Ov2008, ID8), Colon (Colo-26), mastocytoma (P815), lung (M109), renal (RENCA, RD0995), and breast (4T1, JC, MMT06056) cancer cell lines (*19, 20*). Accordingly, we chose biotin as the tumor-targeting molecule for our tumor-targeting DDS bearing a self-immolative disulfide linker with a second-generation taxoid as the cytotoxic agent (*4, 18*).

We describe here a mechanistic study on the validation of tumor-targeting DDS by monitoring (i) the internalization through receptor-mediated endocytosis (RME), (ii) drug release triggered by an intracellular thiol, e.g., glutathione (GSH), and (iii) drug binding to the molecular target in cancer cells, using fluorescent and fluorogenic molecular probes of the biotin-linker-taxoid conjugate. The efficacy and cell-specificity of the biotin-linker-taxoid conjugate is also evaluated based on cytotoxicity of the conjugate against cells that overexpress the biotin receptor, as well as those do not express this vitamin receptor, including normal human cells.

EXPERIMENTAL SECTION

Chemical Syntheses, Procedures, and Characterization Data for Conjugates 1−4. See Supporting Information.

Cell Culture. L1210 (ATCC) and L1210FR (a gift from Dr. Gregory Russell-Jones, Access Pharmaceuticals Australia Pty Ltd., Australia) cell lines were grown as a suspension in the RPMI-1640 cell culture medium (Gibco) in the absence of folic acid (FA) but supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) as well as 1% (v/v) penicillin and streptomycin (P/S) at 37 °C in a humidified atmosphere with 5% CO_2. WI-38 human lung fibroblast cells (ATCC) were cultured as monolayers on 100 mm tissue culture dishes in a DMEM cell culture medium (Gibco) that was supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) P/S at 37 °C in a humidified atmosphere with 5% CO_2. The cells were harvested, collected by centrifugation at 1000 rpm for 6 min, and finally resuspended in fresh cell culture medium containing different cell densities for subsequent biological experiments and analysis.

Incubation of Cells with the Biotin−FITC Conjugate 2. The cell suspension (1 mL) at 5×10^5 cells/mL was added to a 1.5 mL microcentrifuge tube first. The biotin−FITC conjugate **2** (10 μM) in DMSO (10 μL) was then injected into the microcentrifuge tube to provide the final concentration of 100 nM, and the resultant suspension was incubated at 37 °C for 3 h. For low-temperature experiments, the incubation of cells (L1210FR) with conjugate **2** was carried out in the cold room at 4 °C for 3 h. For the assessment of the effect of NaN_3 addition, the cells were initially preincubated at 37 °C for 0.5 h with 0.05% (w/v) of NaN_3, prior to the addition of conjugate **2**. For the assessment of the effect of excess biotin addition, the cells were treated with 2 mM of biotin (at the final concentration) at 37 °C for 1 h prior to incubation with conjugate **2**. After incubation, the cells were washed twice with PBS, collected by centrifugation, and resuspended in 100 μL PBS for imaging.

Incubation of Cells with the Biotin-Linker-Coumarin Conjugate 3. The cell suspension (1 mL: 5×10^5 cells/mL) was added to 1.5 mL microcentrifuge tube. The conjugate **3** (100 μM) in DMSO (10 μL) was added to the microcentrifuge tube at the final concentration of 1 μM. After incubation at 37 °C for 3 h, the cells were washed twice by PBS to remove excess conjugates and resuspended in the medium. To observe the release of coumarin in a short period of time, GSH-OEt (10 μL) was added to the L1210FR cell suspension at the final concentration of 2 mM and incubated for another 2 h. Excess GSH-OEt was removed by washing twice with PBS, and the cells were collected by centrifugation. Then, the cells were resuspended in 100 μL PBS prior to imaging. In the control experiment, DMSO (10 μL) was added to the suspension and incubated for another 2 h. After incubation, cells were washed with PBS twice, collected by centrifugation, and resuspended in 100 μL PBS prior to imaging.

Incubation of Cells with the Biotin-Linker-SB-T-1214-Fluorescein Conjugate 4. The cell suspension (1 mL: 5×10^5 cells/mL) was added to a 1.5 mL microcentrifuge tube. The biotin-linker-SB-T-1214-fluorescein conjugate **4** (2 mM) in DMSO (10 μL) was added to the microcentrifuge tube at the final concentration of 20 μM. After incubation at 37 °C for 2 h, the cells were washed twice with PBS to remove excess conjugates and resuspended in the medium. For observation of the release of taxoid in a short period of time, GSH-OEt (10 μL) was added to the L1210FR cell suspension at the final concentration of 2 mM and incubated for another 1 h. Excess GSH-OEt was removed by washing twice with PBS, and the cells were collected by centrifugation. Then, the cells were resuspended in 100 μL PBS prior to imaging. In the control experiment, DMSO (10 μL) was added to the suspension and incubated for another 1 h. After incubation, cells were washed with PBS twice, collected by centrifugation, and resuspended in 100 μL PBS prior to imaging.

Confocal Microscopy Imaging of the Treated Cells. Cells treated as described above were resuspended in 100 μL of PBS after each experiment, and dropped onto an uncoated glass dish (MatTek Corp.). Confocal fluorescence microscopy (CFM) experiments were performed using a Zeiss LSM 510 META NLO two-photon laser scanning confocal microscope system, operating at a 488 nm excitation wavelength and at 527 ± 23 nm detecting emission wavelength using a 505−550 nm bandpass filter. Images were captured using a C-Apochromat 63×/1.2 water (corr.) objective or a Plan-Apochromat 100×/1.45 oil objective. Acquired data were analyzed using LSM 510 META software.

Flow Cytometry Fluorescent Measurements of the Cells. Flow cytometry analysis of the treated cells was performed with a flow cytometer, FACSCalibur, operating at a 488 nm excitation wavelength and detecting emission wavelengths with a 530/30 nm bandpass filter. Cells treated as described above were resuspended in 0.5 mL of PBS. At least 10 000 cells were counted for each experiment using *CellQuest 3.3* software (Becton Dickinson) and the distribution of FITC fluorescence was analyzed using *WinMDI 2.8* freeware (Joseph Trotter, Scripps Research Institute). Propidium iodide staining was used in all experiments to rule out dead cell count in the analysis.

In Vitro Cytotoxicity Assay. The cytotoxicities of biotin-linker-SB-T-1214 (**1**), biotin-linker-SB-T-1214-fluorescein (**4**), SB-T-1214, SB-T-1214-fluorescein (**24**), and paclitaxel were evaluated in vitro against murine leukemia cell lines, L1210FR and L1210, as well as a human lung blastoma cell line, WI38, by means of a quantitative colorimetric assay using a tetrazolium

salt-based analysis ("MTT assay" (*23*); MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.). The inhibitory activity of each compound is represented by the IC_{50} value, which is defined as the concentration required for inhibiting 50% of the cell growth. Cells were harvested, collected, and resuspended in 100 μL medium at a concentration of $\sim 2 \times 10^4$ cells per well over a 96-well plate. For the adhesive cell type, cells were allowed to descend to the bottom of the plates overnight and fresh medium was added to each well upon removal of the old medium. A conjugate (**1** or **4**), taxoid (SB-T-1214 or **24**), or paclitaxel in DMSO stock solution was diluted to a series of concentrations in the cell culture medium without FBS to prepare test solutions. These test solutions at different concentrations ranging from 0.1 to 100 nM (10 μL each) were added to the wells in the 96-well plate and cells were subsequently cultured for 72 h. After spinning down the cells and removing the old medium, the fresh medium containing MTT (e.g., 100 μL of 0.5 mg/mL) was added and incubated at 37 °C for 4 h. The resulting medium was then removed and as-produced insoluble violet formazan crystals were dissolved into 0.1 N HCl in isopropanol with 10% Triton X-100 to give a violet solution. The spectrophotometric absorbance measurement of each well in the 96-well plate was run at 570 nm. The IC_{50} values and their standard errors were calculated from the viability−concentration curve using the Four Parameter Logistic Model of *Sigmaplot*. The concentration of DMSO per well was ≤1% in all cases. The control experiment, i.e., adding 1% DMSO to the cells, indicated that 1% DMSO was not cytotoxic, and thus, all the cells treated remained virtually 100% positive.

RESULTS AND DISCUSSION

Design of an Efficient Tumor-Targeting DDS and Its Molecular Probes. Our tumor-targeting DDS consists of a tumor recognition moiety (biotin in the present study) and a cytotoxic agent connected through a self-immolative linker. The drug conjugate should be nontoxic systemically but release the cytotoxic agent upon internalization to cancer cells, as mentioned above.

Self-Immolative Disulfide Linker. In order to develop efficacious tumor-targeting DDS's, efficient mechanism-based linkers are critically important since the tumor-targeting DDS should be stable in blood circulation while readily cleavable in the tumor. These linkers should be bifunctional so that the cytotoxic agent can be connected to the linker at one end and the tumor-targeting module at the other end. Previously, we designed and examined mAb−taxoid conjugates as tumor-targeting DDS, which exhibited extremely promising results against human cancer xenografts in SCID mice (*2*). The results clearly demonstrated the tumor-specific delivery of a taxoid anticancer agent, curing all animals tested without any noticeable toxicity to the animals (*2*). As the linker for these mAb-taxoid DDS's, a simple disulfide linker was employed, which was stable in blood circulation but efficiently cleaved by intracellular reducing substances in the tumor. However, in this first-generation mAb-taxoid DDS, the original taxoid molecule was not released because of the compromised modification of the taxoid molecule to accommodate the disulfide linker. Thus, the cytotoxicity of the taxoid released (SB-T-12136-SH) was 8 times weaker than the parent taxoid (SB-T-1213) (*2*).

As a solution to this problem, we devised the second-generation mechanism-based bifunctional disulfide linkers, which can be connected to various cytotoxic agents and tumor-targeting molecules. We designed self-immolative disulfide linkers wherein the intracellular thiol-triggered cascade drug release should generate the original anticancer agent via thiolactone formation and ester bond cleavage (Figure 1) (*24*).

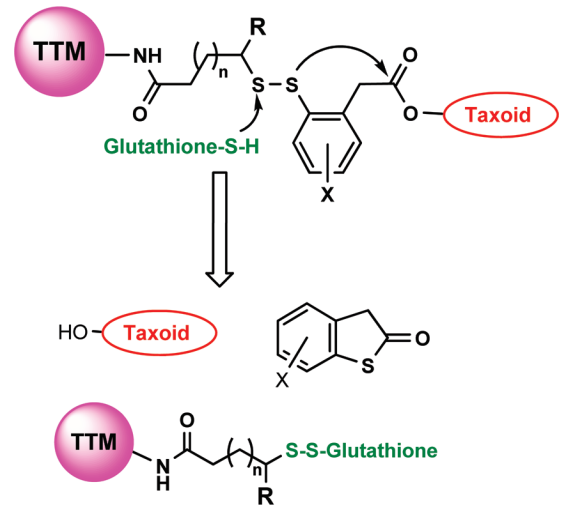


Figure 1. Second-generation self-immolative disulfide linkers (TTM = tumor-targeting module).

The designed mechanism-based drug release has been proven in a fluorine-labeled model system by monitoring the reaction with ^{19}F NMR (*24*). The strategic placement of a phenyl group attached to the disulfide linkage should direct the cleavage of this linkage by a thiol to generate the desirable thiophenolate or sulfhydrylphenyl species for thiolactonization. The disulfide linkage should be cleaved by endogenous thiols, e.g., GSH and thioredoxin. It has been shown that the GSH level in cancer cells is >1000 times higher than that in blood plasma (*25*−*29*). The resulting thiol undergoes an intramolecular nucleophilic substitution at the ester moiety, forming a 5-membered ring thiolactone and releasing the taxoid in its original form. This type of self-immolative disulfide linker can readily be applied to a variety of tumor-targeting drug conjugates. We chose a rather simple linker (R = X = H, $n = 0$) in the present study.

Cytotoxic Agent. Although paclitaxel and docetaxel have brought about significant impact on cancer chemotherapy, mainly because of their unique mechanism of action (*30*), these drugs seriously suffer from the lack of tumor specificity and multidrug resistance (MDR). These taxane drugs are effective against breast, ovary, and lung cancers, while they do not show efficacy against colon, pancreatic, melanoma, and renal cancers. As an example, human colon carcinoma is inherently multidrug-resistant due to the overexpression of P-glycoprotein (Pgp), which is an effective ATP-binding cassette (ABC) transporter and effluxes out hydrophobic anticancer agents, such as paclitaxel and docetaxel (*31*).

We have developed a series of highly potent second- and third-generation taxoids through structure−activity relationship study of taxoids (*32*−*37*). Most of these taxoids exhibited 2−3 orders of magnitude higher potency than those of paclitaxel and docetaxel against MDR cell lines expressing Pgp. Accordingly, these highly potent taxoids have been used as cytotoxic agents of our tumor-targeting DDS's. Among these new-generation taxoids, we chose SB-T-1214, a second-generation taxoid, as the warhead for the present study.

Molecular Probes of the Biotin-Linker-Taxoid Conjugate. The structure of a "guided molecular missile", biotin-linker-SB-T-1214 (**1**), is shown in Figure 2. In order to monitor the sequence of events involved in tumor-targeting drug delivery of conjugate **1**, it was necessary for us to design and synthesize three fluorescence-labeled biotin conjugates, i.e., biotin-fluorescein (**2**), biotin-linker-coumarin (**3**) (fluorogenic probe), and biotin-linker-taxoid-fluorescein (**4**) (Figure 3).

Conjugate **2** was designed to observe RME (Figure 4), while conjugate **3** was designed for confirming the internalization of

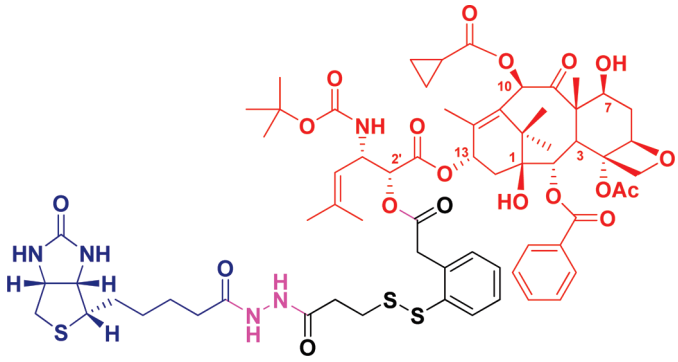


Figure 2. Biotin-linker-SB-T-1214 conjugate (**1**).

the conjugate via RME and the release of coumarin, which becomes fluorescent only when it is released as a free molecule via disulfide cleavage. Fluorogenic molecules, such as coumarin derivatives, have been widely utilized as model systems for probing drug release (*38*). Conjugate **4** was designed to validate the whole internalization by RME and drug release processes, wherein the released fluorescent taxoid should bind to the target protein, microtubules, in the cancer cells (Figure 4).

Figure 4 illustrates the internalization and drug release mechanism of a biotin-linker-taxoid conjugate. Once localized at the tumor site, the drug-conjugate binds to its complementary receptor on the tumor cell surface and is further internalized via RME. The disulfide linkage of the drug-conjugate is cleaved by endogenous reducing agents (e.g., GSH) to generate a thiol group. The resulting thiol group undergoes thiolactonization to release the taxoid warhead in its original form (i.e., SB-T-1214). When a fluorescent taxoid (i.e., SB-T-1214-fluorescein) is used, it should bind to microtubules, visualizing the fluorescent microtubule network.

Chemical Synthesis. The fluorescent probe, biotin-FITC **2**, was synthesized via coupling of fluorescein isothiocyanate (FITC) to biotin-$NHNH_2$ (**5**), which was readily prepared from biotin through esterification and subsequent reaction with hydrazine, as shown in Scheme 1.

The synthesis of the fluorogenic probe, biotin-linker-coumarin **3**, is illustrated in Scheme 2. First, triisopropylsilyl(TIPS) pyridine-2-yldisulfanylpropanoate (**8**) was prepared through thiol-disulfide exchange reaction of pyridine disulfide (**6**) with 3-sulfhydrylpropanoic acid, giving **7**, followed by TIPS protection of the carboxylic acid moiety of **7**. Second, 2-sulfhydrylphenylacetic acid (**11**) was prepared from benzothiophene-2-boronic acid (**9**) through hydrogen peroxide oxidation to benzothiophen-2-one (**10**), followed by hydrolysis with lithium hydroxide. The reaction of **8** with **11** via thiol-disulfide exchange reaction gave 2-(TIPSO-carbonylethyl)disulfanylphenylacetic acid (**12**). The coupling of **12** with 7-hydroxy-4-methylcoumarin (**13**) gave coumarin-linker (TIPS) conjugate (**14**), which was deprotected to afford coumarin-linker conjugate **15**. The DCC-coupling of **15** with *N*-hydroxysuccinimide (HOSu) afforded coumarin-linker-OSu active ester **16**. The amide coupling of **5** with **16** gave conjugate **3**.

The synthesis of biotin-linker-taxoid-fluorescein **4** is illustrated in Schemes 3−5. First, commercially available fluorescein was converted to the corresponding methyl ester **17** (*39*), while succinic acid mono-*tert*-butyl ester (**18**) was reduced to *tert*-butyl 4-hydroxybutanoate (**19**) (*40*). The Mitsunobu coupling of **17** with **19** afforded modified fluorescein diester **20** (*39*). Selective deprotection of the *tert*-butyl group of **20** with trifluoroacetic acid afforded 6-tethered fluorescein methyl ester **21** (Scheme 3). Next, the C2′ hydroxy group of taxoid SB-T-1214 was protected as *tert*-butyldimethylsilyl (TBS) ether to give **22**, which was coupled with **21** under DIC/DMAP conditions, followed by deprotection of the C2′-TBDMS group to afford 7-fluorescein-tethered taxoid **24** (Scheme 4). Subsequently, SB-T-1214-fluorescein **24** was coupled with disulfanylacetic acid **12** using DIC/DMAP to give **25**, which was deprotected with HF-pyridine to afford (SB-T-1214-fluorescein)-linker-CO_2H conjugate **26** (Scheme 5). The carboxylic acid moiety of **26** was converted to the corresponding activated ester **27** via esterification with HOSu in the presence of DIC. Finally, activated ester **27** was reacted with **5** to give biotin-linker-SB-T-1214-fluorescein conjugate **4** in high yield (Scheme 5).

Following the same synthetic protocol, biotin-linker-SB-T-1214 **1** was synthesized by using SB-T-1214 without the fluorescein tether, as shown in Scheme 6.

Probing Receptor-Mediated Endocytosis (RME), Drug Release, and Drug Binding to Target. Internalization of these fluorescent and fluorogenic probes **2**−**4** was monitored by confocal fluorescence microscopy (CFM) and the fluorescence was quantified by flow cytometry, using ca. 10 000 treated live cells. Results are shown in Figures 5−8.

Figure 5A shows the intense fluorescence observed when the L1210FR cell was incubated with the probe **2** (100 nM) at 37 °C for 3 h, followed by thorough washing of the cells by phosphate-buffered saline (PBS) and CFM analysis. The result clearly indicates the efficient internalization of the fluorescent probe **2** into the leukemia cells. It has been shown that endocytosis is an energy-dependent process and should be inhibited either at low temperature (4 °C) or in the presence of the metabolism inhibitor, typically NaN_3 (*41*−*43*). The CFM image of the probe **2**, incubated at 4 °C (100 nM, 3 h) showed dramatically diminished (ca. 6 times by flow cytometry) fluorescence in the cells (Figure 5B). When L1210FR cells were incubated with probe **2** (100 nM, 3 h) in the presence of 0.05% (w/v) NaN_3 at 37 °C, a substantial decrease (ca. 4 times) in fluorescence occurred inside the cells as well (Figure 5C). These results unambiguously show that probe **2** was internalized through endocytosis. To validate that this was a RME process, excess free biotin (2 mM) was preincubated for 1 h to saturate the biotin receptors on the cancer cell surface, and subsequently, probe **2** (100 nM, 37 °C, 3 h) was added. The CFM image revealed a substantial decrease (ca. 4.5 times) in fluorescence. The result confirms that this is indeed the receptor-mediated endocytosis (Figure 5D).

Next, the fluorogenic probe **3** (1 μM) was incubated with L1210FR cells at 37 °C for 3 h, and afterward, the cells were washed thoroughly with PBS. Then, glutathione ethyl ester (GSH-OEt, 2 mM) was added to the medium and cells were incubated for another 2 h at 37 °C. The addition of GSH-OEt was to ensure the cleavage of the self-immolative disulfide linker to release free coumarin, as designed. [Note: It has been shown that GSH-OEt is internalized into cells to generate GSH inside the cell (*44*). GSH-OEt also acts as a reducing agent.] As Figure 6A shows, fluorescent coumarin molecules (blue) are indeed released in L1210FR cells. The result unambiguously demonstrates that the intracellular release of coumarin (a drug surrogate) via cleavage of the disulfide linkage by GSH and the subsequent thiolactonization, took place as designed. In the absence of additional GSH (or GSH-OEt), the observed blue fluorescence was drastically weaker (Figure 6B). This means that the concentration of intracellular GSH in this leukemia L1210FR cell line is rather low, especially in the 3 h time frame, under the in vitro experimental conditions, which are significantly different from those in vivo where the GSH supply in tumor tissues is more than adequate (*26, 27*). On the other hand, this experiment using additional GSH (as GSH-OEt) confirmed that the cleavage of the self-immolative disulfide linkage and subsequent drug release was caused by GSH.

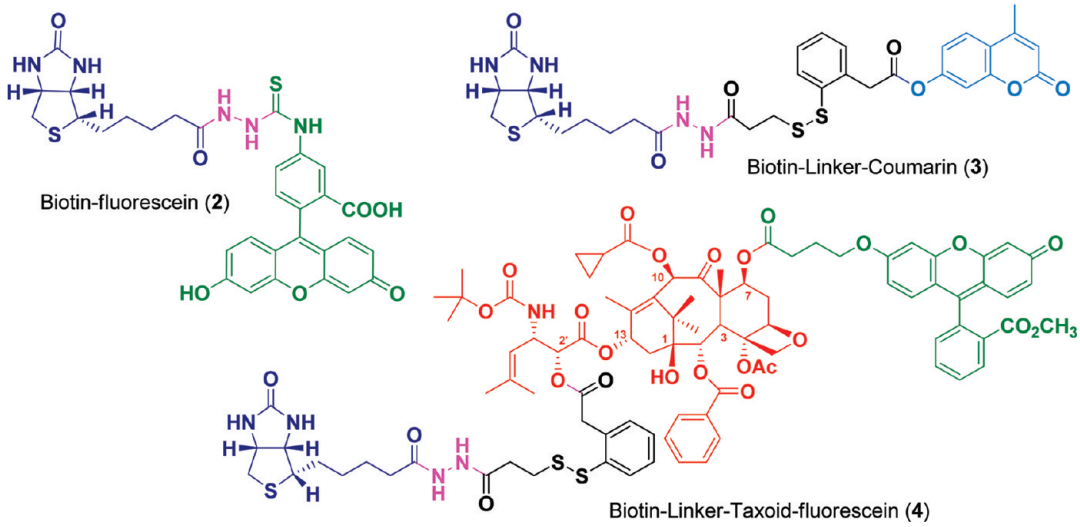


Figure 3. Fluorescent and fluorogenic probes for the internalization and drug-release.

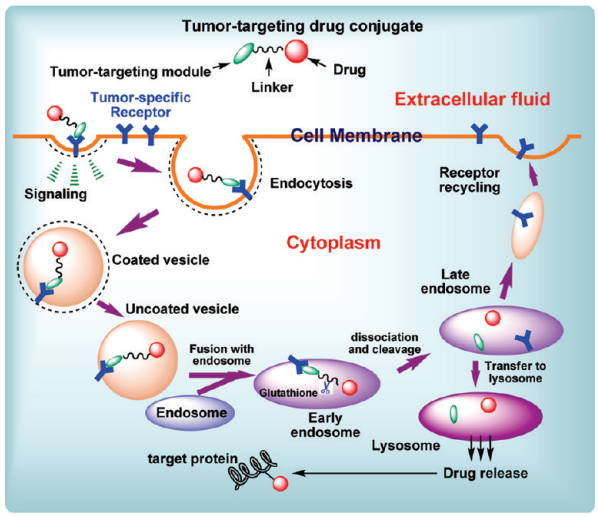
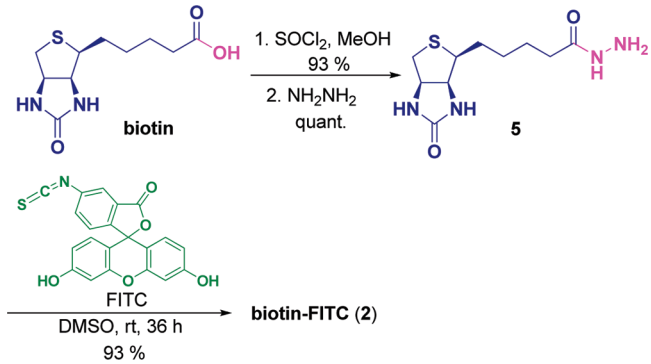


Figure 4. Schematic representation of the RME of a tumor-targeting drug conjugate, drug release, and drug-binding to the target protein.

Scheme 1. Synthesis of Biotin−FITC Conjugate 2



Finally, the internalization and drug release of probe **4** was examined. Probe **4** (20 μM) was incubated with L1210FR cells at 37 °C for 2 h and analyzed by CFM. As Figure 7A shows, the whole conjugate was internalized in the same manner as that described above for the probe **2** (see Figure 5A). Next, GSH-OEt (2 mM) was added to the medium, and the cells were incubated for another 1 h to ensure the drug release. As Figure 7B shows, the CFM image of this system is dramatically different from that shown in Figure 7A. The CFM image in Figure 7B indicates that the released fluorescent taxoid binds to the microtubules that are the drug target of the taxoid and highlights the fluorescence-labeled microtubule bundles. The result confirms that the release of the taxoid proceeded through the designed mechanism (see Figure 1) in the same manner as that observed for the fluorogenic probe **3**. To double-check the morphology of microtubules with the fluorescence-labeled taxoid, the CFM image of the L1210FR cells treated with SB-T-1214-fluorescein (**24**) was taken. As Figure 7C shows, the morphology of treated cells is indeed virtually identical to that treated with the conjugate **4**. This provides additional evidence of efficient release of **24** from the conjugate **4**.

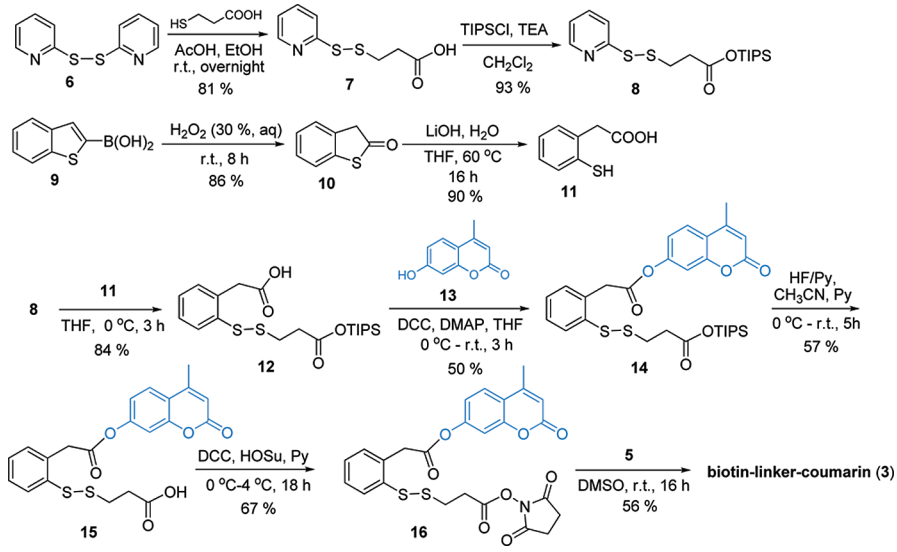
It should be noted that the intracellular GSH in L1210FR would also be able to cleave the disulfide linkage with longer incubation time (vide infra), but the endogenous GSH level in cancer cells varies due to the significant difference in the physiological conditions in the actual leukemia or solid tumors. Accordingly, the extracellular addition of excess GSH-OEt was beneficial for rapid visualization of the drug release inside the leukemia cells.

Consequently, we can safely conclude that the tumor-targeting DDS successfully delivered the active cytotoxic agent to the drug target, as designed, through RME and GSH-triggered intracellular drug release via cleavage of self-immolative disulfide linker and thiolactonization.

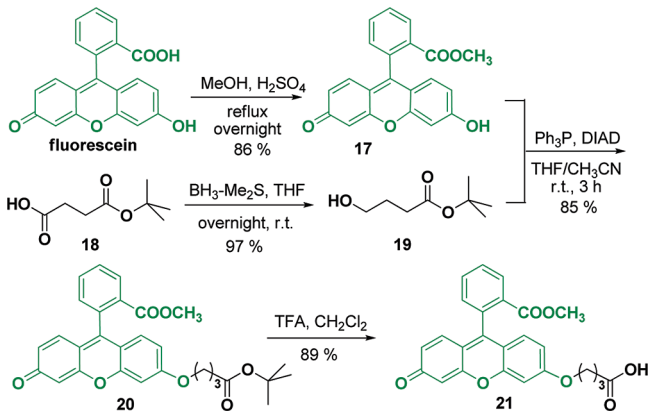
Evaluation of Tumor-Targeting Specificity of Biotin-Linker-Taxoid Conjugates by CFM and Cytotoxicity Assay. To evaluate the tumor specificity of the biotin-mediated RME, two other cell lines, i.e., L1210 murine leukemia cell line and WI38 noncancerous human lung fibroblast cell line, were chosen to compare results with that for L1210FR cell line. Both L1210 and WI38 cell lines do not overexpress biotin receptors on their cell surfaces. Accordingly, this comparison should provide a fair and definitive information about the efficacy of tumor-targeting using biotin as the "guiding" module for our "guided molecular missile".

As Figure 8 clearly shows, much stronger (ca. 12−13 times) fluorescence was indeed observed in L1210FR cells (Figure 8A) as compared to that in L1210 cells (Figure 8B) as well as WI38 cells (Figure 8C) upon incubation with the probe **4** under identical conditions (20 μM at 37 °C for 2 h) by CFM and flow cytometry analyses. Thus, the results confirm the validity of our tumor-targeting design based on this vitamin and its receptors, which can clearly distinguish cancer cells overexpressing the vitamin receptors from other cells which do not, including human normal cells.

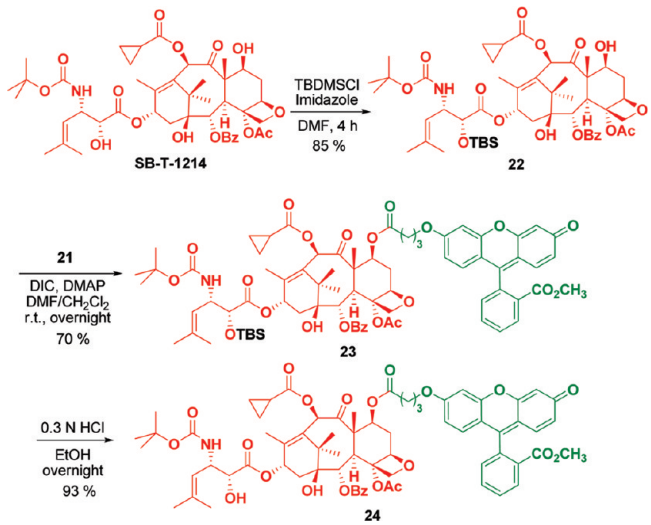
Scheme 2. Synthesis of Biotin-Linker-Coumarin Conjugate (3)



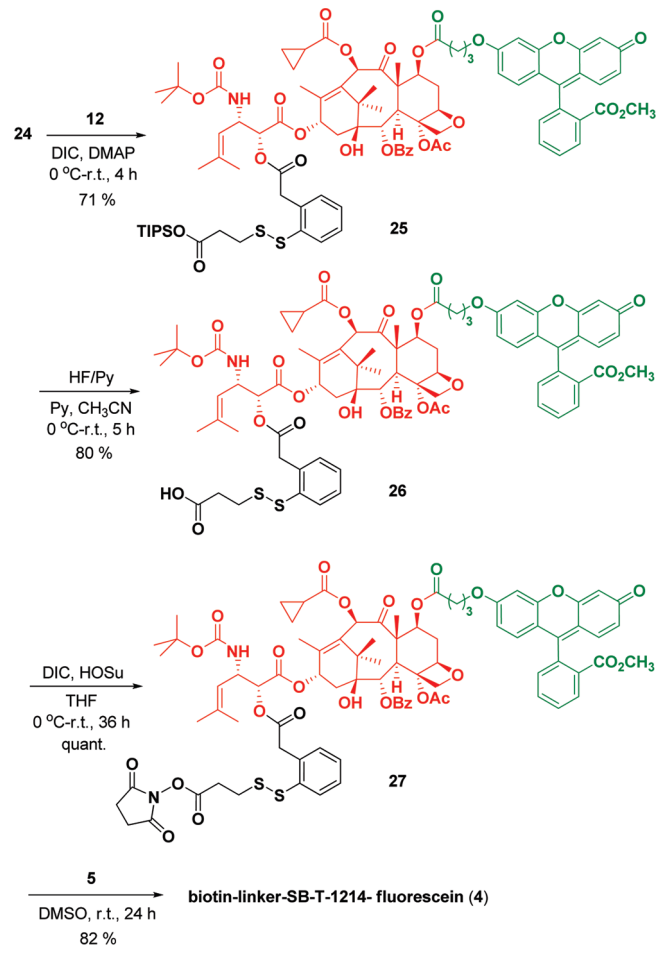
Scheme 3. Preparation of Fluorescein Derivative with Carboxylic Acid Tether



Scheme 4. Synthesis of SB-T-1214 Linked to Fluorescein (24)



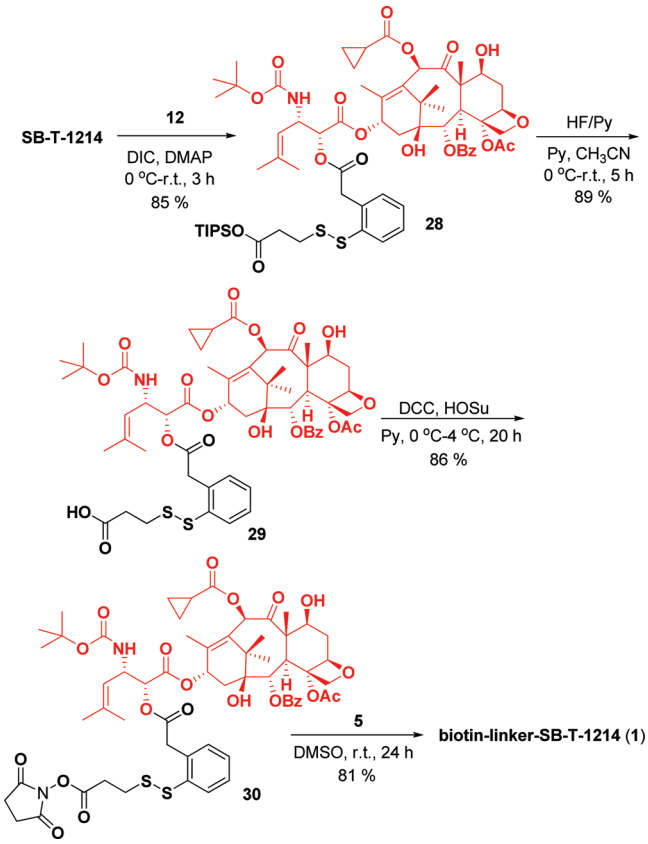
Scheme 5. Synthesis Diagram of Biotin-Linker-SB-T-1214-Fluorescein Conjugate (4)



As described above, the mechanistic investigation into the cancer cell specificity, RME, drug release, and drug binding to the target protein with the use of fluorescent and fluorogenic molecular probes were successfully executed by means of CFM and flow cytometry. However, these observations should be correlated to the cancer cell specific cytotoxicity of the originally designed biotin-linker-taxoid conjugate **1** against three cell lines examined by CFM and flow cytometry.

Accordingly, the cytotoxicity assays (MTT method (*23*)) of conjugate **1** against L1210FR, L1210, and WI38 cell lines were performed. For comparison purpose, the parent taxoid (SB-T-1214), its fluorescein conjugate **24**, and paclitaxel were assayed as well. It should be noted that, in these assays, no GSH-OEt was added to the medium, since the exposure time is much longer (72 h) than that for CFM and flow cytometry analyses

Scheme 6. Synthesis of Biotin-Linker-SB-T-1214 (1)



(2−3 h), and hence, the amount of endogenous GSH should be sufficient to release the taxoid warhead (SB-T-1214) from conjugate **1**. Results are summarized in Table 1.

As Table 1 shows, conjugate **1** exhibited high potency (IC_{50} 8.8 nM) against L1210FR, overexpressing biotin receptors, while those against L1210 (IC_{50} 522 nM) and WI38 (IC_{50} 570 nM) cell lines were 59 times and 65 times less. For comparison, the parent taxoid (SB-T-1214) was also assayed against those three cell lines. As anticipated, SB-T-1214 cannot distinguish these cell lines, resulting in very similar IC_{50} values, i.e., 9.5 nM for L1210FR, 9.7 nM for L1210, and 10.7 nM for WI38. The results clearly indicate that highly efficient vitamin-receptor specific drug delivery to cancer cells has been achieved by conjugate **1**, and also, the drug release by endogenous GSH (and possibly by some other thiols) is efficient as well in 72 h incubation.

As Table 1 shows, the IC_{50} values of SB-T-1214, SB-T-1214-fluorescein, (**24**) and paclitaxel against L1210FR are 9.5 nM, 87.6 nM, and 122 nM, respectively. Thus, SB-T-1214 is ca. 13 times more potent than paclitaxel, and fluorescently labeled **24** is ca. 9 times less potent than the parent SB-T-1214 against L1210FR. As mentioned above, SB-T-1214 exhibited very similar IC_{50} values against these three cell lines. SB-T-1214-fluorescein **24** and paclitaxel showed slight to small differences in their IC_{50} values against the three cell lines, but differences are less than 45% at most (see Table 1). Thus, it is safe to say that these two taxanes cannot distinguish targeted cancer cells from normal cells, as anticipated.

We also assayed the cytotoxicity of the probe **4** against the same three cell lines. As Table 1 shows, virtually the same differences in IC_{50} values were observed, i.e., 81.7 nM for L1210FR and >5 μM for L1210 as well as WI38 (>61 times difference), as those for the conjugate **1**. Thus, the drug delivery to L1210FR is highly specific through vitamin-receptor recognition and RME in this conjugate (probe **4**), as well. As mentioned above, the cell specificity of probe **4** quantified by flow cytometry was 11.7 for L1210FR/L1210 and 13.2 for L1210FR/WI38, which is more modest as compared to that based on cytotoxicity. It is most likely that this difference is attributed to the substantially shorter exposure time for the flow cytometry analysis (2 h) than that for the cytotoxicity assay (72 h), i.e., the internalization of the probe **4** has not been completed in 2 h. At any rate, the observation by CFM and flow cytometry corresponds well to the cytotoxicity assay results.

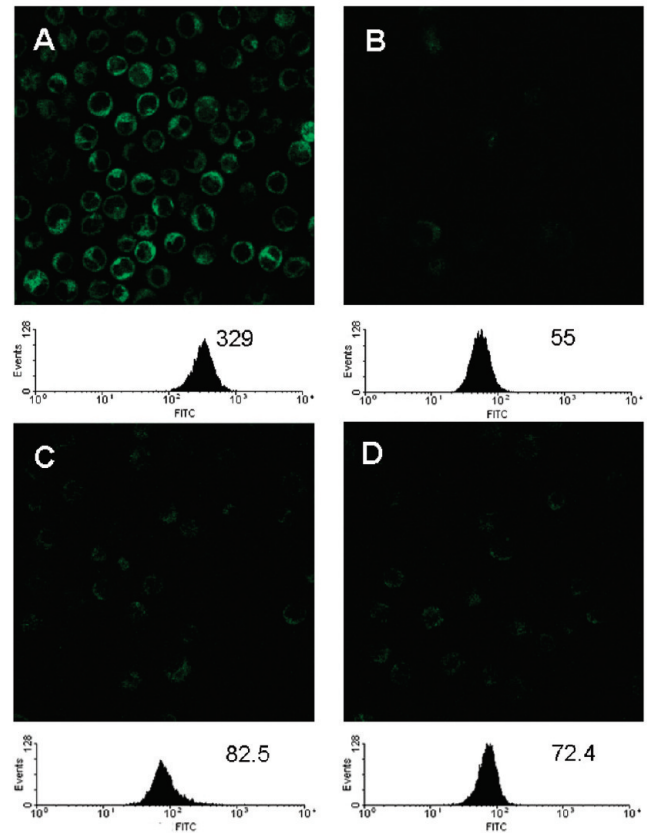


Figure 5. CFM images and flow cytometry analysis of L1210FR cells after incubation with the probe **2** under different conditions: (A) 100 nM, 37 °C, 3 h; (B) 100 nM, 4 °C, 3 h; (C) 100 nM, 37 °C, 0.05% NaN_3, 3 h; (D) 100 nM, 37 °C, 3 h, pretreated with excess biotin (2 mM).

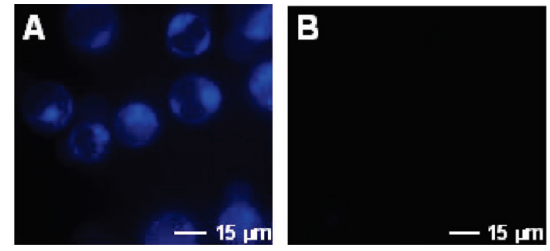


Figure 6. (A) Epifluorescence image of L1210FR cells that were initially incubated with the probe **3** (nonfluorescent form), followed by treatment with GSH-OEt to trigger the self-immolation of the linker. The released coumarin showed blue fluorescence. (B) Epifluorescence image of L1210FR cells after incubation with the probe **3** (nonfluorescent form).

CONCLUSIONS

A highly efficient mechanism-based tumor-targeting DDS, consisting of a tumor-targeting molecule, self-immolative linker, and a cytotoxic agent, was designed and successfully developed. The proof of concept was obtained by using a drug conjugate, bearing biotin as the cell-targeting moiety, a mechanism-based

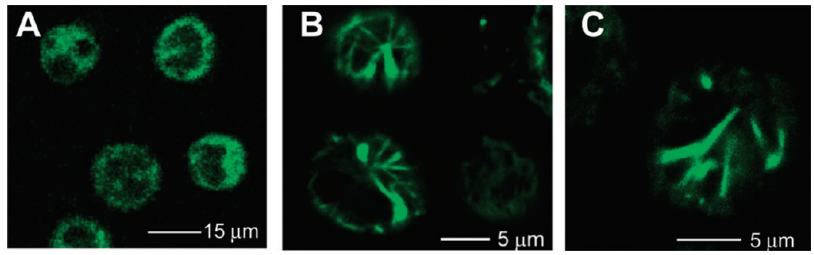


Figure 7. (A) CFM image of L1210FR cells after incubation with the probe **4**. (B) CFM image of L1210FR cells that were initially incubated with the probe **4**, followed by treatment with GSH-OEt to release the fluorescent taxoid **24**. Then, the microtubule network in the cells was fluorescently labeled by **24** and visualized. (C) CFM image of L1210FR cells after incubation with **24** as a control experiment.

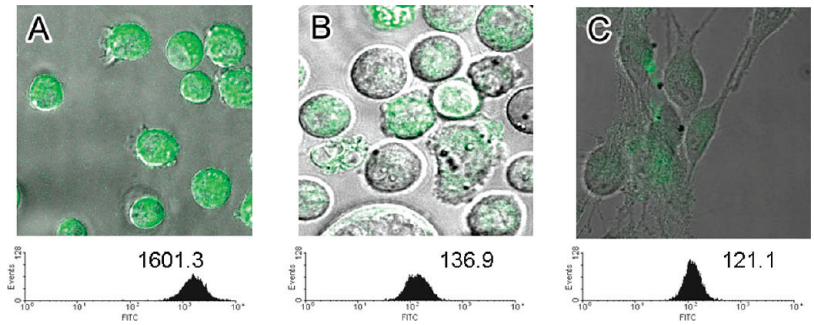


Figure 8. CFM images and flow cytometry analysis of different types of cells after incubation with the probe **4** at the final concentration of 20 μM at 37 °C for 2 h: (A) L1210FR that overexpressed biotin receptors; (B) L1210; and (C) WI38 human lung fibroblast cells.

Table 1. Cytotoxicities (IC_{50}, nM) of Paclitaxel, SB-T-1214, and Its Conjugates

	L1210FR	L1210	WI38
paclitaxel	121	151	157
SB-T-1214	9.50	9.72	10.7
24	87.6	107	127
1	8.80	522	570
4	81.7	>5000	>5000

disulfide linker, and a second-generation taxoid linked to fluorescein by means of CFM and flow cytometry. The result was confirmed by cytotoxicity assay using the drug conjugates with and without a link to fluorescein. Both conjugates exhibited excellent cell-specificity against three cell lines, L1210FR (biotin receptor +++), L1210 (biotin receptor ±), and WI38 (biotin receptor ±) based on the expression of biotin receptors on the cell surfaces. Efficient internalization of the drug conjugate into the targeted cancer cells via RME, smooth drug release via GSH-triggered self-immolation of disulfide linker, and drug binding to the target protein (microtubules) were monitored and validated by means of CFM using three fluorescent/fluorogenic molecular probes. This tumor-targeting DDS is applicable to a variety of drug conjugates consisting of various tumor-targeting molecules, e.g., monoclonal antibodies, aptamers, vitamins, peptides, and proteins, as well as cytotoxic agents. Further studies along this line are actively underway in these laboratories (*18*).

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Supporting Information Available: Chemical synthesis of conjugates **1**−**4** and intermediates, including procedures and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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