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## Tumor-targeting drug delivery of new-generation taxoids

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### Abstract

A long-standing problem of conventional cancer chemotherapy is the lack of tumor specificity. Tumor-targeting drug-delivery systems have been explored to overcome this problem. These systems combine a powerful cytotoxic anticancer agent with a tumor-targeting molecule via a ‘smart’ linker to form highly efficacious drug conjugates. These drug conjugates can deliver potent cytotoxic drugs specifically to tumors and tumor cells with minimal systemic toxicity. This review describes our groups’ research on the molecular approaches to the design and development of a novel drug-delivery system bearing highly potent new-generation taxoids for tumor-targeting chemotherapy in our laboratory.

Cancer is both the leading cause of death in developed countries and the second leading cause in developing countries [1]. Currently, one out of every four deaths in the USA is caused by cancer. It has been estimated that there will be approximately 1.6 million new cancer cases and over half a million deaths from cancer in the USA in 2011 [2]. Despite the significant advances in cancer detection, prevention, surgical oncology, chemotherapy and radiation therapy, there is still no common cure for this disease. Traditional chemotherapy relies on the premise that rapidly proliferating tumor cells are more likely to be destroyed than normal cells by cytotoxic agents. However, in reality, these cytotoxic agents have little or no specificity, leading to severe and dose-limiting side effects such as neutropenia, anemia, hair loss, damage to liver, kidney and bone marrow. Therefore, extensive efforts have been made to develop highly efficacious tumor-targeting drug conjugates to overcome the shortcomings of conventional chemotherapy in the last few decades [3,4]. These drug conjugates recognize and take advantage of intrinsic morphological and physiological differences between normal and cancerous cells/tissues. For example, rapidly growing cancer cells overexpress cancer-specific receptors to enhance the uptake of nutrients and vitamins. These receptors can be used as targets to deliver cytotoxic drugs specifically to cancer cells through receptor-mediated endocytosis (RME). A variety of ligands to those cancer-specific receptors, such as monoclonal antibodies (mAbs), polyunsaturated fatty acids (PUFAs), folic acid, transferrin, oligopeptides, hyaluronic acid and aptamers, have been exploited as the tumor-targeting modules (TTMs) to construct tumor-targeting drug conjugates, and a numbers of those drug conjugates are currently in clinical and preclinical development [3–6]. Tumor- targeting drug conjugates typically consist of a TTM connected to an anticancer agent directly or through a suitable ‘smart’ linker. These drug conjugates should be nontoxic and stable in blood circulation to minimize systemic toxicity, but should

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be effectively internalized inside the tumor cells and efficiently release the anticancer agent without loss of potency [3,7–9].

We describe an account of our research on the design and development of novel tumor-targeting drug-delivery systems for new-generation taxoid anticancer agents.

## New-generation taxoids to be delivered specifically to tumors

Paclitaxel and docetaxel have had a significant impact on current cancer chemotherapy, but seriously suffer from the lack of tumor specificity and multidrug resistance (MDR). Paclitaxel and docetaxel are effective against solid tumors, such as 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 due to the overexpression of P-gp, which is an effective ATP-binding cassette transporter. P-gp effluxes hydrophobic anticancer agents, including paclitaxel and docetaxel, out of cancer cells [10]. On the basis of our extensive structure–activity relationship study of taxoids, we have developed a series of highly potent new-generation taxoids [11–19], including ‘ortataxel’ which has advanced to Phase II human clinical trials [20]. Most of these new-generation taxoids exhibited two-to-three orders of magnitude higher potency than those of paclitaxel and docetaxel against drug-resistant cell lines expressing MDR phenotypes. Accordingly, we have used these highly potent taxoids as the cytotoxic agents for tumor-targeting drug-delivery systems. Selected new-generation taxoids are listed in **Table 1** and their potencies in **Table 2**.

Recently, it has been shown that the ineffectiveness of standard chemotherapeutic agents could be attributed to the existence of relatively rare, highly drug resistant, quiescent or slowly proliferating tumor-initiating cells (i.e., cancer stem cells [CSCs]) [21,22]. These cells have heightened expression of many stem cell-related genes, such as those in the Notch and Wnt signaling pathways, exhibit similar **pluripotency**, and are notoriously malignant. CSCs are exclusively endowed with tumor-initiating capacity in the majority of cancer types, and are responsible for sustaining tumor growth, meta stasis and relapse.

One of the new-generation taxoids, SB-T-1214, exhibited remarkable efficacy against highly drug-resistant (P-gp+) colon tumor **xeno-graft** in severe combined immune deficiency (SCID) mice, inducing complete regression in all surviving mice with tumor growth delay >187 days [18]. The observed total suppression of tumor recurrence by SB-T-1214 might indicate that this taxoid can kill or regulate CSCs by inhibiting stem cell-related gene expression of CSCs and/or promoting their differentiation. Thus, we examined the activity of SB-T-1214 against colon CSCs from HCT116, HT-29 and DLD-1 cell lines using cancer spheroids in 3D cultures [23]. Administration of 100 nM SB-T-1214 to the HCT116, HT-29 and DLD-1 spheroids for 48 h resulted in marked suppression of the growth of the secondary spheroids in all cells (**Figure 1**). Most importantly, viable cells that survived this treatment regimen significantly lost the ability to form secondary spheroids, which indicates that colon CSC population was critically affected. In fact, the cells that survived drug treatment displayed profound morphological abnormalities, indicating a clear sign of the mitotic catastrophe.

Also, it was found that the treatment of HCT-116, DLD-1 and HT-29 CSCs with SB-T-1214 led to the downregulation of a number of stem cell-related genes and significant inhibition of genes involved in retaining pluripotency [23]. Relatively low concentrations of SB-T-1214 (100 nM for 24 or 48 h) inhibited the majority of stem cell-related genes in all colon CSCs examined. It is worthy of note that many of these genes are related to self-renewal, regulation of symmetric/asymmetric division and pluripotency.

These results provided additional strong support for the use of new-generation taxoids as the highly potent cytotoxic anti-tumor agents for novel tumor-targeting drug-delivery systems.

## PUFA as a TTM of drug-delivery system

**Omega-3 PUFA** such as docosahexaenoic acid (DHA), eicosapentaenoic acid and linolenic acid are naturally occurring compounds found in vegetable oils, cold-water fish and meat. In a single-arterial perfusion study, it was shown that some PUFAs are taken up more rapidly by tumor tissue than normal cells [24]. In addition, some omega-3 PUFAs have shown anticancer and drug-sensitizing activity against various cancers in both clinical and preclinical trials [25,26]. It has also been shown that PUFAs are readily incorporated into the lipid bilayer of tumor cells, which disrupts the morphology of the cell and presumably influences the susceptibility of the tumor cells to anticancer agents [27,28].

PUFAs are utilized as cellular membrane components, energy resources and signaling molecules. It has also been shown that PUFAs and their metabolites affect a number of cellular processes involved in carcinogenesis including inflammation, apoptosis and cell division. Furthermore, PUFAs are readily taken up by malignant cells, thus making them ideal TTMs for drug conjugation. The conjugation of cytotoxic drugs to PUFAs has been found to substantially alter their pharmacokinetics (PK), resulting in the tumor-specific accumulation of the cytotoxic agent [29].

The PUFA component of the PUFA–drug conjugate readily binds to **human serum albumin** (HSA) to form a complex, which helps improve solubility in blood plasma [30]. There is evidence to suggest that gp60 and secreted protein, which is acidic and rich in cysteine, play a crucial role in the transport of HSA-bound complexes from blood vessels to the tumor interstitium leading to an accumulation of the HSA-bound complexes in the tumor tissue (**Figure 2**) [31–33].

It is very likely that PUFA–drug conjugates are accumulated into the tumor tissue, followed by uptake to tumor cells, wherein the PUFA–drug linkage is cleaved, releasing the PUFA and the free drug. It is possible that once internalized, certain PUFAs and their metabolites may result in synergism with cytotoxic drugs due to their ability to modulate several key intracellular processes [34]. Thus, conjugation of drugs to PUFAs results in tumor-specific drug accumulation, lower volume of distribution to benign tissues and enhanced efficacy.

DHA–paclitaxel (**Figure 3**), which has advanced to Phase III clinical evaluation against several cancer types [101], has been shown to have better PK properties and increased efficacy than its parent compound paclitaxel [29]. DHA–paclitaxel exhibits less systemic toxicity than the parent compound by one or two orders of magnitude, until the ester linkage is cleaved. In addition, the high levels of serum protein binding significantly reduces clearance rate and volume of distribution, resulting in less systemic toxicity, hence reduced side effects [35]. Another advantage of DHA–paclitaxel over paclitaxel is that the DHA–drug conjugate exhibits a wider therapeutic window, and therefore allows the delivery of higher concentrations of paclitaxel to the tumor. It is unlikely, however, that DHA–paclitaxel would be effective against MDR tumors that overexpress P-gp, as paclitaxel is a well-documented P-gp substrate.

As mentioned above, a number of new-generation taxoids exhibit two to three orders of magnitude better activity compared with paclitaxel against MDR cancer cell lines [11,12,18]. Thus, PUFA conjugates that bear a new-generation taxoid should be more efficacious than DHA–paclitaxel against MDR cell lines. Accordingly, novel PUFA–taxoid conjugates were synthesized and assayed *in vivo* for their efficacy against different drug-resistant and drug-sensitive human tumor xenografts in SCID mice (**Figure 4**) [36]. Several

of these conjugates led to a complete remission in all surviving mice with minimal systemic toxicity. Remarkably, DHA-SB-T-1214 led to a complete regression of the highly drug-resistant DLD-1 colon tumor xenograft in five out of five mice without recurrence of tumor growth for more than 190 days after treatment. Again, little systemic toxicity was observed, implying the tumor specificity of these conjugates (**Figure 4**). DHA-SB-T-1214 is currently undergoing the last stage of preclinical evaluations, and an Investigational New Drug application to the US FDA will be filed in the near future.

## mAb as a TTM of drug-delivery system

Cancer cells overexpress certain antigens on the cell surface, and these (tumor-specific) antigens can be used as a biomarker to differentiate tumor tissues from normal tissues [37]. mAbs with high binding specificity to tumor-specific antigens can be developed, which can be used as drug-delivery vehicles to carry a payload of cytotoxic agents specifically to the tumor site. Upon binding to the cell surface antigen, an mAb–drug conjugate is internalized via RME and anticancer agents would be released inside the tumor cell. For example, brentuximab vedotin is a mAb–drug conjugate targeting CD30 and recently approved by the FDA for the treatment of Hodgkin's lymphoma and several other mAb–drug conjugates are currently in human clinical trials. Brentuximab vedotin consists of an anti-CD30 mAb and monomethyl auristatin E, a potent antimetabolic agent, which are conjugated through a citrulline–valine dipeptide linker (**Figure 5**) [38,39]. This linker, containing unnatural amino acid citrulline, was chosen to confer added stability to the mAb–drug conjugate in the blood circulation, while maintaining the ability to release monomethyl auristatin E by the action of cathepsin B upon internalization into CD30-expressing tumor cells.

The efficacy of the mAb–drug conjugate depends not only on the specificity of the mAb and potency of the cytotoxic drug, but also on the linker. For the synthesis of a mAb–taxoid conjugate, we chose a cleavable disulfide linker due to the observation that the concentration of glutathione (GSH) in cancer tissues is three orders of magnitude greater than that in the blood circulation [40]. In this first-generation mAb–taxoid conjugate (**Figure 6**), modification of a taxoid structure was necessary to form a disulfide linkage to the mAb via a thiol–disulfide exchange reaction [41]. We successfully conjugated highly cytotoxic C-10 methylthioethylpropanoyl taxoids to different IgG class mAbs recognizing the EGFR (**Figure 6**) [41]. These immunoconjugates showed exceptional target specificity *in vitro*. For example, KS78-SB-T-12136 showed high potency ( $IC_{50} = 1.5$  nM) against the A431 cell line, while a negative control, mN901-SB T-12136, exhibited no cytotoxicity against the A431 cell line at least up to 100 mM. Moreover, KS61-SB-T-12136 showed excellent anti-tumor activity *in vivo* against A431 human epidermoid carcinoma xenografts in SCID mice, resulting in complete inhibition of tumor growth in all treated mice with no noticeable toxicity to the animals (**Figure 7**) [41]. Necropsy on day 75, followed by histopathological examination, showed residual calcified material at the tumor site but no evidence of tumor cells. It is noteworthy that treatment with free taxoid SB-12136 at the equi-molar dose to the immunoconjugate showed no therapeutic effect at all (**Figure 7**). The results unambiguously indicated that targeted delivery of the taxoid using a tumor-specific mAb was essential for the activity. The results also indicated the aliphatic disulfide linker was stable in blood circulation long enough for delivery to the tumor, but efficiently cleaved by intracellular reducing substances in cancer cells.

Although the first-generation mAb–taxoid immunoconjugates exhibited impressive results, the potency of SB-T-1213, a parent taxoid, was compromised eightfold by the introduction of a sulfhydryl group at the C10 acyl position (i.e., SB-T-12136-SH) in order to conjugate to mAbs [41]. Therefore, novel self-immolative linkers were designed to efficiently release unmodified taxoids to fully exploit their exceptional potency [7,8].

## Self-immolative disulfide linkers for a tumor-targeting drug-delivery system

The drug-release mechanism of the second-generation self-immolative bifunctional disulfide linkers involves a cascade process, featuring thiolactonization in the key step, which is triggered by the nucleophilic attack of an intracellular thiol (e.g., GSH) on the disulfide moiety. To promote the thiolactonization process, a phenyl moiety was strategically placed to direct the cleavage of the disulfide bond by an intracellular thiol, generating a thiophenolate or sulfhydrylphenyl species that attacks at the ester linkage to the drug molecule (**Figure 8**). This drug-release mechanism was validated in a model system using fluorine-labeling and monitoring by  $^{19}\text{F}$  NMR spectroscopy [7] as well as in a real system with cancer cells using fluorescence labeling and **confocal fluorescence microscopy (CFM)** [8,9,42].

The self-immolative disulfide linker module can release a taxoid (or other anticancer agents) efficiently inside cancer cells by taking advantage of the 1000-times higher concentration of GSH in tumor as compared with that in blood plasma [40]. When the TTM navigates the drug conjugate to the target receptors on the tumor surface, the whole conjugate is internalized via RME (**Figure 9**). Then, an intracellular thiol-triggered cascade drug release takes place through thiolactonization and the released potent anticancer agent attacks its target protein, for example, microtubules for taxoids. These second-generation disulfide linkers have been successfully incorporated into various tumor-targeting drug conjugates and their efficacy evaluated in cancer cells [8,9,42].

## Vitamins as TTMs for a drug-delivery system

Vitamins are required by all living cells for survival, but cancer cells particularly need certain vitamins and nutrients to sustain their rapid growth and proliferation; for example, vitamin B<sub>12</sub>, folic acid, biotin and riboflavin are essential for cell division. Therefore, the vitamin receptors are overexpressed on the cancer cell surface for the uptake of necessary vitamins. Accordingly, those vitamin receptors serve as useful targets for tumor-targeting drug delivery as well as biomarkers for identification and imaging of cancer cells. Among those vitamin receptors, folate and biotin receptors have been shown to be important and relevant targets.

Folic acid (vitamin B<sub>9</sub>), mostly in the form of folate, is essential for DNA synthesis, DNA repair, DNA methylation and RNA synthesis, and acts as a cofactor in certain biological processes [43]. Folate is not endogenously produced by animals. Thus, most mammalian cells obtain folate through low-affinity folate carriers, proton-coupled folate transporters or folate receptor on the cell surface [5,44]. The folate receptor was recognized as excellent target for tumor-targeting drug delivery, and considerable advances have been made [5,44–49]. It is worthy of note that folate–drug conjugates retain a high binding affinity to the folate receptor, but do not have affinity to the low-affinity folate carriers and proton-coupled folate transporters found on the majority of normal cells [5]. Biotin (vitamin B<sub>7</sub>) is essential for cell division, cell growth, fatty acid production, metabolism of fats and amino acids and plays a role in energy production [50], which makes it essential for rapidly dividing cancer cells. In contrast to folate receptors, biotin receptors had not been studied until it was reported in 2004 that those receptors were even more overexpressed than the folate and vitamin B<sub>12</sub> receptors in a variety of cancer cells [51]. Thus, the biotin receptor has emerged as a new target for tumor-targeting drug delivery.

Vitamin–drug conjugates should be internalized into cancer cells via RME (**Figure 9**) [8,9]. The binding of the vitamin module to its receptor initiates a signaling cascade, which leads to the formation of a membrane invagination. The vitamin–receptor complex is encapsulated into a coated vesicle and individual vesicles fuse to form the early endosome. In the

endosome, the vitamin–drug conjugate is released from the receptors and the receptors are recycled to the cell surface. In the endosome or lysosome, the vitamin–drug linkage can be cleaved by endogenous enzymes, lower pH or reducing substances, such as GSH, to release the drug in its active form. Then, the drug released from lysosome attacks its target, microtubules for taxoids.

We chose biotin as the TTM and conjugated it to a second-generation taxoid, SB-T-1214, with a self-immolative disulfide linker, successfully constructing biotin–linker–SB-T-1214 (**1**; **Figure 10**) [8,9]. The target specificity and potency of this drug conjugate **1** was evaluated using three cell lines, L1210FR (murine leukemia cell line, overexpressing folate and biotin receptors), L1210 (parent murine leukemia cell line) and WI-38 (normal human lung fibroblast cell line) (**Table 3**) [9]. Both L1210 and WI38 cell lines do not overexpress biotin receptors on their cell surfaces. As **Table 3** shows, conjugate **1** exhibited high potency ( $IC_{50} = 8.8$  nM) against L1210FR cell line. In sharp contrast,  $IC_{50}$  values against L1210 and WI38 cell lines were 59-times (522 nM) and 65-times (570 nM) larger, respectively. SB-T-1214 was also assayed against those three cell lines for comparison. As anticipated, the free taxoid was not able to distinguish these cell lines, resulting in very similar  $IC_{50}$  values [9]. The results clearly indicate that highly efficient vitamin–receptor-specific drug delivery to cancer cells has been achieved by conjugate **1**.

In order to validate the mechanism of tumor-targeting drug delivery by conjugate **1**, we monitored (i) RME, (ii) intracellular drug release, and (iii) drug-binding to the target protein (i.e., microtubules) using three fluorescently labeled probes (**Figure 10**) [8,9]. These probes are:

- Biotin–fluorescein (Probe A) designed to observe RME;
- Biotin–linker–coumarin conjugate (Probe B) designed to confirm internalization via RME and release of coumarin from the fluorogenic system via disulfide cleavage and thiolactonization;
- Biotin–SB-T-1214–fluorescein conjugate (Probe C) designed to validate the internalization of the drug conjugate via RME and drug release followed by the binding of free fluorescent taxoid to microtubules.

The cellular uptake of these probes was monitored by CFM and **fluorescence-activated cell sorting (FACS) flow cytometry** [8,9]. Probe A (100 nM) was added to L1210FR cells at 37°C and incubated for 3 h. After thorough washing of the cells with phosphate-buffered saline (PBS), CFM analysis showed the efficient internalization of Probe A into the leukemia cells (**Figure 11A**). Endocytosis is an energy-dependent process and should be inhibited at low temperature (4°C) or in the presence of the metabolism inhibitor, typically  $NaN_3$  [52]. The CFM image and FACS of L1210FR cells incubated with Probe A at 4°C (100 nM, 3 h) showed dramatically diminished fluorescence in the cells (**Figure 11b**). In a similar manner, when L1210FR cells were incubated with Probe A (100 nM, 3 h) in the presence of 0.05% (w/v)  $NaN_3$  at 37°C, a significant decrease in fluorescence inside the cells was also observed (**Figure 11C**). The results unambiguously indicate that the Probe A was internalized through endocytosis. To further validate that the internalization of Probe A was exclusively through a RME process, excess free biotin (2 mM) was pre-incubated for 1 h to saturate the biotin receptors on the cancer cell surface, followed by the addition of Probe A (100 nM) at 37°C for 3 h. Both the CFM and FACS analysis showed drastic decrease in fluorescence (**Figure 11D**). The results confirm that Probe A was indeed internalized through RME.

Next, Probe B (1  $\mu$ M) was incubated with L1210FR at 37°C for 3 h. After washing the cells thoroughly with PBS, GSH ethyl ester (GSH-OEt, 2 mM) was added to the medium to

assure cleavage of the disulfide bond, and cells were incubated for an additional 2 h at 37°C. As shown in the epifluorescence image of the treated cells (**Figure 12a**), coumarin molecules were released inside L1210FR cells, as indicated by the blue fluorescence. The result clearly indicates that the intracellular release of coumarin (a drug surrogate) via cleavage of the disulfide linkage by GSH and the subsequent thiolactonization proceeded as designed. In the absence of additional GSH-OEt, the observed blue fluorescence was markedly weaker (**Figure 12B**). The result indicates that the concentration of intracellular GSH in the L1210FR cell line is not sufficient for full cleavage of the disulfide linkage in 3 h *in vitro*, which are significantly different from those *in vivo* where the GSH supply in tumor tissues is more than adequate. Also, it should be noted that this experiment using additional GSH (as GSH-OEt) confirmed that the cleavage of the self-immolative disulfide linker and drug release was caused by GSH.

Finally, Probe C (20  $\mu$ M) was incubated with L1210FR cells at 37°C for 2 h and analyzed by CFM. As **Figure 13A** shows, the whole conjugate was internalized in the same manner as that for Probe B. Then, GSH-OEt (2 mM) was added to the medium and the cells were incubated for another 1 h to ensure the drug release through disulfide cleavage and thiolactonization. As **Figure 13B** shows, the CFM image of the cells is dramatically different from that in **Figure 13A**. **Figure 13b** clearly indicates that the released fluorescent taxoid binds to the microtubules and highlights the microtubule bundles with green fluorescence. The result validates that the internalization and release of the taxoid proceeded through the designed mechanism, involving RME via biotin receptors and drug release via GSH-mediated self-immolative disulfide linker cleavage (**Figure 9**).

Since we synthesized SB-T-1214–fluorescein (**2**) and Probe C, we examined their tumor specificity and potency as well. As shown in **Table 3**, the IC<sub>50</sub> values of SB-T-1214, **2** and paclitaxel against L1210FR are 9.5, 87.6 and 122 nM, respectively. As mentioned above, SB-T-1214 exhibited comparable IC<sub>50</sub> values against these three cell lines. Fluorescence-labeled taxoid **2** and paclitaxel showed slight to small differences in their IC<sub>50</sub> values against the three cell lines. Thus, these three taxanes practically cannot distinguish targeted cancer cells from non-targeted cells (L1210) as well as normal cells (WI38), as anticipated.

We also examined the specificity and potency of the Probe C against the same three cell lines. As **Table 3** shows, similar differences in IC<sub>50</sub> values were observed to those for conjugate **1**, albeit that the values for L1210 and WI38 were too high to determine. Thus, it is safe to conclude that the delivery of Probe C as well as the conjugate **1** to L1210FR is highly specific through vitamin–receptor recognition and RME based on both CFM/FACS and cytotoxicity analyses.

#### ■ Taxoid conjugate with dual-targeting modules & that with dual anti-tumor agents

As an extension of the tumor-targeting drug conjugates bearing a vitamin as the TTM, a self-immolative disulfide linker and a taxoid described above, we have designed and successfully synthesized a novel drug conjugate bearing dual-targeting modules (**3**), such as biotin and folic acid, a trisubstituted 1,3,5-triazine splitter, a self-immolative disulfide linker and SB-T-1214, as well as another novel drug conjugate bearing dual anti-tumor agents (**4**); SB-T-1214 and topotecan, with self-immolative disulfide linkers, a biotin or folic acid as the TTM and a trisubstituted 1,3,5-triazine splitter (**Figure 14**) [53]. It is reasonable to hypothesize that the tumor-targeting efficacy would be enhanced if the drug conjugates possess different types of vitamins. Since tumors are heterogeneous (i.e., tumor cells are substantially different from each other), the relative levels of vitamin receptor overexpression differ from cell to cell in the tumor. Therefore, it would be beneficial if the tumor-targeting drug conjugates bear dual-vitamin–TTMs, which can target both vitamin receptors. Furthermore, it is also reasonable to assume that the anti-tumor efficacy would be

substantially enhanced if two or more different anti-tumor agents were present in the same drug conjugate. This type of drug conjugate can deliver a combination of drugs in a single molecule, circumventing possible complications and PK issues associated with the standard combination drug therapy. The evaluation of the efficacy of these novel drug conjugates will be reported elsewhere in due course.

## Single-walled carbon nanotubes as a unique vehicle for tumor-targeting drug delivery

Single-walled **carbon nanotubes** (SWNTs) have been attracting extensive interest for their potential application in biomedical research as unique drug-delivery vehicles [54,55]. Functionalized SWNTs can incorporate multiple units of TTMs and drug molecule, which can penetrate the cell membrane, accumulate in the cytoplasm and release the drug molecules [54–56]. Building upon the successful construction of highly tumor-specific biotin–linker–taxoid conjugates (**1** and Probe C) described above, we designed and devised a novel SWNT-based drug-delivery system [42] SWNTs can be functionalized with numerous units of TTM as well as the conjugate of a self-immolative linker and an anti-tumor agent to provide a novel tumor-targeting drug-delivery system (**Figure 15**), which can undergo mass delivery of cytotoxic payloads into cancer cells through tumor-specific RME.

This SWNT-based drug-delivery system possesses the following advantages:

- Multivalent ligand–receptor interactions on the tumor cell surface [57,58];
- Increased drug-loading capacity;
- Capability of controlled drug release inside tumor cells.

This unique drug-delivery system is multi-functional and multicomponent, consisting of TTMs (e.g., biotin) and cytotoxic payloads (e.g., second-generation taxoids) covalently linked to a SWNT scaffold to form a TTM–SWNT– linker–drug conjugate.

To evaluate the specificity and efficacy of the SWNT-based drug-delivery system, we designed and synthesized three fluorescently labeled SWNT-conjugates **5**, **6** and **7**, as molecular probes (**Figure 14**) [42]. Those SWNT conjugates were labeled by using either fluorescein isothiocyanate (FITC) (for conjugates **5** and **6**) or fluorescein with a tether (for conjugate **7**) and biotin was used as the TTM for conjugates **6** and **7**. The fluorescent probes, SWNT– FITC (**5**) and biotin–SWNT–FITC (**6**), were designed to track the internalization of SWNT and biotin–SWNT, respectively, into the cancer cells. Biotin–SWNT–linker–taxoid–fluorescein (**7**) was designed to verify the internalization of the conjugate via RME and intracellular drug release in a manner similar to the conjugate **1** and Probe 3 described above (**Figure 10**).

We examined cellular uptake of SNWT conjugates **5** and **6** using L1210FR leukemia cells [42] in a manner similar to that for fluorescent Probe A described above. **Figure 16A & B** show CFM images of L1210FR cells after treatment with 10 µg/ml of conjugates **5** and **6**, respectively, for 3 h at 37°C, followed by thorough washing with PBS and analysis [42]. It is apparent that L1210FR cells treated with conjugate **6** yielded far more intense fluorescence than those incubated with conjugate **5**. This observation was quantified by FACS analysis, which clearly showed that the fluorescence intensity of the cells treated with conjugate **6** increased by one order of magnitude as compared with conjugate **5** (**Figure 16C**) [42]. Also, the RME mechanism was confirmed [42] by the temperature dependence, sodium azide inhibition test and saturation of the vitamin receptors with pre-incubation of excess biotin in the same manner as those performed for Probes A–C described above. The results indicate markedly enhanced internalization of conjugate **6** into the cancer cells



through highly efficient RME that can be attributed to multivalent binding of the biotin modules to the vitamin receptors. At the same time, the result practically eliminates the 'piece through mechanism' known for unfunctionalized and lightly functionalized SWNTs [59].

Next, we examined the efficacy of conjugate **7** for cellular uptake and drug release inside the leukemia cells [42] in a manner similar to that for fluorescent Probe C described above. To evaluate the efficacy of **7** for its internalization and drug release, L1210FR cells were incubated with **7** (50  $\mu\text{g}/\text{ml}$ ) for 3 h at 37°C, followed by thorough washing with PBS and analysis. The CFM image of the cells shows intense fluorescence, confirming the efficient internalization of **7** (**Figure 17a**) [42]. Then, the cells were treated with GSH-OEt for an additional 2 h at 37°C in order to ensure cleavage of the disulfide bond covalently linking the taxoid to the biotin–SWNT moiety. **Figure 17b** shows that the fluorescent taxoid binds to the target protein (microtubules) fluorescently activating large bundles of microtubules [42], which provides ultimate proof of the designed drug release in the same manner as that for Probe C described above.

We also evaluated the cell-specificity of **7** against L1210FR, L1210 and WI-38 cell lines [42]. As **Table 4** shows, after 72 h of incubation, the  $\text{IC}_{50}$  value of **7** against the L1210FR cell line was 0.36  $\mu\text{g}/\text{ml}$ , whereas those for L1210 and WI-38 cell lines were more than 50  $\mu\text{g}/\text{ml}$ . As a control, the  $\text{IC}_{50}$  values of conjugates **5** and **7** against all three cell lines were determined, which were consistently  $>100 \mu\text{g}/\text{ml}$  [42]. Therefore, the observed cytotoxicity is unambiguously attributed to fluorescent taxoid **2** released from **7** internalized. It should also be noted that taxoid–fluorescein molecules appear to be sufficiently released by endogenous GSH in L1210FR cells after 72 h of incubation in contrast to just 3 h exposure for the CFM study described above (see **Figure 13**).

Based on further analysis, the  $\text{IC}_{50}$  value (0.36  $\mu\text{g}/\text{ml}$ ) of **7** corresponds to approximately 51 nM of taxoid **2** against the L1210FR cell line by assuming that all taxoid molecules attached to SWNTs are released. This means that the apparent cytotoxicity per taxoid is substantially increased by using the biotin–SWNT-based drug-delivery system ( $\text{IC}_{50}$  of taxoid **2** is 87.6 nM, see **Table 3**) [42]. The results clearly indicate that the mass drug delivery into the cytosol of the cancer cells using this unique drug-delivery system is superior to simple exposure of the drug itself to the same cancer cells. It is also worthy of note that the cell-specificity of SWNT–conjugate **7** is substantially higher than that of conjugate **1** (**Table 3**). When taxoids get into the cancer cells through the mass drug-delivery system via RME, the released taxoids can quickly and tightly bind to microtubules so that the effective intracellular drug concentration is substantially higher than that achieved by extracellular exposure of the drug.

## Future perspective

Numerous mAb-based anticancer drug immuno conjugates have been developed and are currently in human clinical trials, but still only a few drug immunoconjugates have become FDA-approved drugs. Nevertheless, it appears that the time has come for these tumor-targeting immunoconjugates to be clinically relevant drugs. Also, several folate–drug conjugates are currently undergoing clinical evaluations, and some of them might become FDA-approved drugs in a few years time. Since there are numerous cancer-specific receptors and bio-markers, tumor-targeting drug-delivery systems have a bright future for developments with a wise combination of TTMs, potent anticancer drugs and mechanism-based 'smart' linkers. Our future efforts will be concentrated on the design and synthesis of 'tailor-made nano-medicines', consisting of a well-defined vehicle, multiple targeting modules (including dual-targeting molecules), self-immolative smart linkers, highly potent

cytotoxic agents (including a combination of different anticancer drugs) and imaging modules (for MRI, positron emission tomography and fluorescence imaging), which would enable us to perform diagnostics and therapy in the real time. We are certain that a variety of new and efficacious tumor-targeting drug conjugates will emerge in the coming years that specifically target different and challenging tumors. We predict that a number of such drug conjugates will go into clinical trials and eventually become powerful drugs in cancer chemotherapy with minimum adverse effects.

## Acknowledgments

Financial & competing interests disclosure

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### Key Terms

**Pluripotency:** The potential of a cell to develop into more than one type of mature cell, depending on the environment.

**Xenograft:** The transplantation of living cells, tissues or organs from one species to another.

**Omega-3 polyunsaturated fatty acids:** Essential unsaturated fatty acids with a double bond (C=C) after the third carbon atom from the end of the carbon chain.

### Key Term

**Human serum albumin:** The most abundant protein in human blood plasma. It is produced in the liver. Albumin constitutes approximately half of the blood serum protein. It is soluble and monomeric, and transports hormones, fatty acids, and other compounds, buffers pH, and maintains osmotic pressure, among other functions.

### Key Term

**Confocal fluorescence microscopy:** An optical imaging technique used to increase optical resolution and contrast of microscopy by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of 3D structures from the images obtained.



### Key Terms

**Fluorescence-activated cell sorting:** A specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell.

**Flow cytometry:** A technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second.

### Key Term

**Carbon nanotubes:** Allotropes of carbon with a cylindrical nanostructure. These cylindrical carbon molecules have unusual properties, which are valuable for nanotechnology, electronics, optics and other fields of materials science and technology. Most single-walled nanotubes have a diameter of close to 1 nm, with a tube length that can be many millions of times longer. The structure of a single-walled nanotubes can be conceptualized by wrapping a one-atom-thick layer of graphite called graphene into a seamless cylinder.

## Executive summary

### **Background**

- There is a critical need to develop highly efficacious tumor-targeting drug conjugates to overcome the shortcomings of conventional chemotherapy.
- Polyunsaturated fatty acids, vitamins (e.g., biotin and folic acid) and monoclonal antibodies can be used as tumor-targeting modules (TTMs) and single-walled carbon nanotubes can be used as unique vehicles for tumor-targeting drug-delivery systems.
- Various cancer cells overexpress vitamin receptors and tumor-specific antigens such as growth factor receptors, which can be exploited as targets for tumor-specific drug delivery.

### New-generation taxoids as the drugs to be delivered specifically to tumors

- A number of new-generation taxoids possess two to three orders of magnitude greater potency than paclitaxel and docetaxel, against multidrug-resistant cancer cell lines.
- Treatment of colon cancer stem cells with a new-generation taxoid (SB-T-1214) led to effective suppression of cell growth and significant downregulation of the pluripotency gene expression in cancer stem cells.

### Polyunsaturated fatty acid as a TTM of drug-delivery systems

- Several polyunsaturated fatty acid–taxoid drug conjugates exhibited high efficacy *in vivo*. Among those conjugates, docosahexaenoic acid–SB-T-1214 demonstrated exceptional activity against highly drug-resistant DLD-1 colon cancer xenograft in severe-combined immune deficiency mice with minimal systemic toxicity. Investigational New Drug application will be filed for FDA approval shortly and this drug conjugate will go into clinical trials in the near future.

### Monoclonal antibody as a TTM of drug-delivery systems

- A taxoid–mAb immunoconjugate showed excellent antitumor activity *in vivo* against A-431 human epidermoid carcinoma xenografts in severe-combined immune-deficiency mice without noticeable toxicity to the animals, and there was no trace of cancer cells based on histopathological analysis at the end of the experiment.

### Self-immolative disulfide linkers for tumor-targeting drug-delivery system

- Highly efficient self-immolative disulfide linkers have been designed and successfully incorporated into various tumor-targeting drug conjugates.

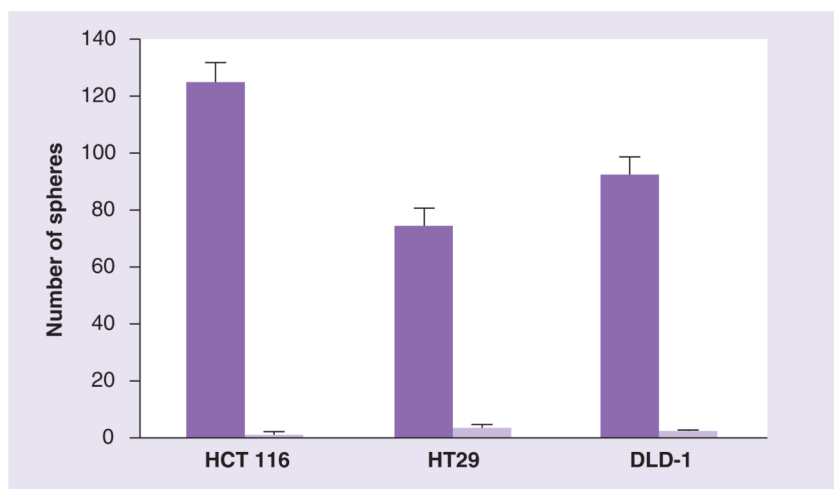
### **Vitamins as TTMs for drug-delivery systems**

- A novel biotin–linker–taxoid conjugate exhibited excellent target-specific activity via receptor-mediated endocytosis (RME) for a leukemia cell line as compared with a normal human cell line. The mechanism of internalization (i.e., validation of RME), drug release and drug binding to the target protein (i.e., microtubules) was unambiguously confirmed using fluorescent probes and monitoring by confocal fluorescence microscopy and fluorescence-activated cell sorting.

### Single-walled carbon nanotubes as a unique vehicle for tumor-targeting drug delivery

- The use of multifunctionalized single-walled carbon nanotubes as a unique drug-delivery system bearing multiple cytotoxic payloads and multiple targeting modules has shown highly promising results, demonstrating the benefit of mass delivery of

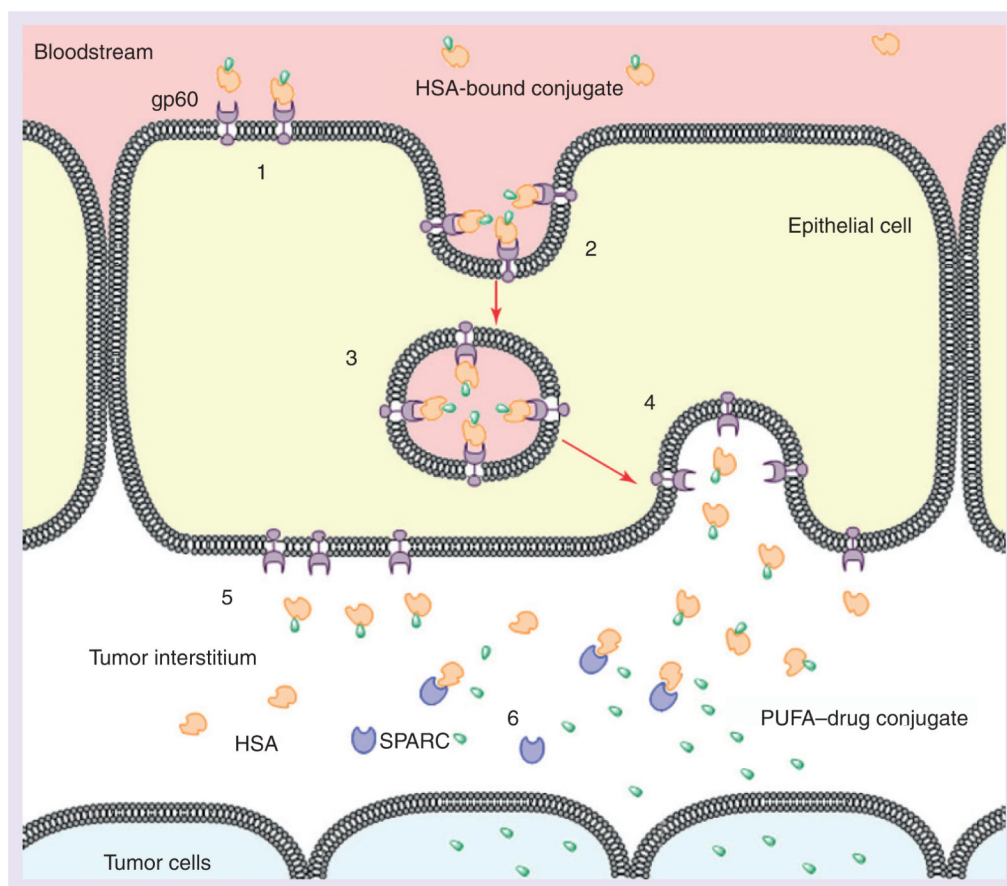
anticancer drug molecules to cancer cells with high specificity via enhanced RME by multivalent binding of TTM. The mechanism of internalization, drug release and drug binding to the target protein was verified using fluorescent probes and monitoring by confocal fluorescence microscopy and fluorescence-activated cell sorting.



**Figure 1. Effects of SB-T-1214 on colon cancer stem cells (HCT 116, HT29 and DLD-1)**

To evaluate possible alterations in sphere-forming capacity, control and drug-treated (with 100 nM SB-T-1214) spheroids/cells were dissociated to a single-cell suspension and an equal number of viable cells (400 cells) were plated on ultra-low attachment six-well plates in MSCB medium containing 10% Matrigel. The number of secondary spheroids was counted 10 days after plating. Untreated cells (dark purple) and SB-T-1214-treated cells (light purple).

Adapted with permission from [23].

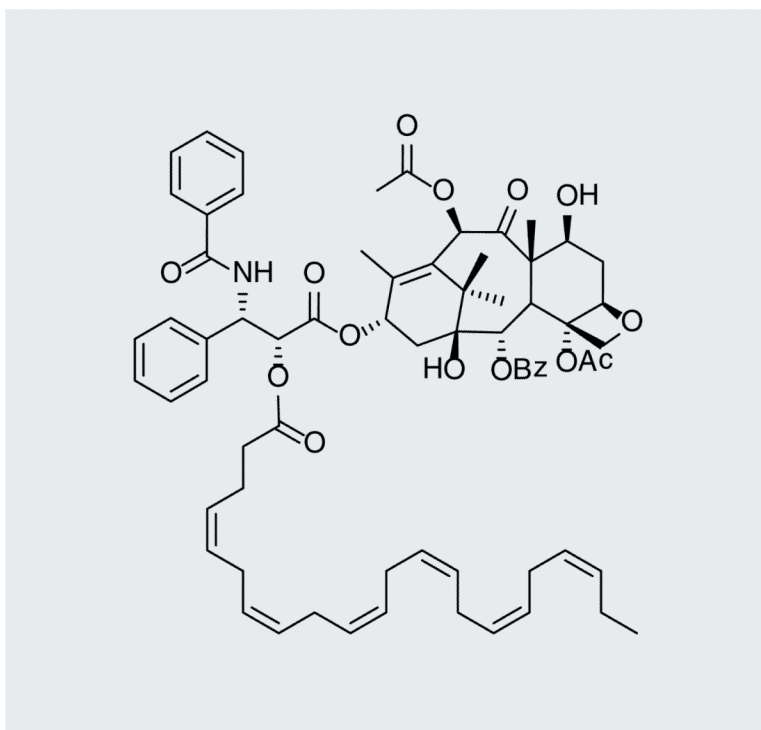


### Figure 2. gp60-mediated transcytosis

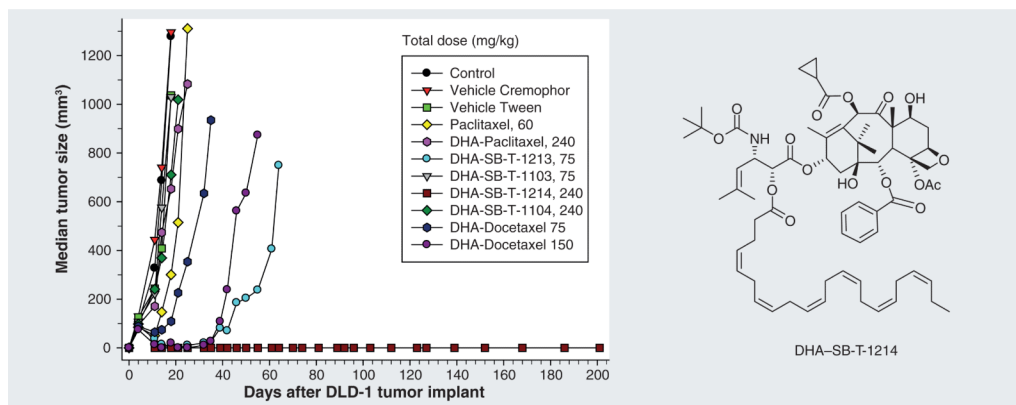
(1) HSA (orange) bound to drug conjugate (green) travels through blood stream and binds to gp60 (purple) on the tumor epithelial cell surface. (2) Ligand binding initiates transcytosis. (3) Vesicles containing drug conjugate-protein complex are transported across the cell. (4) Fusion of the vesicle to the interstitial cell wall occurs. (5) Drug conjugate-HSA complex is released into the tumor interstitium. (6) Binding of SPARC to HSA causes release of a free drug conjugate, which permeates into tumor cells.

HSA: Human serum albumin; PUFA: Polyunsaturated fatty acid; SPARC: Secreted protein acidic and rich in cysteine.

Reproduced with permission from [33].



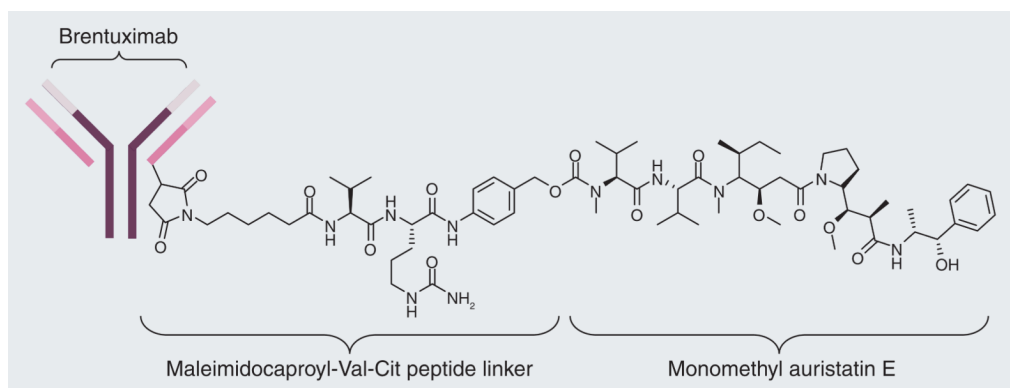
**Figure 3.**  
Docosahexaenoic acid– paclitaxel (Taxoprexin®).



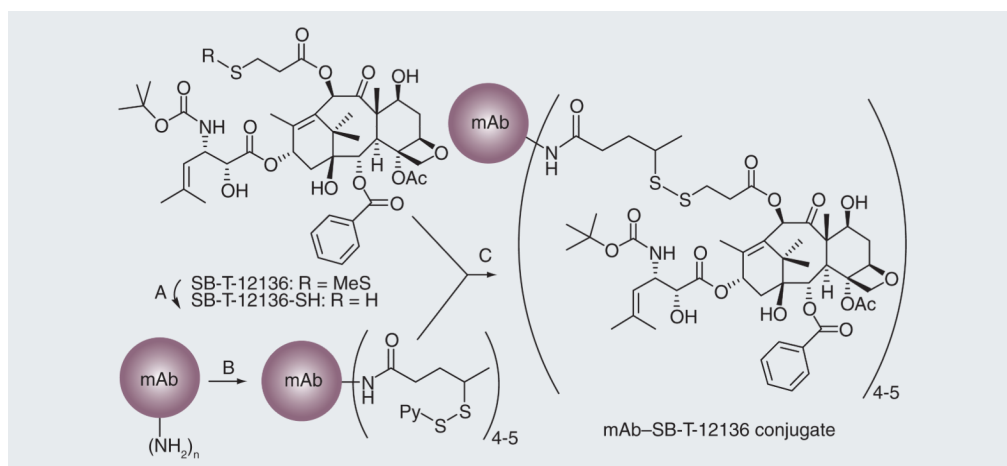
**Figure 4. Effect of docosahexaenoic acid–taxoid conjugates on human colon tumor xenograft DLD-1**

Adapted with permission from [36].





**Figure 5.**  
Brentuximab vevotin.

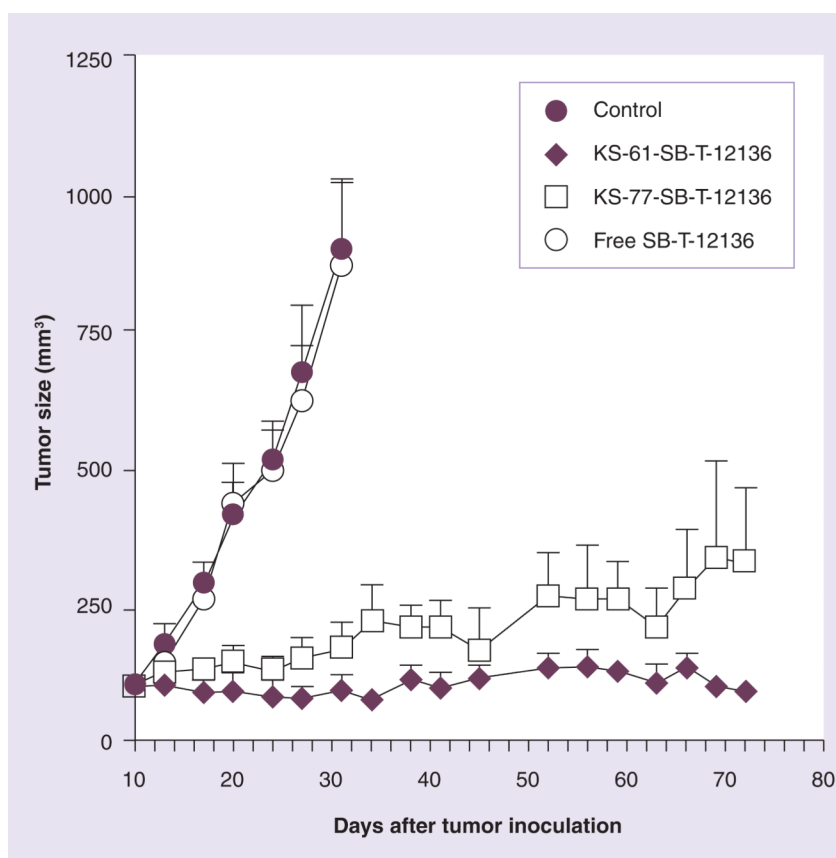


**Figure 6. Synthesis of first-generation monoclonal antibody-taxoid conjugates**

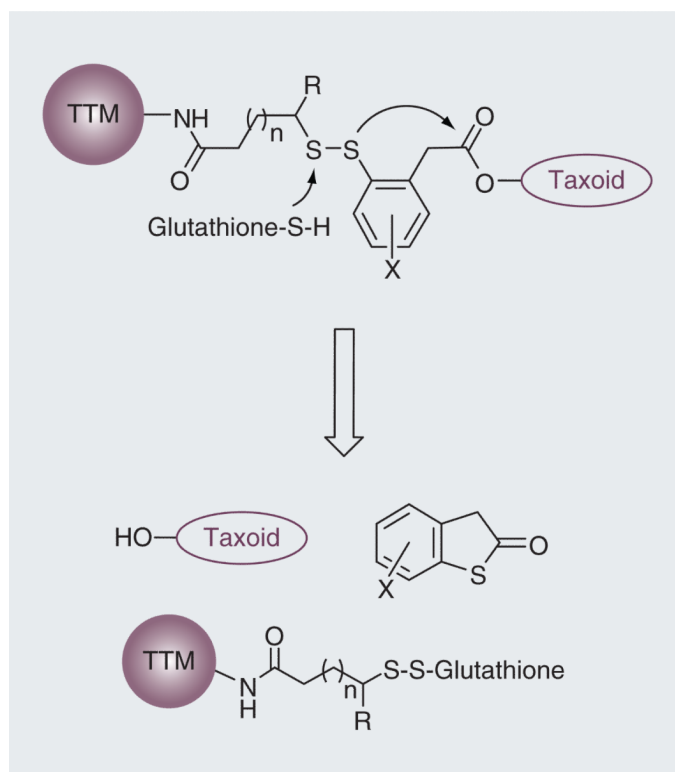
(A) Dithiothreitol (B) *N*-succinimidyl-4-(2-pyridyldithio) pentanoate (SPP, 10 equivalent in ethanol), potassium phosphate buffer (50 mM), pH 6.5, NaCl (50 mM), EDTA (2 mM), 90 min. (C) Potassium phosphate buffer (50 mM), pH 6.5; NaCl (50 mM); EDTA (2 mM); SB-T-12136-SH (1.7 equivalent per dithiopyridyl group, in EtOH), 24 h.

mAb: Monoclonal antibody.

Adapted with permission from [41].



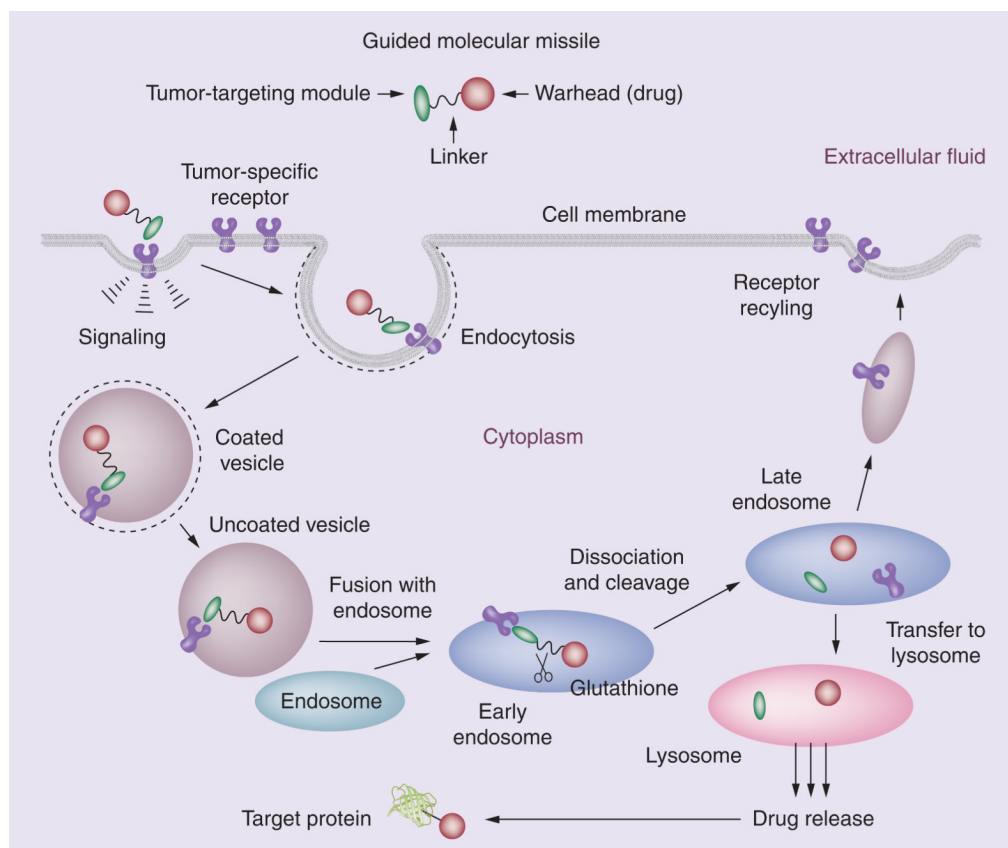
**Figure 7. Anti-tumor activity of anti-EGFR mAb–taxoid conjugates against A431 xenografts in severe combined immunodeficient mice**  
Adapted with permission from [41].



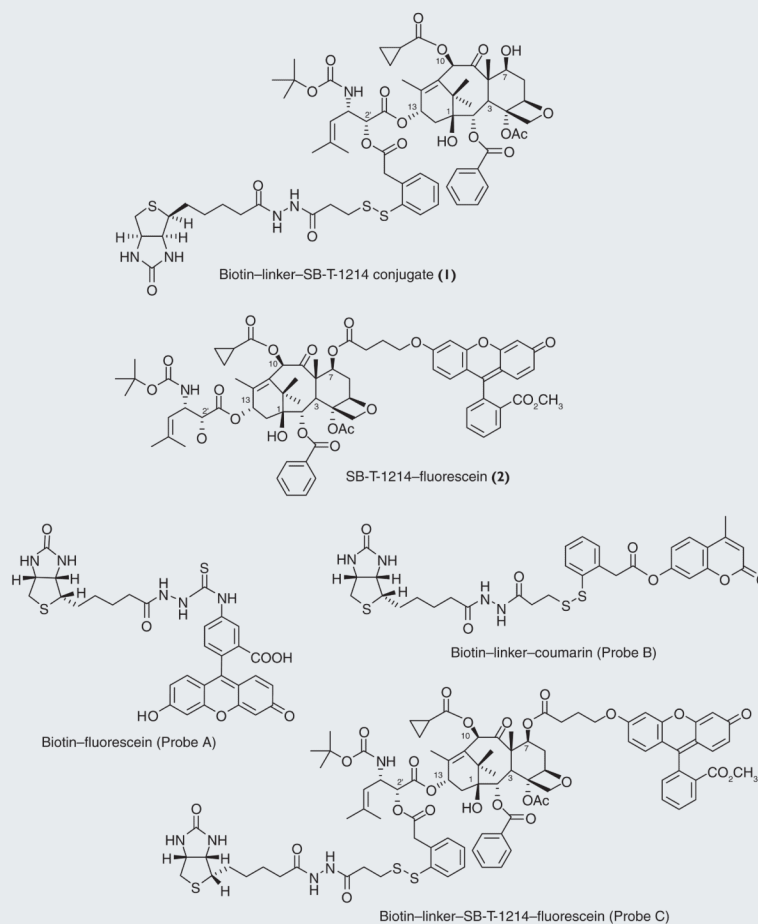
**Figure 8. Second-generation self-immolative disulfide linkers and their drug-release mechanism**  
TTM: Tumor-targeting module.

Adapted with permission from [8].

Adaptive with permission from [8].

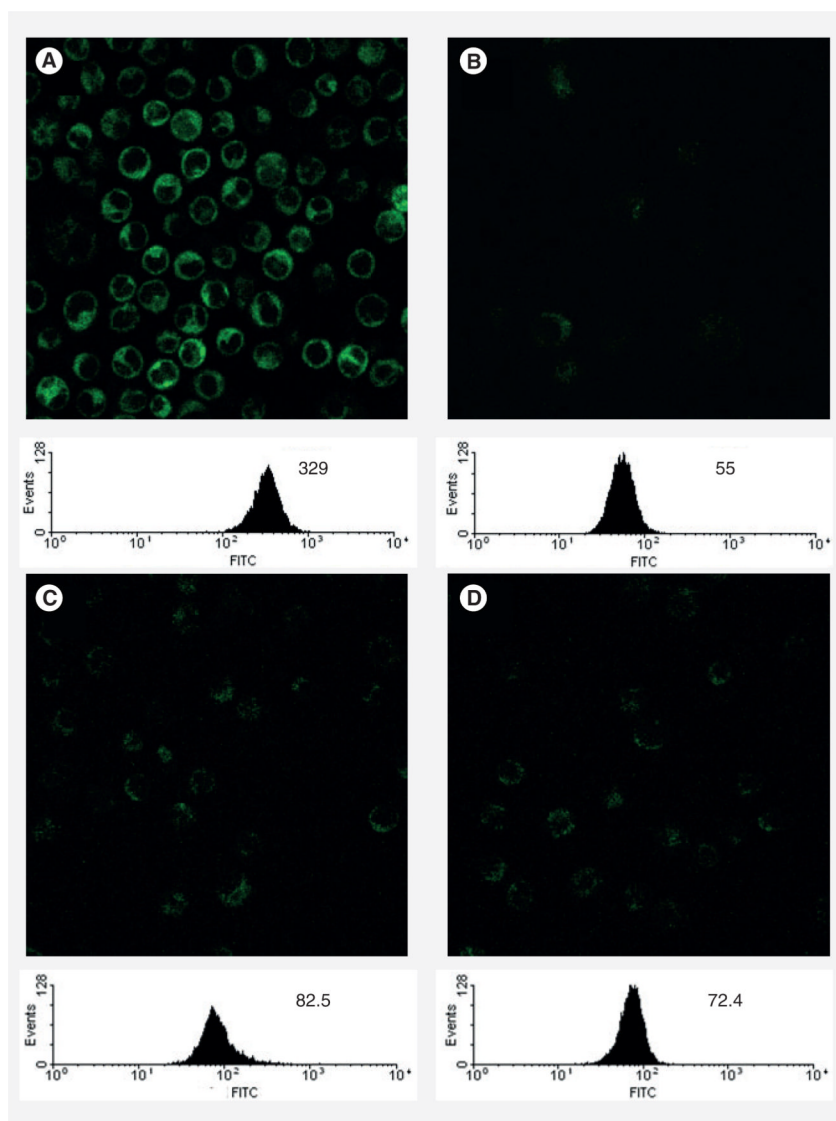


**Figure 9. Receptor-mediated endocytosis of a tumor-targeting drug conjugate, drug release and binding of the released drug to the target protein**  
Adapted with permission from [8].

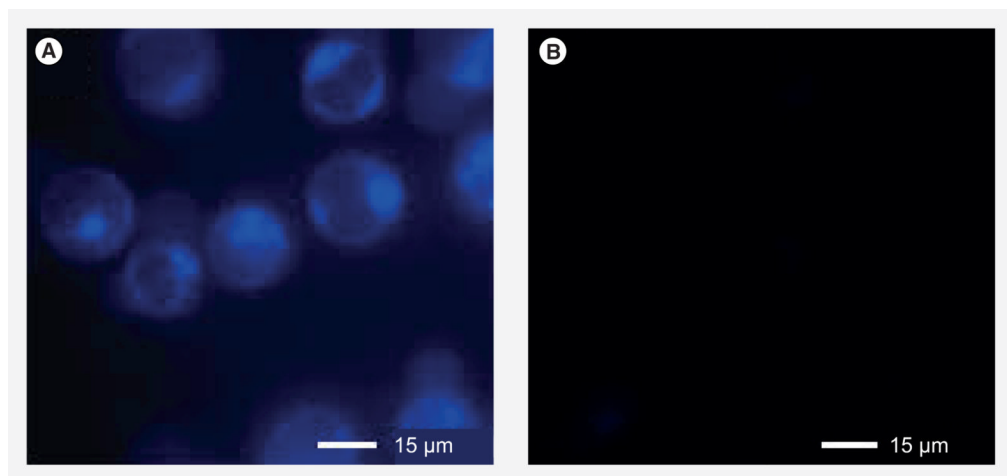


**Figure 10. Vitamin-linker-taxoid conjugate (1), fluorescence-labeled taxoid (2) and fluorescent probes (A-C)**

Adapted and modified with permission from [9].

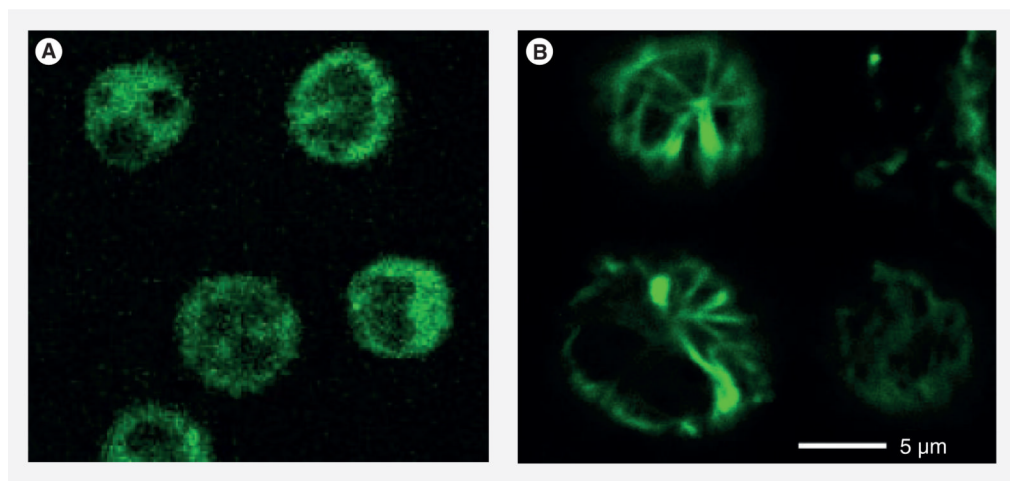


**Figure 11. Confocal fluorescence microscopy images and fluorescence- activated cell sorting analysis of L1210FR cells after incubation with Probe A under different conditions (A) 100 nM, 37°C, 3 h. (B) 100 nM, 4°C, 3 h. (C) 100 nM, 37°C, 0.05% NaN<sub>3</sub>, 3 h. (D) 100 nM, 37°C, 3 h, pretreated with excess biotin (2 mM). Adapted with permission from [8].**

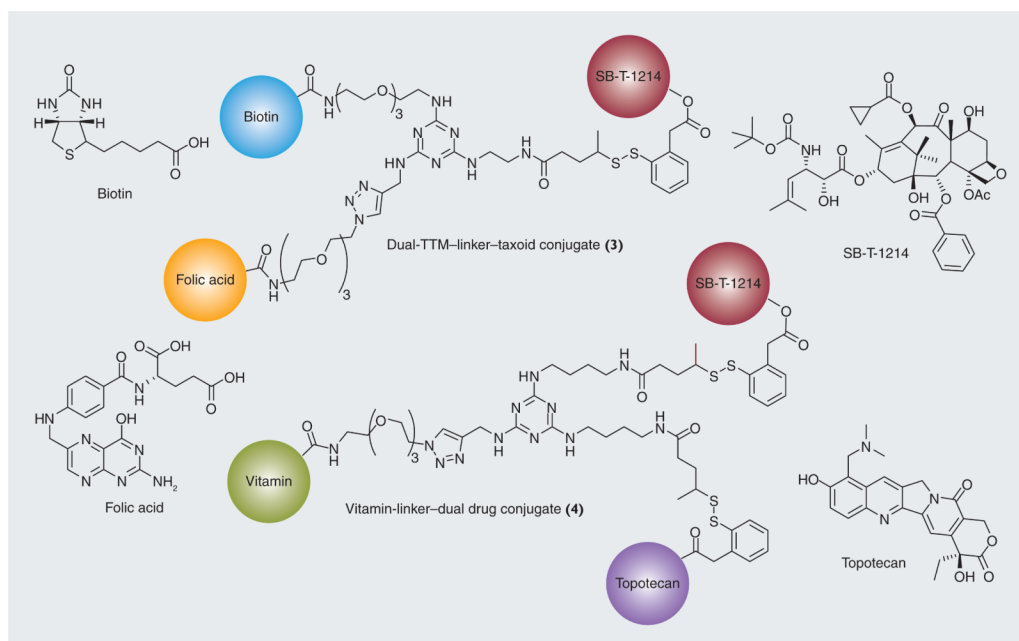


**Figure 12. Confocal fluorescence microscopy analysis of L1210FR cells treated with Probe B** (A) Confocal fluorescence microscopy image of L1210FR cells that were incubated with Probe B (nonfluorescent form), followed by treatment with glutathione-OEt to trigger the self-immolation of the linker. The coumarin released showed blue fluorescence. (B) Confocal fluorescence microscopy image of L1210FR cells after incubation with Probe B (nonfluorescent form) as control. Adapted with permission from [8].

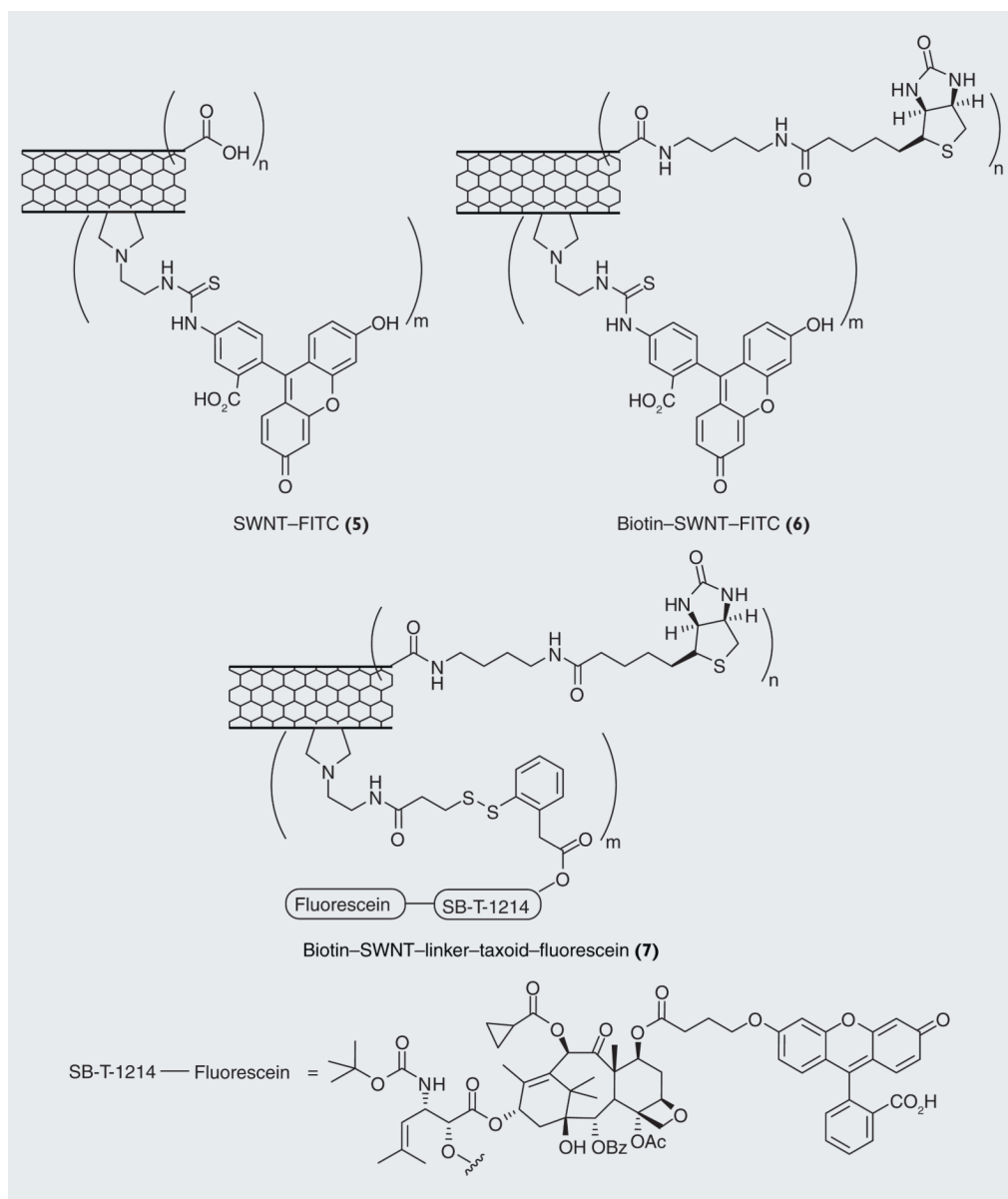




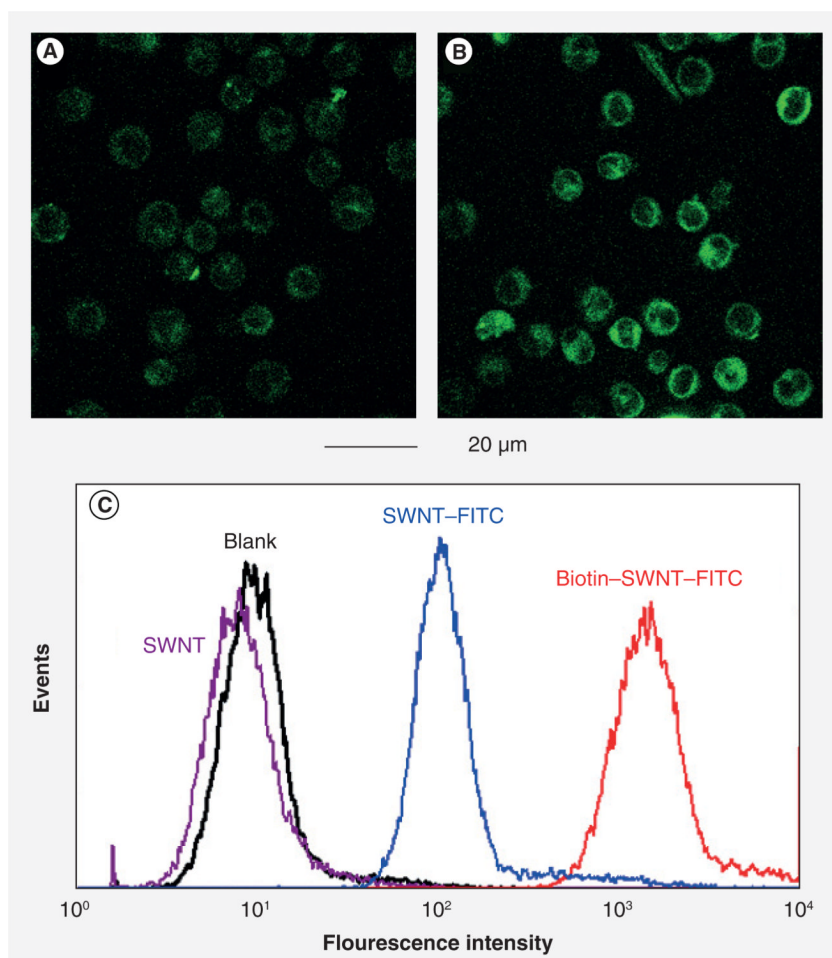
**Figure 13. Confocal fluorescence microscopy analysis of L1210FR cells treated with Probe C** (A) Confocal fluorescence microscopy image of L1210FR cells after incubation with Probe C. (B) Confocal fluorescence microscopy image of L1210FR cells were incubated with Probe C, followed by treatment with glutathione-OEt to release the fluorescent taxoid **2**. Then, the microtubule network in the cells was fluorescently labeled by **2** and visualized. Adapted with permission from [8].



**Figure 14. Tumor-targeting drug conjugates bearing dual-TTM or dual-cytotoxic drugs**  
TTM: Tumor-targeting module.



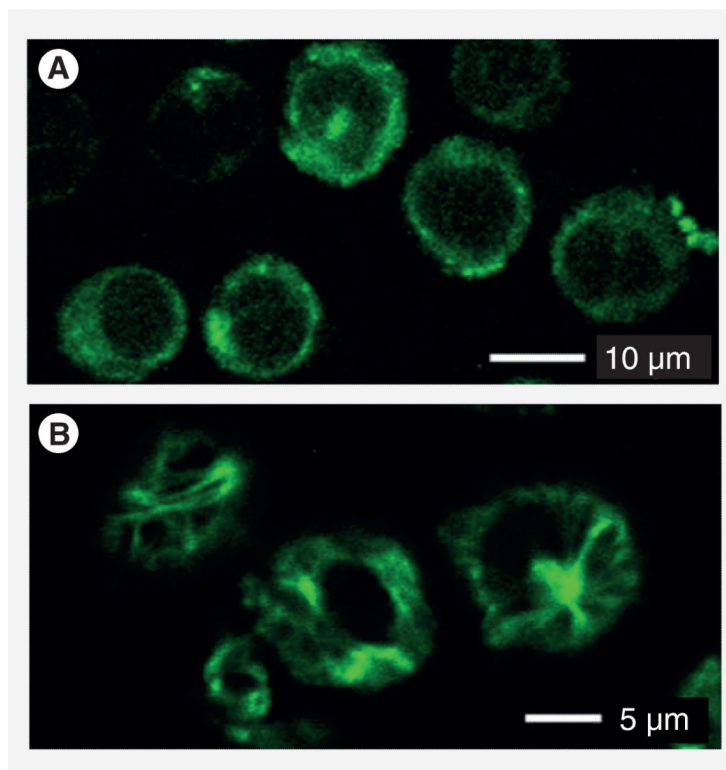
**Figure 15.** Fluorescently labeled single-walled carbon nanotubes conjugates 5, 6 and 7 as probes.



**Figure 16. Confocal fluorescence microscopy and fluorescence-activated cell sorting analysis of L1210FR cells treated with single-walled carbon nanotube-conjugates 5 and 6**  
**(A)** Confocal fluorescence microscopy image after incubation with SWNTs-FITC (**5**) and **(B)** that with biotin-SWNTs-FITC (**6**) at the final concentration of 10  $\mu\text{g}/\text{ml}$  at 37°C for 2 h. **(C)** Comparison of fluorescence intensities of L1210FR cells by fluorescence-activated cell sorting upon treatment with pristine SWNTs 0 (purple), conjugate **5** (blue) and **6** (red) at the final concentration of 10  $\mu\text{g}/\text{ml}$  in each case. Background data for untreated cells are plotted in black.

FITC: Fluorescein isothiocyanate; SWINT: Single-walled carbon nanotube.

Adapted with permission from [42].



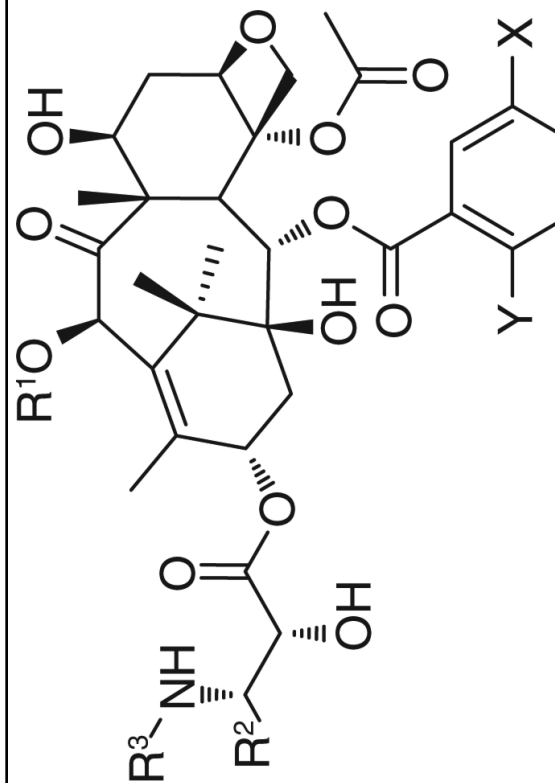
**Figure 17. Confocal fluorescence microscopy analysis of L1210FR cells treated with single-walled carbon nanotube conjugate 7**

Confocal fluorescence microscopy image of L1210FR cells incubated with conjugate 7 before (A) and after (B) the addition of glutathione (GSH)-OEt. Image (B) clearly highlights the presence of fluorescent microtubule networks in the living cells generated by the binding of the fluorescent taxoid 2 released by cleavage of the disulfide bond in the linker by either GSH or GSH-OEt.

Adapted with permission from [42].

Table 1

Selected new-generation taxoids.



The chemical structure shows a complex polycyclic taxoid core. It features a central ring system with a double bond and a hydroxyl group. A side chain is attached to the core, containing a secondary amine (R<sup>3</sup>-NH), a hydroxyl group (OH), and a carbonyl group (C=O). Another side chain is attached to the core, containing a hydroxyl group (OH) and a carbonyl group (C=O). The core also has a hydroxyl group (OH) and a carbonyl group (C=O). The substituents R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>10</sup> are defined in the table below. The substituents X and Y are also defined in the table below.

Taxane	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	X	Y
Paclitaxel	Ac	Ph	PhCO	H	H
Docetaxel	H	Ph	<i>t</i> -Boc	H	H
SB-T-1213	EtCO	<i>i</i> -butenyl	<i>t</i> -Boc	H	H
SB-T-1214	<i>c</i> -PrCO	<i>i</i> -butenyl	<i>t</i> -Boc	H	H
SB-T-1216	Me <sub>2</sub> NCO	<i>i</i> -butenyl	<i>t</i> -Boc	H	H
SB-T-11033	EtCO	<i>i</i> -Bu	<i>t</i> -Boc	MeO	H
SB-T-121303	EtCO	<i>i</i> -butenyl	<i>t</i> -Boc	MeO	H
SB-T-121313	EtCO	<i>i</i> -butenyl	<i>t</i> -Boc	MeO	MeO
SB-T-121602	Me <sub>2</sub> NCO	<i>i</i> -butenyl	<i>t</i> -Boc	Me	H

**Table 2**

Cytotoxicity (IC<sub>50</sub>; nM) of new-generation taxoids against selected cancer cell lines.

Taxane	MCF7 <sup>†</sup>	NCI/ADR <sup>‡</sup>	LCC6-MDR <sup>§</sup>	CFPAC-1 <sup>¶</sup>	HT-29 <sup>#</sup>	DL-D-1 <sup>††</sup>
Paclitaxel	1.7	550	346	68	12	300
Docetaxel	1.0	723	120	-	-	-
SB-T-1213	0.18	4.0	-	4.6	0.37	3.9
SB-T-1214	0.20	3.9	-	0.38	0.73	3.8
SB-T-1216	0.13	7.4	-	0.66	0.052	5.4
SB-T-11033	0.36	0.61	0.80	-	-	-
SB-T-121303	0.36	0.79	0.90	0.89	-	-
SB-T-121313	0.30	-	-	0.025	0.56	-
SB-T-121602	0.08	-	-	0.31	0.003	0.46

MDR: Multidrug resistance.

<sup>†</sup> Human mammary cancer cell line (P-gp<sup>-</sup>).

<sup>‡</sup> Human ovarian cancer cell line (P-gp<sup>+</sup>).

<sup>§</sup> mdrl-transduced human breast cancer cell line (P-gp<sup>+</sup>).

<sup>¶</sup> Human pancreatic cancer cell line.

<sup>#</sup> Human colon cancer cell line (P-gp<sup>-</sup>).

<sup>††</sup> Human colon cancer cell line (P-gp<sup>+</sup>).

**Table 3**

Cytotoxicities (IC<sub>50</sub>, nM) of taxoids and vitamin–linker–taxoid drug conjugates against leukemia and normal human lung fibroblast cells.

<b>Taxane/cojugate</b>	<b>L1210FR</b>	<b>L1210</b>	<b>WI38</b>
Paclitaxel	121	151	157
SB-T-1214	9.50	9.72	10.7
Biotin–linker–SB-T-1214 (1)	8.80	522	570
SB-T-1214–fluorescein (2)	87.6	107	127
Biotin–linker–SB-T-1214–fluorescein (Probe C)	81.7	>5000	>5000



**Table 4**

Cytotoxicity (IC<sub>50</sub>) of biotin–single-walled carbon nanotube–taxoid–fluorescein (7) against leukemia and normal human lung fibroblast cells.

SWNT-conjugate	L1210	L1210FR	WI38
7	>50 µg/ml	0.36 µg/ml	>50 µg/ml