doi:10.2533/chimia.2008.918 CHIMIA **2008**, *62*, No. 11

CONFERENCE REPORT

Chimia 62 (2008) 918–934

ISSN 0009–4293

Drug Targeting

Mini-Symposium of the Division for Medicinal Chemistry (DMC) of the Swiss Chemical Society (SCS), at the Department of Chemistry, University of Basel, May 29, 2008

Ruth Duncan[a], Iwao Ojima[b], Dario Neri[c], Nobuo Shimma[d], Hisafumi Okabe[d], Hideo Ishitsuka[e], and Hans Peter Märki*[f]

Abstract: Specific targeting of drugs to their respective target organs or tissues is challenging. Substantial research efforts have been undertaken in the recent past to develop target specific drugs or drug conjugates. Such concepts are most relevant in rather severe diseases like cancer since it helps to reduce the concentration of frequently rather toxic drugs outside the tumor tissue. Various techniques can be used to specifically direct a drug or a drug conjugate to a specific tumor tissue such as using antibodies directed against tumor specific proteins, as nanoparticles or nano-sized polymer conjugates carrying tumor-specific recognition elements or by applying the active drug principle in a prodrug form designed to be liberated specifically in tumor tissue. Three speakers from the academia and one speaker from industry described different approaches and their respective potentials from various perspectives in the lectures entitled: 'Polymer Therapeutics and other Nanomedicines as Targetable Cancer Therapies', 'Design, Application, and Chemical Biology of Tumor-Targeting Drug Conjugates', 'Antibody-Based Vascular Tumor Targeting: From the Bench to the Clinic', and 'Discovery of Capecitabine, a Rationally Designed and Tumor-Activated Oral Prodrug of 5-FU, and Beyond'.

Keywords: Antibody-based vascular tumor targeting · Capecitabine (Xeloda) · Conference report · Drug conjugates · 5-Fluorouracil (5-FU) · Nanomedicines · Polymer therapeutics · Prodrug

*Correspondence: Dr. H. P. Märki[f]
Tel.: +41 61 688 5055
Fax: +41 61 688 6459
E-mail: hans_p.maerki@roche.com
[a]Centre for Polymer Therapeutics
Welsh School of Pharmacy, Cardiff University
Cardiff CF10 3NB, UK
E-mail: duncanr@cf.ac.uk
[b]Institute of Chemical Biology & Drug Discovery
State University New York
Stony Brook, USA
E-mail: iojima@notes.cc.sunysb.edu
[c]Department of Chemistry and Applied Biosciences
ETH Zurich
Wolfgang-Pauli-Str. 10, CH-8093 Zurich
E-mail: dario.neri@pharma.ethz.ch
[d]Kamakura Research Institute
Chugai Pharmaceutical Co., Ltd. Kajiwara
Kamakura, Kanagawa, Japan 247-8530
E-mail: shinmanbo@chugai-pharm.co.jp
[e]Roche Diagnostics K.K. Shiba 2-6-1
Minato City, Tokyo, Japan 105-0014.
[f]F. Hoffmann-La Roche Ltd
Pharmaceuticals Division
Discovery Chemistry
Building 092/3.92B
CH-4070 Basel

Polymer Therapeutics and other Nanomedicines as Targetable Cancer Therapies

Ruth Duncan

Nanomedicines (or nanopharmaceuticals as they are sometimes called) have been defined as nanometer size scale complex systems consisting of at least two components, one of which is biologically active, and it is agreed that they can be developed either as drug delivery systems or biologically active drug products.[1] Over the last three decades European R&D has been at the forefront of development of nanomedicines in the form of liposomes, nanoparticles, antibodies and their conjugates, and polymer conjugates. Although not widely appreciated, progress in the development of such nano-sized hybrid therapeutics and nano-sized drug delivery systems for the treatment of cancer has already made a significant contribution.[2] Ruth Duncan gave an introduction to the field of anticancer nanomedicines, and has underlined the fact that the first systems already entered routine clinical use as early as 1989, and since then there has been a growing number of products securing Regulatory Authority approval with, in turn, a healthy clinical development pipeline in the sector. Many of these products have been designed as anticancer treatments.

Polymers have been increasingly used, both as components of drug delivery systems (polymer-coated liposomes, nanoparticles, antibody conjugates) and moreover, as polymeric drugs and polymer-drug conjugates which are nanomedicines in their own right. In 1994 Ruth coined the descriptor 'polymer therapeutics' an umbrella term

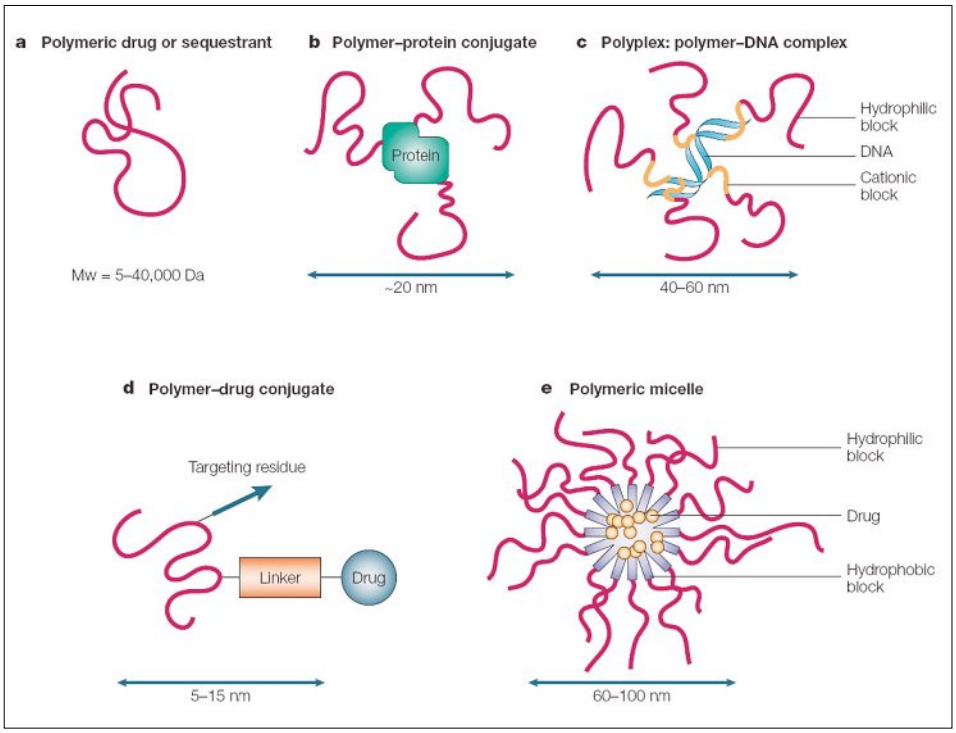


Fig. 1. Schematic representation of polymer therapeutics now in, or progressing towards, clinical development. The nano-sized and frequently multicomponent nature of these structures is visible. Mw, molecular weight.

to describe polymeric drugs, polymer–drug conjugates, polymer–protein conjugates, polymeric micelles to which the drug is covalently bound, and multi-component polyplexes being developed as non-viral vectors[3,4] (Fig. 1). The current status and rationale for design of anticancer polymer therapeutics was reviewed. From the industrial standpoint, these complex, nano-sized medicines are more like new chemical entities than conventional 'drug delivery systems or formulations' which simply entrap, solubilize or control drug release without resorting to chemical conjugation. Conceptually, polymer therapeutics share many features with other macromolecular drugs and the versatility of synthetic chemistry, which allows tailoring of molecular weight and addition of biomimetic features. Interdisciplinary research has proved essential to design therapeutically useful systems that are tailored using advanced polymer chemistry and precision engineering at a molecular level with due appreciation of the patho-physiology of normal and diseased tissue, and the ultimate practicality of industrial development and safe clinical use.[5]

Since 1990 an increasing number of polymer–protein conjugates have been transferred to market[6] including PEG-asparaginase for the treatment of acute lymphoblastic leukemia (ALL), PEG-GCSF (as an adjunct to chemotherapy), and PEG-interferon-alpha. More than 14 polymer-anticancer drug conjugates have progressed into clinical development. The most successful have been rationally designed in respect of their molecular weight, drug content and most importantly the polymer drug linker. Drug conjugation radically changes the drug's pharmacokinetics at whole body and the cellular level.[3,4] Prolonged circulation times promote passive tumor targeting by the enhanced permeability and retention (EPR) effect, and receptor-targeting ligands can be incorporated (*e.g.* galactose has been used to target the hepatocyte asialoglycoprotein receptor for polymer-anthracycline targeting in the context of hepatocellular carcinoma treatment). Once in the tumor interstitium the polymer–drug conjugate enters cells by the endocytic route leading to lysosomotropic drug delivery.

Initially the anticancer drug conjugates incorporated well-known chemotherapeutic agents such as doxorubicin, paclitaxel, camptothecins and most recently platinates. A polyglutamic acid (PGA)-paclitaxel conjugate (XYOTAX^{TM}/OPAXIO^{TM}; Cell Therapeutics Inc) is currently undergoing pivotal Phase III clinical trials in non-small cell lung cancer (NSCLC) patients and has been submitted to EMEA for Regulatory Authority review. However, now that clinical proof of concept has been established efforts are trying to develop more sophisticated second-generation systems that will exploit either tumor, or tumor vasculature-specific targeting,[7] improved delivery of novel natural product anticancer agents and also polymer–drug combinations.[8] Whilst the first-generation polymer–drug conjugates have used lysosomotropic delivery as the route of intracellular delivery, bioresponsive dextrin-phospholipase A2 constructs have recently been designed to attack tumor cells from the cell surface using a novel polymer–protein masking technique called 'PUMPT'.[9]

There are significant challenges for characterization of nano-sized, complex polymer-based constructs, both in the context of validated preclinical development[10] and to define their changes in conformation in biological environments for improved understanding of structure–activity relationships. New techniques are being developed and recently small angle neutron scattering (SANS) has been used for this purpose.[11] The promising beginnings for polymer therapeutics (current market size >$5 billion) suggest much more to come and it is clear that this growing family of agents can make a significant contribution to cancer therapy in the 21st century.

[1] European Science Foundation, 'Forward Look on Nanomedicine 2005', *http://www.esf.org/research-areas/medical-sciences/activities/esf-research-conferences-old/nanomedicine/european-science-foundation-publishes-forward-look-report-on-nanomedicine.html*
[2] R. Duncan, in 'Encyclopedia of Molecular Cell Biology and Molecular Medicine', Ed. R.A. Meyers, Wiley-VCH, Weinheim, Germany, **2005**, p. 163.
[3] R. Duncan, *Nat. Rev. Drug Disc.* **2003**, *2*, 347.
[4] R. Duncan, *Nat. Rev. Cancer* **2006**, *6*, 688.
[5] R. Duncan, H. Ringsdorf, R. Satchi-Fainaro, *Adv. Polymer Sci.* **2006**, *192*, 1.
[6] *Adv. Drug Delivery Rev*. **2008**, *60*, pp. 1-87, whole issue.
[7] R. Satchi-Fainaro, R. Duncan, C. M. Barnes, *Adv. Polymer Sci.* **2006**, *193*, 1.
[8] M. J. Vicent, F. Greco, R. I. Nicholson, R. Duncan, *Angew. Chem., Int. Ed.* **2005**, *44*, 2.
[9] R. Duncan, H. R. P. Gilbert, R. J. Carbajo, M. J. Vicent, *Biomacromolecules* **2008**, *9*, 1146.
[10] R. Duncan, in 'Handbook of Anticancer Drug Development', Eds D. Budman, H. Calvert, E. Rowinsky, Lippincott, Williams & Wilkins, Philadelphia, **2003**, p. 239.
[11] A. Paul, M. J. Vicent, R. Duncan, *Biomacromolecules* **2007**, *8*, 1573.

Design, Application, and Chemical Biology of Tumor-Targeting Drug Conjugates

Iwao Ojima

Key Issues in the Conventional Chemotherapy

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

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

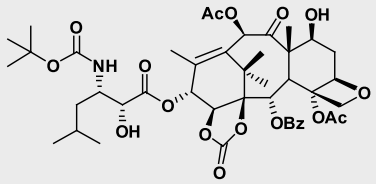
A rapidly growing tumor requires various nutrients and vitamins. Therefore, tumor cells overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors.[1] For example, monoclonal antibodies,[2–6] polyunsaturated fatty acids,[7,8] folic acid,[9,10] apatamers,[11]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

*Use of New Generation Taxoids as '**Warhead**'*

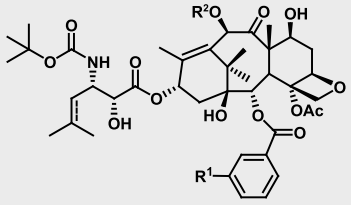
Paclitaxel and docetaxel have had a significant impact on current cancer chemotherapy, mainly because of their unique mechanism of action[14] but seriously suffer from the lack of tumor specificity and multidrug resistance (MDR). Paclitaxel and docetaxel are effective against breast, ovary, and lung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

Table. Cytotoxicity (IC_{50}, nM) of selected new generation taxoids against human cancer cell lines

Taxane	*MCF7*[a]	*NCI/ ADR*[b]	*R/S Ratio*[c]
Paclitaxel	1.7	550	324
Docetaxel	1.0	723	432
Ortataxel	1.1	66	60
SB-T-1103	0.35	5.1	15
SB-T-1104	0.51	7.9	15
SB-T-1213	0.18	4.0	22
SB-T-1214	0.20	3.9	20
SB-T-1216	0.13	7.4	57
SB-T-1217	0.14	9.7	69
SB-T-11033	0.36	0.61	1.7
SB-T-121303	0.36	0.79	2.2



Ortataxel (SB-T-101132; IDN5109) Phase II



R^1 = EtCO, c-Pr-CO-, Me_2NCO, MeOCO; R^2 = H or MeO

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

On the basis of our structure-activity relationship study of taxoids, we have developed a series of highly potent new generation taxoids.[16–20] Most of these taxoids exhibited 2–3 orders of magnitude higher potency than those of paclitaxel and docetaxel against drug-resistant cell lines expressing MDR phenotypes. New generation taxoids are also highly efficacious against paclitaxel-resistant cell lines based on point mutations, pancreatic cancer cell lines and tumor xenografts, and a variety of other drug-sensitive and drug-resistant cell lines, including GI cancer stem cells. Accordingly, these highly potent taxoids have been used as the warhead of our 'guided molecular missiles'. Selected new generation taxoids are listed in the Table.

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

Polyunsaturated fatty acids (PUFAs) are ideal candidates for tumor-specific guiding molecules. Representative naturally occurring PUFAs possess 18, 20, and 22 carbons and 2–6 unconjugated *cis*-double bonds separated by one methylene, such as linolenic acid (LNA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs are included in vegetable oils, cold-water fish and meat. DHA is classified as a nutritional additive by the FDA in the U.S. Thus, DHA and its metabolites are considered to be safe to humans.[21,22] PUFAs have exhibited anticancer activity against CFPAC, PANC-1 and Mia-Pa-Ca-2 pancreatic and HL-60 leukemia cell lines, and their anti-tumor activities have been evaluated in preclinical and clinical studies.[23,24] Perfusion studies demonstrated that some PUFAs are taken up more rapidly by tumor cells than by normal cells.[25,26] In addition, PUFAs are readily incorporated into the lipid bilayer of tumor cells, which results in disruption of membrane structure and fluidity.[27] This has been suggested to influence the chemosensitivity of tumor cells.[28] These findings strongly suggest the benefit in the use of PUFAs for tumor-targeting drug delivery.

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

Although Taxoprexin® exhibits an impressive antitumor activity against drug-sensitive tumors, this conjugate would not be effective against multidrug-resistant (MDR) tumors since the released paclitaxel would be caught by the Pgp efflux pump and eliminated from the cancer cells. As mentioned above, many of the second-generation taxoids developed in our laboratory showed 2–3 orders of magnitude higher activity against drug-resistant cancer cells and tumor xenografts in mice.[16–19] Thus, we hypothesized that the PUFA conjugates

of the second-generation taxoids would be efficacious against drug-resistant tumors for which DHA-paclitaxel is ineffective. To prove this hypothesis, the conjugates of DHA, LNA and LA with the second-generation taxoids were synthesized and their efficacy assayed *in vivo* against human tumor xenografts.

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

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

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

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

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

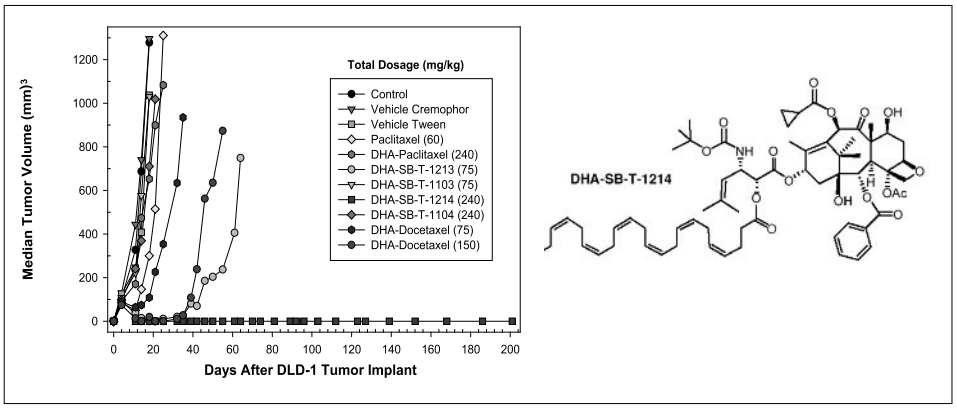


Fig. 1. Effect of DHA–taxoid conjugates on human colon tumor xenograft DLD-1

Use of Monoclonal Antibodies as Tumor-targeting Module

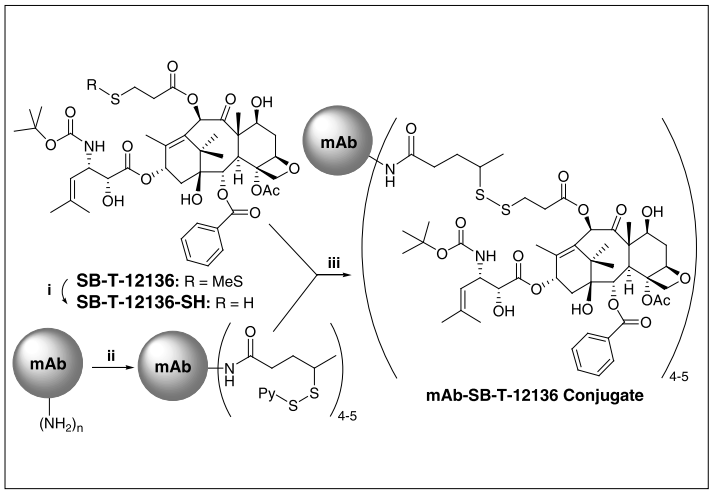
Monoclonal antibodies (mAbs), which have shown high binding specificity for tumor-specific antigens, are ideal to deliver cytotoxic drugs selectively to tumor cells.[6] A mAb-drug immunoconjugate would target the tumor cells and should be internalized to release the original cytotoxic agent in its active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

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

Design of mAb-taxoid Conjugates[4]

Use of an appropriate linker between a taxoid and an mAb is crucial for the efficacy of the resulting immunoconjugate. It is required that the linker is stable for an extended period of time upon storage and also in circulation *in vivo*, while it is readily cleavable inside of cancer cells. Among possible linker units reported, we chose to employ a disulfide linker unit because of its favorable characteristics.[6,32,36] The use of disulfide linkers is attractive by taking into account the fact that the concentration of glutathione is much higher (>1,000 times) in tumor cells than in blood plasma.[37] It is expected that the mAb module of the conjugate binds to the specific antigens on tumor surfaces and the whole conjugate is internalized *via* endocytosis. The disulfide bond is then cleaved by an intracellular thiol such as glutathione to release taxoid in its active form.

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



Scheme 1. (i) dithiothreitol (DTT); (ii) N-succinimidyl-4-(2-pyridyldithio) pentanoate (SPP, 10 equiv in ethanol), 50 mM potassium phosphate buffer, pH 6.5, NaCl (50 mM), EDTA (2 mM), 90 min; (iii) 50 mM potassium phosphate buffer, pH 6.5, NaCl (50 mM), EDTA (2 mM), SB-T-12136-SH (1.7 equiv per dithiopyridyl group, in EtOH), 24 h.

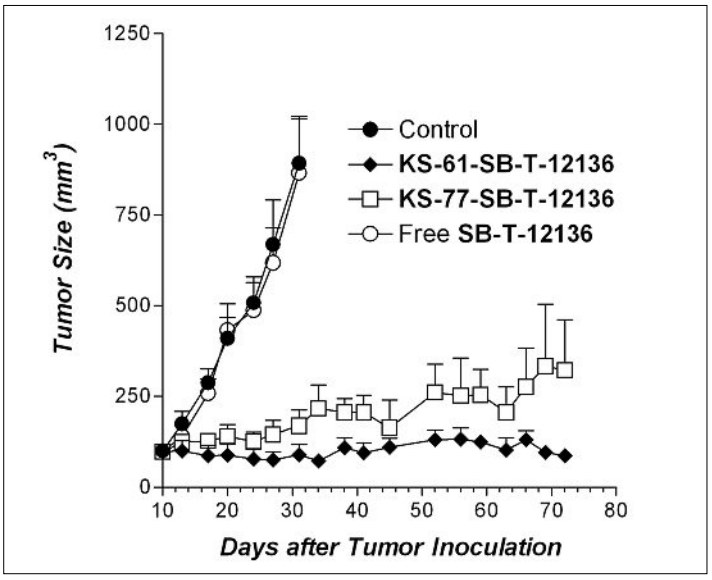


Fig. 2. Antitumor activity of anti-EGFR mAb-taxoid conjugates against A-431 xenografts in SCID mice

mental to the potency. This was an important finding, which was totally unexpected. Accordingly, a 10-MDS-propanyl-taxoid, SB-T-12136, was selected as the warhead precursor.

Conjugation of Taxoid with mAbs Targeting EGFR[4]

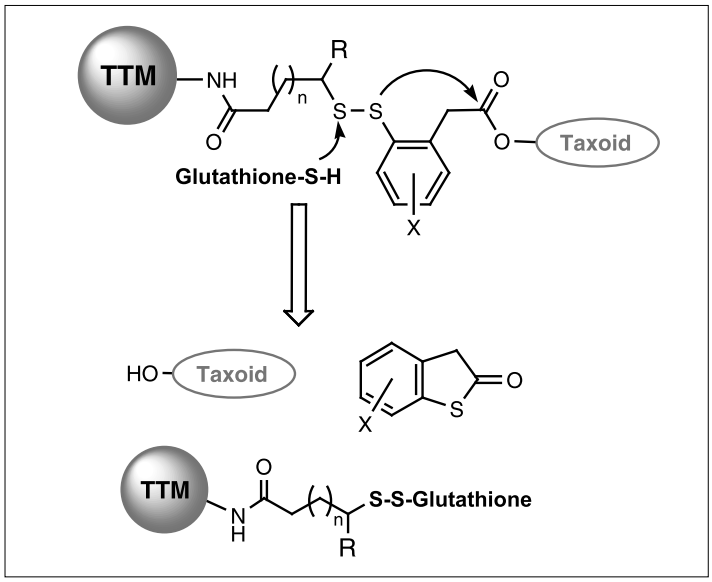
The epidermal growth factor receptor (EGFR) is known to be over-expressed in several human squamous cancers such as head and neck, lung and breast cancers. Murine monoclonal antibodies directed against the human EGFR were used as the tumor-targeting moieties in immunoconjugates. Three such immunoglobulin G class monoclonal antibodies, *KS61* (IgG2a), *KS77* (IgG1) and *KS78* (IgG2a), were linked to SB-T-1216 *via* disulfide bonds. The preparation of mAb-taxoid conjugates is illustrated in Scheme 1. SB-T-12136 was treated with dithiothreitol to generate SB-T-12136-SH bearing a free thiol functionality. The anti-EGFR mAb was modified with *N*-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP) to attach 4-pyridyldithio(PDT)-pentanoyl groups. Recovery of the antibody was about 90%, with 4~5 PDT-propanoyl groups linked per antibody molecule on average. Then, the modified mAb was conjugated with SB-T-12136-SH (2 equiv.), which proceeded with virtually complete conversion. The mAb-taxoid conjugates were purified by gel filtration, which separated aggregates from monomeric species and only the fractions corresponding to the monomeric conjugates were collected. Recovery of the conjugate was 65–70%. Three immunoconjugates, *KS61*-, *KS77*- and *KS78*-taxoid, were thus prepared. Preliminary MALDI-TOF analyses of the *KS77*-taxoid conjugate in comparison with *KS-77* strongly support that 4~5 taxoids, on average, are attached to the mAb. The final formulation of the conjugate was in phosphate-buffer saline (PBS), containing 20% propylene glycol and 0.1 % Tween 80 (v/v). A conjugate of SB-T-12136 with monoclonal antibody *mN901* that does not bind to EGFR was also prepared in a similar manner for comparison.

In vitro *Cytotoxicity Assay*[4]

In vitro cytotoxicity was determined in a clonogenic assay after a continuous exposure of the cells to the conjugates. It is expected that antigen-expressing cancer cells could only be targeted by an immunoconjugate bearing a mAb specific to the antigen. In fact, *mN901*-taxoid exhibited no cytotoxicity against the A431 cell line, expressing EGFR. In sharp contrast, *KS78*-taxoid showed high potency (IC_{50} = 1.5 nM) against the same A431 cell line. It should be noted that the addition of an excess of unconjugated anti-EGFR antibody, *e.g. KS-61* at 3×10^{-8} M to the *KS-61*-taxoid conjugate, abolished its cytotoxicity against A-431 cells, indicating that cytotoxicity depended on the specific binding of the conjugate to the antigen on cells. These results demonstrate that the binding of anti-EGFR mAb-taxoid conjugate to EGFR is highly specific. Moreover, it is strongly indicated that the immunoconjugate *KS78*-taxoid generates the highly cytotoxic agent SB-T-12136-SH upon binding to EGFR, followed by internalization and the subsequent cleavage of the disulfide linkage.

In vivo *Tumor Growth Inhibition Assay*[4]

The anti-tumor activities of two anti-EGFR-mAb-taxoid conjugates, *KS61*-taxoid and *KS77*-taxoid, were evaluated against human tumor xenografts in severe combined immune deficiency (SCID) mice (Fig. 2). Each mouse was inoculated with 1.5×10^6 A431 human squamous cancer cells and the tumors were allowed to grow for 11 days to an average size of 100 mm^3. The mice were then randomly divided into four groups. The first group received *KS61*-taxoid conjugate (10 mg/kg, qd × 5, administered *i.v.*). The second group received *KS77*-taxoid conjugate in the same manner. The third group received free taxoid (0.24 mg/kg, qd × 5, *i.v.*) at the same dose as that is present in the conjugate. A control group of mice received PBS using the same treatment schedule as in the groups 1–3. The weights of the mice and tumor sizes were measured twice weekly. The results are shown in Fig. 2. The tumors in the control group of mice grew to a size of nearly 1000 mm^3 in 31 days. Treatment with free taxoid (same dose as that in the mAb-taxoid conjugates) showed no therapeutic effect. Also, treatment with mAb *KS-77* (2 × dose of the mAb-taxoid conjugate) exhibited only a moderate delay in tumor growth, *i.e.* the tumor size reached 800 mm^3 within 25 days after the treatment (data not shown). In contrast, both anti-EGFR-mAb-taxoid conjugates, especially *KS61*-SB-T-12136, showed remarkable antitumor activity, resulting in complete inhibition of tumor growth and elimination of tumor cells in all the treated animals for the duration of the experiment. Necropsy on day 75, followed by histopathological examination showed residual calcified material at the tumor site, but no evidence of tumor cells. These data also indicate that targeted delivery of the taxoid using a tumor-specific mAb is essential for the activity since an equivalent dose of unconjugated taxoid shows no antitumor activity. Notably, the doses of mAb-taxoid conjugates used are non-toxic to the



Scheme 2. Second-generation self-immolative disulfide linkers (TTM = tumor-targeting mAb)

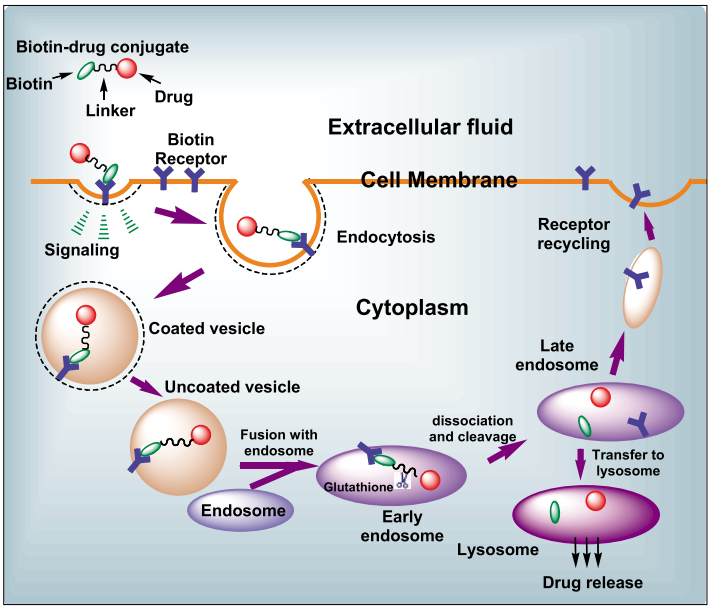


Fig. 3. Receptor-mediated endocytosis of biotin-drug conjugates

mice as demonstrated by the absence of any weight loss. The results clearly indicate that the 'guided molecular missiles' combining the second-generation taxoids with mAbs highly specific to the antigen on tumor cell surfaces are very promising as potential chemotherapeutic agents with few side effects.

Second-generation Self-immolative Disulfide Linkers

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

In order to solve this problem, we have been developing the second-generation mechanism-based bifunctional disulfide linkers, which can be connected to various warheads as well as tumor-targeting modules. One of our approaches is to use self-immolative disulfide linkers wherein the glutathione-triggered cascade drug release takes place to generate the original anticancer agent *via* thiolactone formation and ester bond cleavage (Scheme 2). This mechanism-based drug release concept was nicely proven in a model system by monitoring the reaction with ^{19}F NMR using fluorine-labeled compounds.[39] The strategic design of placing a phenyl group attached to the disulfide linkage directs the cleavage of this linkage by a thiol to generate the desirable thiophenolate or sulfhydrylphenyl species for thiolactonization. This type of self-immolative disulfide linkers is readily applicable to a range of tumor-targeting drug conjugates.

Use of Biotin as Tumor-targeting Module of Anticancer Drug Conjugates

All living cells require vitamins for survival, but the rapidly dividing cancer cells require certain vitamins to sustain their rapid growth. Accordingly, the receptors involved in uptake of the vitamins are overexpressed on the cancer cell surface. Thus, these vitamin receptors serve as useful biomarkers for the imaging and identification of tumor cells as well as for 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 tumor cells. The folate receptors were recognized as potentially excellent biomarkers for targeted drug delivery and significant advancement has been made to date.[9,10,40,41] However, the biotin receptors were not studied for this purpose until recently. Biotin (vitamin H or vitamin B7) is a growth promoter at the cellular level, and its content in tumors is substantially higher than in normal tissues. Recently it has been shown that the biotin receptors are even more overexpressed than the folate and/or vitamin B12 receptors in many cancer cells, *e.g.* leukemia (L1210FR), ovarian (Ov 2008, ID8), Colon (Colo-26), mastocytoma (P815), lung (M109), renal (RENCA, RD0995), and breast (4T1, JC, MMT06056) cancer cell lines.[42]

Accordingly, we chose biotin as the tumor-targeting molecule for our molecular missile bearing the second-generation disulfide linker with SB-T-1214 as the warhead. We also performed a mechanistic study on the validation of tumor-targeting drug delivery by monitoring the internalization through receptor-mediated endocytosis (Fig. 3) and drug release, using fluorescent and fluorogenic molecular probes for the biotin-taxoid conjugate.

We designed and synthesized three fluorescence-labeled biotin conjugates, *i.e.* biotin-flurorescein (A), biotin-linker-coumarin (B) (fluorogenic probe), and biotin-linker-taxoid-fluorescein (C) (Fig. 4). The conjugate A was designed to observe the receptor-mediated endocytosis (RME, Fig. 5), while the conjugate B was designed for confirming the internalization *via* RME of the biotin-linker unit and the release of coumarin, which becomes fluorescent only when it is released as free molecule (it is conjugated to the linker *via* an ester bond) *via* disulfide cleavage by endogenous thiol, glutathione in particular. The conjugate C was designed to validate the whole internalization by RME and drug release processes, in which the freed fluorescent taxoid should bind to the target protein, microtubules, in the cancer cells.

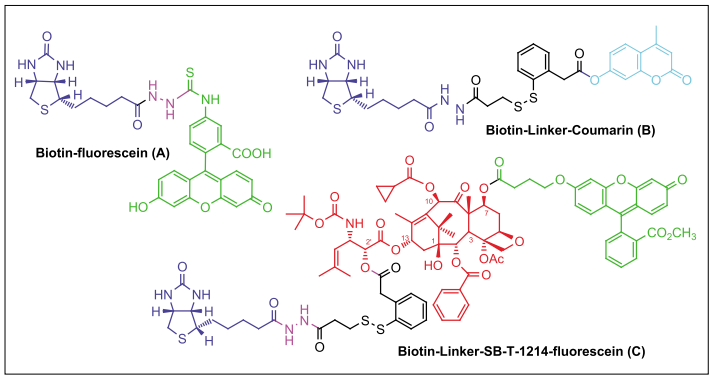


Fig. 4. Fluorescent and flurogenic probes for the internalization and drug-release

Cellular uptake of these three fluorescent and fluorogenic probes was monitored by confocal fluorescence microscopy (CFM). Fig. 5(a) shows the observation of intense fluorescence when the L1210FR cell was incubated with biotin-fluorescein (probe A) (100 nM) at 37 °C for 3 h, followed by thorough washing of the cells by phosphate-buffered saline (PBS) and analysis. The result confirms the internalization of this fluorescent probe A into the leukemia cells. It has been shown that endocytosis is an energy-dependent process. Thus, endocytosis should be inhibited at low temperature (4 °C). The CFM image of the probe A, incubated at 4 °C (100 nM, 3 h) showed greatly diminished fluorescence in the cells, which clearly indicates that the probe A was internalized through endocytosis. To further confirm that this was a receptor-mediated endocytosis process, the cancer cell surface was pre-incubated for 1 h with excess of free biotin molecules (2 mM) to saturate the biotin receptors, followed by the addition of the probe A (100 nM, 37 °C, 3 h). The CFM image revealed the virtually total absence of fluorescence, which confirms that this is indeed the receptor-mediated endocytosis.

Next, the fluorogenic probe B, biotin-linker-coumarin, (1 μM) was incubated with L1210FR at 37 °C for 3 h. After washing thoroughly with PBS, glutathione methyl ester (2 mM) was added to the medium and cells were incubated for another 2 h at 37 °C. The addition of glutathione methyl ester was to ensure the cleavage of the self-immolative disulfide linker to release free coumarin (fluorescent), as designed. As Fig. 5(b) shows, fluorescent coumarin molecules (blue) are indeed released in the leukemia cells. The result confirms that the intracellular drug release *via* cleavage of the disulfide linkage by glutathione and the subsequent thiolactonization, took place as designed. In the absence of additional glutathione, the observed blue fluorescence was substantially weaker. This means that the concentration of intracellular glutathione in this leukemia L1210FR cell line is small under the *in vitro* experimental conditions, which are significantly different from *in vivo* conditions where the glutathione supply in tumor tissues is more than adequate. On the other hand, this experiment using additional glutathione (ester) obviously demonstrates that the cleavage of the self-immolative disulfide linkage and drug release was caused by glutathione. Thus, this result confirms that the designed drug release using the fluorogenic molecule in place of the taxoid warhead has worked well.

Finally, the internalization and drug release of the biotin-linker-SB-T-1214-fluorescein conjugate (probe C) was investigated. First, the probe C (20 μM) was incubated with L1210FR cells at 37 °C for 3 h and analyzed. As Fig. 6(a) shows, the whole conjugate was internalized in the same manner as that described above for the probe A (see Fig. 5(a)). Next, glutathione methyl ester (2 mM) was added to the medium and the cells were incubated for another 1 h to ensure the drug release. Fig. 6(b) shows the CFM image of this system, which is dramatically different from Fig. 6(a). This CFM image indicates that the released fluorescent taxoid binds to the microtubules, which are the drug target of the taxoid, highlighting the fluorescence-labeled microtubule bundles. Accordingly, the release of the taxoid warhead through the designed mechanism is shown to have taken place in the same manner as that observed for the fluorogenic probe B. In addition, the binding of the released fluorescent taxoid to microtubules is observed. Thus, it is concluded that the 'guided molecular missile' successfully delivered the active warhead to the drug target, as designed, through receptor-mediated endocytosis and glutathione-triggered intracellular drug release *via* cleavage of a self-immolative disulfide linker and thiolactonization.

To confirm the specificity of the biotin-mediated endocytosis, two other cell lines, a leukemia L1210 cell line and a WI38 normal human lung fibroblast cell line, were used to compare results with those for the L1210FR cell line. L1210 and WI38 cell lines do not overexpress biotin receptors on their surfaces. Thus, the *in vitro* cytotoxicity assays (MTT) of biotin-linker-SB-T-1214 conjugate (BLT-1214) (Fig. 7) were performed against L1210FR, L1210 and WI38

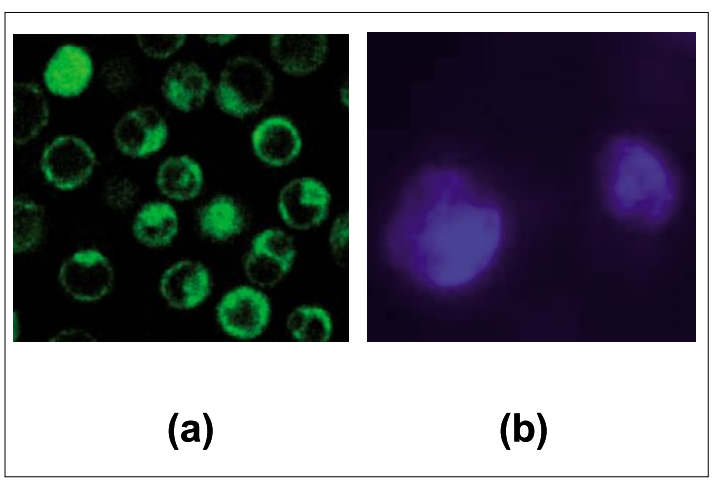


Fig. 5. CFM images of L1210FR cells incorporating (a) biotin-fluorescein (probe A); (b) biotin-linker-coumarin (probe B) after the addition of glutathione Me ester

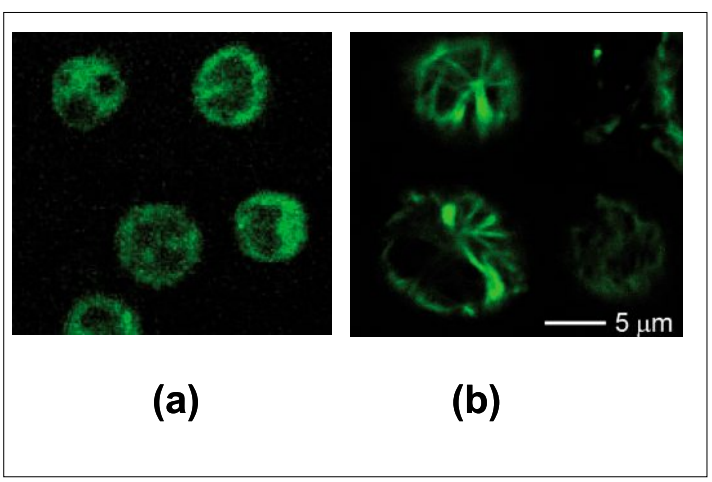


Fig. 6. CFM images of L1210FR cells incorporating biotin-linker-SB-T-1214-fluorescein (probe C): (a) prior to the additions of glutathione; (b) after the addition of glutathione Me ester

cell lines. Paclitaxel and SB-T-1214 (warhead of the conjugate) were also assayed for comparison. The IC_{50} values against L1210FR are as follows: BLT-1214, 8.80 nM; SB-T-1214, 10.1 nM; paclitaxel, 121 nM. It is apparent that the internalization process was enhanced by receptor-mediated endocytosis and the drug release was efficient with endogenous GSH during the incubation time (72 h) applied to the MTT assay. It is worth mentioning that the conjugate BLT-1214 exhibited slightly more potent activity than the parent SB-T-1214. The IC_{50} values of BLT-1214 against L1210 and WI38 cell lines are 522 nM and 570 nM, respectively. Accordingly, the tumor-targeting specificity of BLT-1214 to L1210FR is 59.3 times and 64.8 times higher than to L1210 and WI38, respectively, due to the overexpression of the biotin receptors on L1210FR. (*Note:* the IC_{50} values of SB-T-1214 against L1210 and WI38 are 9.72 nM and 10.7 nM, respectively, showing the same level of cytotoxicity to all three cell lines, as expected.)

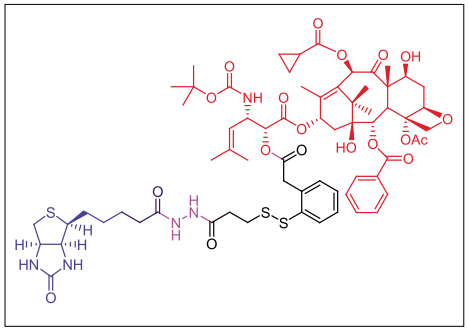


Fig. 7. Biotin-linker-SB-T-1214 conjugate (BLT-1214)

Use of Functionalized Single-walled Carbon Nanotubes as Novel Vehicles for Tumor-targeting Drug Delivery

As an application of the tumor-targeting biotin-linker-drug conjugates described above, a novel single-walled carbon nanotube (SWNT)-based tumor-targeting drug delivery system (DDS) has been developed, which consists of a functionalized SWNT linked to tumor-targeting modules as well as prodrug modules. We have successfully designed and synthesized biotin-functionalized SWNT conjugates (Fig. 8) as a novel and efficient DDS for potential use in tumor-targeting chemotherapy.[43] The key features of this DDS are (i) the presence of tumor-targeting modules (*i.e.* biotin) and (ii) a self-immolative disulfide linkage connecting the anticancer drug warhead to the biotin-SWNT conjugate, which is efficiently cleaved by intracellular thiols such as GSH upon internalization into cancer cells through receptor-mediated endocytosis. We have unambiguously observed the occurrence of the designed cancer-specific receptor-mediated endocytosis of the whole conjugate, followed by efficient drug release and binding of the drug to the target microtubules by confocal fluorescence microscopy analysis in the same manner as that for BLT-1214 described above. The conjugate shows the specificity to the cancer cells, overexpressing biotin receptors on their surface. The cytotoxicity of the conjugate is solely ascribed to the released taxoid molecules in the cytosol of the cancer cells. (IC_{50} values of biotin-SWNT-linker-SB-T-1214 conjugate against L1210FR, L1210 and WI38 are 0.36, >50, and >50 μg/ml, respectively, *i.e.* two orders of magnitude more specific to L1210FR, overexpressing the biotin receptors.) We also have found that the mass drug delivery into the cytosol of the cancer cells using this drug delivery system is superior to the simple exposure of the drug itself to the same cancer cells. [The estimated maximum IC_{50} value for SB-T-1214-fluorescein delivered by this SWNT-based DDS is 51 nM, while that of the same fluorescent-taxoid itself is 87.5 nM.] These results strongly suggest that the functionalized SWNT-based DDS can serve as a highly promising drug delivery platform, which offers (i) biomarker-targeted drug delivery, (ii) possible delivery of greater therapeutic payloads and (iii) possible use of multiple, complementary drug warheads for combination, thereby forming a solid basis for further development.

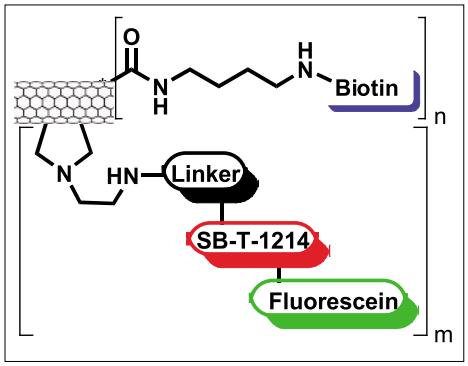


Fig. 8. Biotin-SWNT-linker-SB-T-1214-fluorescein DDS

[1] S. Jaracz, J. Chen, L. V. Kuznetsova, I. Ojima, *Bioorg. Med. Chem.* **2005**, *13*, 5043.
[2] J. Chen, S. Jaracz, X. Zhao, S. Chen, I. Ojima, *Exp. Opin. Drug Deliv.* **2005**, *2*, 873.
[3] X. Wu, I. Ojima, *Curr. Med. Chem.* **2004**, *11*, 429.
[4] I. Ojima, X. Geng, X. Wu, C. Qu, C. P. Borella, H. Xie, S. D. Wilhelm, B. A. Leece, L. M. Bartle, V. S. Goldmacher, R. V. J. Chari, *J. Med. Chem.* **2002**, *45*, 5620.
[5] P. R. Hamaan, L. M. Hinman, I. Hollander, C. F. Beyer, D. Lindh, R. Holcomb, W. Hallett, H.-H. Tsou, J. Upeslacis, D. Shochat, A. Mountain, D. A. Flowers, I. Bernstein, *Bioconjugate Chem.* **2002**, *13*, 47.
[6] R. V. J. Chari, *Adv. Drug Delivery Rev.* **1998**, *31*, 89.
[7] M. O. Bradley, N. L. Webb, F. H. Anthony, P. Devanesan, P. A. Witman, S. Hemamalini, M. C. Chander, S. D. Baker, L. He, S. B. Horowitz, C. S. Swindell, *Clin. Cancer Res.* **2001**, *7*, 3229.
[8] L. V. Kuznetsova, J. Chen, L. Sun, X. Wu, A. Pepe, J. M. Veith, P. Pera, R. J. Bernacki, I. Ojima, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 974.
[9] C. P. Leamon, J. A. Reddy, *Adv. Drug Deliv. Rev.* **2004**, *56*, 1127.
[10] Y. Lu, P. S. Low, *Adv. Drug Deliv. Rev.* **2002**, *54*, 675.
[11] T. C. Chu, J. W. Marks III, L. A. Lavery, S. Faulkner, M. G. Rosenblum, A. D. Ellington, M. Levy, *Cancer Res.* **2006**, *66*, 5989.
[12] A. Nagy, A. V. Schally, G. Halmos, P. Armatis, R.-Z. Cai, V. Csernus, M. Kovacs, M. Koppan, K. Szepeshazi, Z. Kahan, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1794.
[13] Y. Luo, N. J. Bernshaw, Z.-R. Lu, J. Kopecek, G. D. Prestwich, *Pharmaceutical Res.* **2002**, *19*, 396.
[14] P. B. Schiff, J. Fant, S. B. Horwitz, *Nature* **1979**, *277*, 665.
[15] M. R. Vredenburg, I. Ojima, J. Veith, P. Pera, K. Kee, F. Cabral, A. Sharma, P. Kanter, R. J. Bernacki, *J. Nat'l. Cancer Inst.* **2001**, *93*, 1234.
[16] I. Ojima, J. C. Slater, E. Michaud, S. D. Kuduk, P.-Y. Bounaud, P. Vrignaud, M.-C. Bissery, J. Veith, P. Pera, R. J. Bernacki, *J. Med. Chem.* **1996**, *39*, 3889.
[17] I. Ojima, J. S. Slater, S. D Kuduk, C. S. Takeuchi, R. H. Gimi, C.-M. Sun, Y. H. Park, P. Pera, J. M. Veith, R. J. Bernacki, *J. Med. Chem.* **1997**, *40*, 267.
[18] I. Ojima, T. Wang, M. L. Miller, S. Lin, C. Borella, X. Geng, P. Pera, R. J. Bernacki, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3423.
[19] I. Ojima, S. Lin, T. Wang, *Curr. Med. Chem.* **1999**, *6*, 927.
[20] R. Geney, J. Chen, I. Ojima, *Med. Chem.* **2005**, *1*, 125.
[21] W. C. Heird, A. Lapillonne, *Annu. Rev. Nutr.* **2005**, *25*, 549.
[22] W. E. Hardman, *J. Nutrition* **2002**, *132*, 3508S.
[23] S. J. Wigmore, J. A. Ross, J. S. Falconer, C. E. Plester, M. J. Tisdale, D. C. Carter, K. C. H. Fearon, *Nutrition* **1996**, *12*, S27.
[24] R. A. Hawkins, K. Sangster, M. J. Arends, *J. Pathol.* **1998**, *185*, 61.
[25] L. A. Sauer, R. T. Dauchy, D. E. Blask, *Cancer Res.* **2000**, *60*, 5289.
[26] L. A. Sauer, R. T. Dauchy, *Brit. J. Cancer* **1992**, *66*, 297.
[27] S. I. Grammatikos, P. V. Subbaiah, T. A. Victor, W. M. Miller, *Brit. J. Cancer* **1994**, *70*, 219.
[28] L. Diomede, F. Colotta, B. Piovani, F. Re, E. J. Modest, M. Salmona, *Int. J. Cancer Res.* **1993**, *53*, 124.
[29] M. S. Ernstoff, 'Phase III Study of Taxoprexin Injection vs Dacarbazine in Patients with Metastatic Malignant Melanoma,' Norris Cotton Cancer Center,

2003, *http://www.cancer.dartmouth.edu/clinicaltrials/trials/F0239.shtml.*
[30] H. Sunada, B. E. Magun, J. Mendelsohn, C. L. MacLeod, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 3825.
[31] H. Bender, H. Takahashi, K. Adachi, P. Belser, S. Liang, M. Prewett, M. Schrappe, A. Sutter, U. Rodeck, D. Herlyn, *Cancer Res.* **1992**, *52*, 121.
[32] C. Liu, B. M. Tadayoni, L. A. Bourret, K. M. Mattocks, S. M. Derr, W. C. Widdison, N. L. Kedersha, P. D. Ariniello, V. S. Goldmacher, J. M. Lambert, W. Blättler, R. V. J. Chari, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8618.
[33] M. N. Saleh, A. F. LoBuglio, P. A. Trail, *Basic Clin. Oncol.* **1998**, *15*, 397S.
[34] V. Guillemard, H. U. Saragovi, *Cancer Res*. **2001**, *61*, 694.
[35] J. Jaime, M. Page, *Anticancer Res.* **2001**, *21*, 1119.
[36] R. V. J. Chari, K. A. Jackel, L. A. Bourret, S. M. Derr, B. M. Tadayoni, K. M. Mattocks, S. A. Shah, C. Liu, W. A. Blattler, V. S. Goldmacher, *Cancer Res*. **1995**, *55*, 4079.
[37] J. Kigawa, Y. Minagawa, Y. Kanamori, H. Itamochi, X. Cheng, M. Okada, T. Oishi, N. Terakawa, *Cancer* **1998**, *82*, 697.
[38] I. Ojima, I. Habus, M. Zhao, M. Zucco, Y. H. Park, C.-M. Sun, T. Brigaud, *Tetrahedron* **1992**, *48*, 6985.
[39] I. Ojima, *Chem. Bio. Chem.* **2004**, *5*, 628.
[40] J. A. Reddy, E. Westrick, I. Vlahov, S. J. Howard, H. K. Santhapuram, C. P. Leamon, *Cancer Chemother. Pharmacol.* **2006**, *58*, 229.
[41] C. P. Leamon, J. A. Reddy, I. R. Vlahov, M. Vetzel, N. Parker, J. S. Nicoson, L.-C. Xu, E. Westrick, *Bioconjugate Chem.* **2005**, 803.
[42] G. Russell-Jones, K. McTavish, J. McEwan, J. Rice, D. Nowotnik, *J. Inorg. Biochem.* **2004**, *98*, 1625.
[43] J. Chen, S. Chen, X. Zhao, L. V. Kuznetsova, S. S. Wong, I. Ojima, *J. Am. Chem. Soc*. **2008** in press.

Antibody-Based Vascular Tumor Targeting: From the Bench to the Clinic

Dario Neri

Conventional pharmaceutical agents currently in use for the treatment of cancer often suffer from a lack of specificity, leading to the undesired damage of normal tissues. The development of more selective and better tolerated cancer therapeutics is possibly one of the most important goals in modern oncology.

The search for more selective drugs has led to a growing interest in the use of monoclonal antibodies and their derivatives as innovative biopharmaceuticals. Antibodies can recognize their target antigen with high affinity and exquisite specificity: a property which has been exploited for the generation of agents capable of blocking soluble factors relevant for disease (*e.g.* TNF in arthritis, VEGF-A in cancer) or of binding to markers on target cells (*e.g.* tumor-associated membrane antigens). Such antibodies may confer a benefit to patients (and may become approved pharmaceutical products), but are unlikely to cure cancer or other serious conditions (such as rheumatoid arthritis) because of fundamental limitations which are intimately related to the use of unmodified antibodies as drugs. On one hand, when an antibody is used to block a soluble factor, such a product is likely to have a limited efficacy if the target antigen is not the sole cause of disease. On the other hand, antibodies which recognize a tumor-associated antigen on the surface of a tumor cell may be unable to mediate cell killing if they do not manage to localize on the target cell with sufficient affinity (Fig. 1).

In an alternative approach, scientists may prefer to use antibodies (or better, recombinant antibody fragments) to deliver bioactive agents to the tumor environment, thus achieving an increased selectivity and sparing normal tissues. In particular, 'vascular targeting' monoclonal antibodies, which discriminate between a mature blood vessel and a tumor blood vessel, may serve as ideal 'delivery vehicles' in light of the accessibility of tumor neo-vasculature for agents in the bloodstream and of the relevance of angiogenesis for aggressive types of cancer.

There is a fundamental conceptual difference between the inhibition of angiogenesis and the concept of vascular targeting. In the first case, one aims at preventing the growth of new blood vessels (typically by blocking a pro-angiogenic factor). In the second situation, one delivers bioactive molecules to blood vessels at the tumor site.[1,2]

Our laboratory has developed and validated a range of human monoclonal antibodies specific to markers of angiogenesis. In particular, we have generated and extensively studied antibodies specific to splice isoforms of fibronectin and tenascin-C, with a special focus on the extra-domains EDA and EDB of fibronectin, and A1 and C of tenascin-C (Fig. 2).[3–6]

The tumor targeting properties of L19, F8, F16 and G11 have been extensively characterized in animal models of cancer by quantitative biodistribution studies and by microscopic analysis, using both radiolabeled antibody preparations and fluorescently-labeled derivatives (Fig. 3).

In addition, over 100 patients with cancer have been imaged using radioiodinated derivatives of the L19 antibody either in scFv format[7,8] or in SIP format.[9–11]

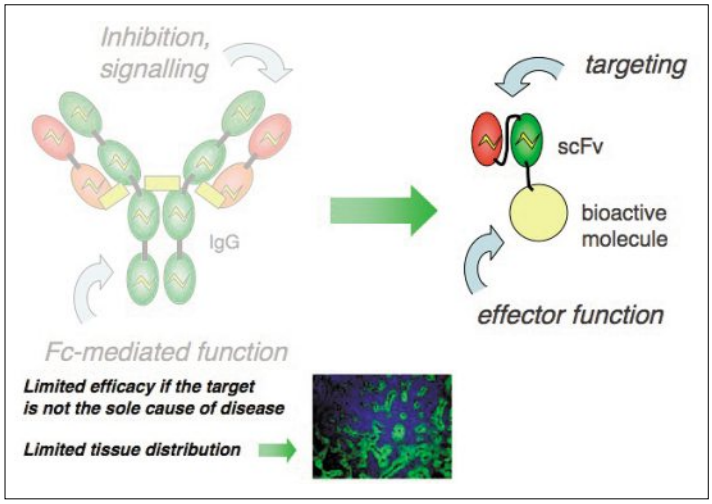


Fig. 1. Schematic representation of different uses of antibody molecules for the development of therapeutic agents. Specific limitations of antibody-based products in IgG format are outlined. The use of antibody fragments for the selective delivery of bioactive agents to the tumor environment may represent a valuable alternative.

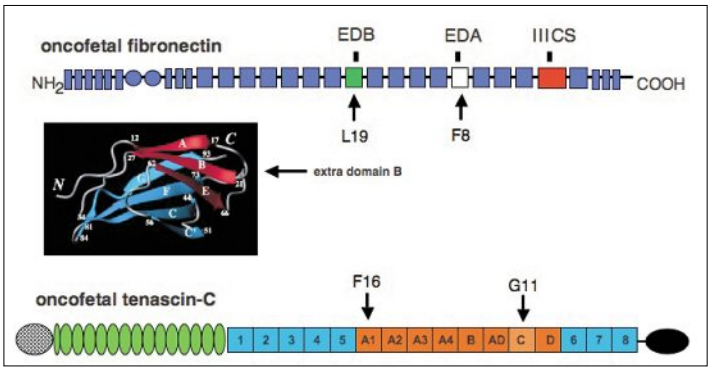


Fig. 2. Schematic representation of the domain structure of fibronectin and tenascin-C. The human monoclonal antibody L19 recognizes the EDB domain (whose 3D structure is depicted in the figure). Similarly, F8 recognizes EDA, while F16 and G11 bind to domains A1 and C of tenascin-C, respectively.

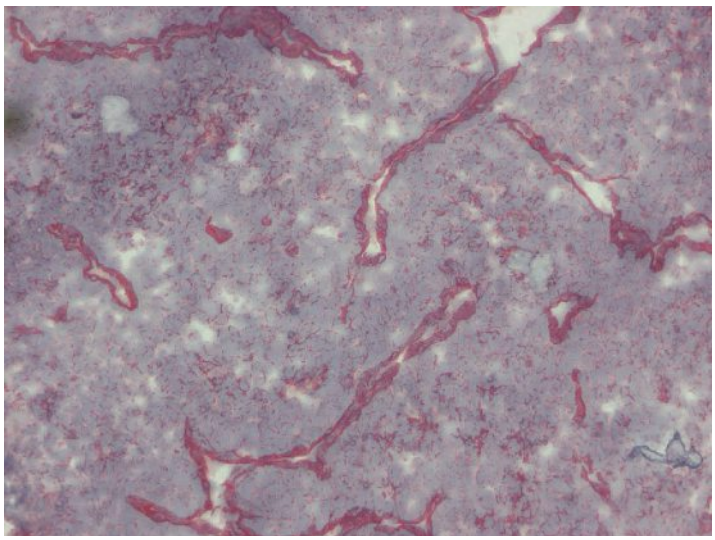


Fig. 3. Immunohistochemical staining of a tumor section using the human monoclonal antibody F8. The tumor blood vessels are stained in red.

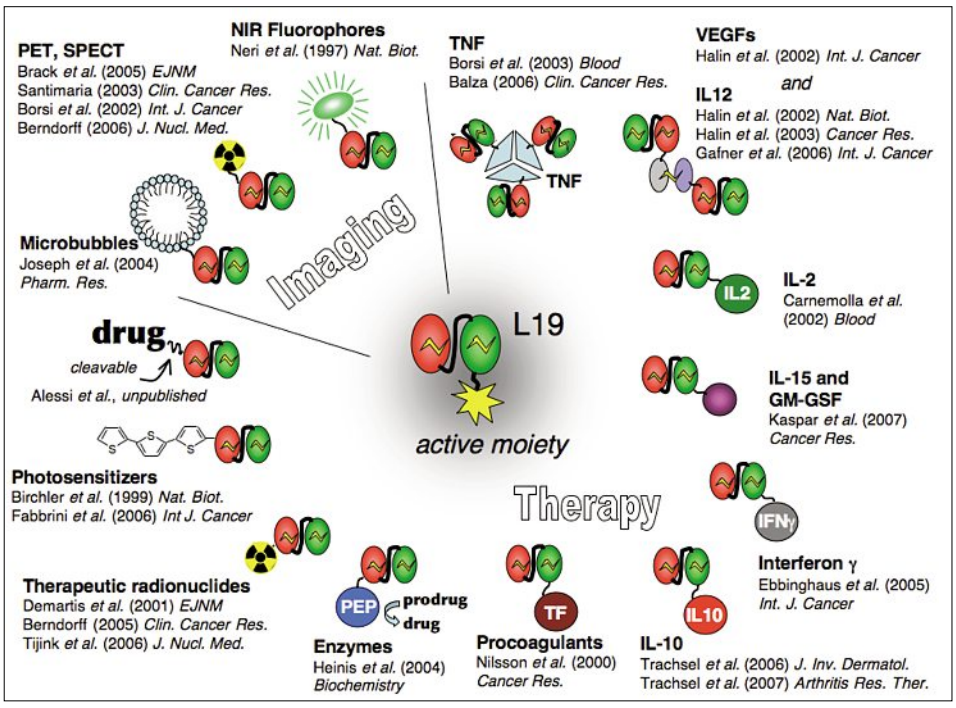


Fig. 4. Schematic representation of some of the derivatives of the L19 antibody, which have been produced and tested *in vivo* for imaging or therapeutic applications. References for published articles have been indicated next to the individual L19 derivatives.

The L19 antibody has been used as a model to systematically investigate, *in vitro* and *in vivo*, the relative merits of different antibody functionalization strategies, both for imaging purposes and for therapeutic applications (Fig. 4). For imaging, antibodies can be coupled to near-infrared fluorophores, microbubbles or can be radiolabeled. For therapy, antibody conjugates with drugs using cleavable linkers, photosensitizers, radionuclides, or derivatives in which the antibody moiety is fused to enzymes, pro-coagulant factors or cytokines can be considered.

Five derivatives of the L19 antibody (the radiolabeled product L19-^{131}I and the immunocytokines L19-IL2 and L19-TNF, developed in a collaboration between Philogen and Bayer Schering) and of the F16 antibody (F16-^{131}I and F16-IL2, developed by Philogen) are currently being investigated in twelve multicenter clinical trials, while two derivatives of the F8 antibody are expected to begin clinical trials shortly.

[1] D. Neri, R. Bicknell, *Nat. Rev. Cancer* **2005**, *5*, 436.
[2] C. Schliemann, D. Neri, *Biochim. Biophys. Acta* **2007**, *1776*, 175.
[3] A. Villa, E. Trachsel, M. Kaspar, C. Schliemann, R. Sommavilla, J.-N. Rybak, C. Roesli, L. Borsi, D. Neri, *Int. J. Cancer* **2008**, *122*, 2405.
[4] A. Pini, F. Viti, A. Santucci, B. Carnemolla, L. Zardi, P. Neri, D. Neri, *J. Biol. Chem.* **1998**, *273*, 21769.
[5] S. S. Brack, M. Silacci, M. Birchler, D. Neri, *Clin. Cancer Res.* **2006**, *12*, 3200.
[6] M. Silacci, S. S. Brack, N. Spaeth, A. Buck, S. Hillinger, S. Arni, W. Weder, L. Zardi, D. Neri, *Protein Eng., Des. Sel.* **2006**, *19*, 471.
[7] M. Santimaria, G. Moscatelli, G. L. Viale, L. Giovannoni, G. Neri, F. Viti, A. Leprini, L. Borsi, P. Castellani, L. Zardi, D. Neri, P. Riva, *Clin. Cancer Res.* **2003**, *9*, 571.
[8] M. T. Birchler, C. Thuerl, D. Schmid, D. Neri, R. Waibel, A. Schubiger, J. Stoeckli Sandro, S. Schmid, W. Goerres Gerrhard, *Otolaryngol. Head Neck Surg*. **2007**, *136*, 543.
[9] L. Borsi, E. Balza, M. Bestagno, P. Castellani, B. Carnemolla, A. Biro, A. Leprini, J. Sepulveda, O. Burrone, D. Neri, L. Zardi, *Int. J. Cancer* **2002**, *102*, 75.
[10] D. Berndorff, S. Borkowski, S. Sieger, A. Rother, M. Friebe, F. Viti, C. S. Hilger, J. E. Cyr, L. M. Dinkelborg, *Clin. Cancer Res.* **2005**, *11*, 7053s.
[11] B. M. Tijink, D. Neri, C. R. Leemans, M. Budde, L. M. Dinkelborg, M. Stigter-van Walsum, L. Zardi, G. A. M. S. van Dongen, *J. Nucl. Med.* **2006**, *47*, 1127.

CHIMIA **2008**, *62*, No. 11

Discovery of Capecitabine, a Rationally Designed and Tumor-Activated Oral Prodrug of 5-FU, and Beyond

Nobuo Shimma, *Hisafumi Okabe, and Hideo Ishitsuka*

Introduction

In recent years, significant progress has been made in development of new anticancer agents with high tumor selectivity. The following three major approaches have been explored.

The first approach aims to target proteins constitutively activated by gene alternation or over-expression in tumors: examples are cell growth factor (receptor) antibodies and inhibitors of kinases involved in major signal transduction pathways. In a second approach tumor vascularization is inhibited by antiangiogenic agents. The third approach consists in tumor selective delivery of cytototoxic agents by antibody–drug conjugates, nano-particle technologies or prodrugs that can be activated by enzymes over-expressed in tumor tissues.

We have been working on tumor targeting by cytotoxic agents carrying prodrugs. In this conference, following three topics were reviewed.

i) Discovery of the tumor-activated prodrug of 5-fluorouracil (5-FU), capecitabine (Xeloda®)
ii) Discovery of a tumor-activated prodrug of a dihydropyrimidine dehydrogenase (DPD) inhibitor for enhancement of capecitabine efficacy.
iii) Search for prodrug activation enzymes in human tumor tissues by DNA microarray, and its application for prodrug design.

Drug Design and Discovery of Capecitabine

Capecitabine (N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) is a tumor-activated oral prodrug of 5-FU with good safety profile, enabling home-based therapy with lower medical care costs. It is now prescribed in more than 90 countries for treatment of metastatic breast, colorectal, and gastric cancers either as a single agent or in combination with other anticancer agents (see below).

5-FU is an old drug that was first introduced into market in the 1960s, but it is still a standard drug for treatment of gastrointestinal tumors. However, 5-FU has the following drawbacks: lack of tumor selectivity resulting in strong bone marrow (BM) and gastrointestinal (GI) toxicity, lack of oral bioavailability and extremely short plasma half life (10 min only). Thus, in order to maximize its efficacy, 5-FU is given by continuous infusion with a modulator, leucovorin. Therefore, patients have to be hospitalized for the treatment, resulting in high total treatment costs, and reduced quality of life. Therefore, we aimed to develop an oral 5-FU prodrug with better efficacy, and less bone marrow and intestinal toxicity as compared to 5-FU infusions, enabling patients to be treated at home.

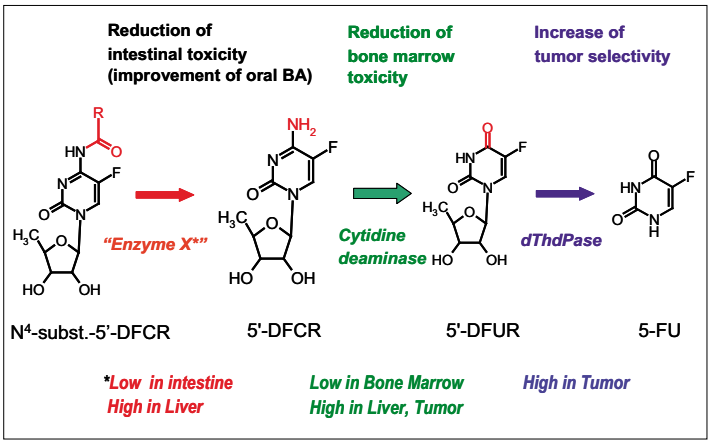


Fig. 1. Design of tumor-activated prodrug: N^4-substituted-5'-DFCR

5'-DFUR as a Lead Compound of Capecitabine

Our strategy was to develop a 5-FU prodrug that generates 5-FU selectively in tumors after sequential conversion by enzymes uniquely localized in normal and tumor tissues.

We selected furtulon® (5'-deoxy-5-fluorouridine; 5'-DFUR) as a first lead compound because of the following reasons. Furtulon was originally synthesized by A. Cock[1] in Roche USA, and developed in Japan for treatment of breast and gastrointestinal cancers.[2] H. Ishitsuka *et al.* discovered that furtulon itself is inactive, but works as a prodrug. Namely, furtulon is selectively converted into 5-FU in tumors, in which thymidine phosphorylase (dThdPase; TP) is over-expressed. Furtulon, however, generates 5-FU also in the intestine to some extent, since this enzyme also exists in the GI tract. Thus, high doses of furtulon cause dose limiting toxicity, diarrhea, and BM toxicity. Therefore, we tried to improve these drawbacks by a prodrug approach.[3–5]

5'-DFCR Derivatives

To minimize the BM toxicity and to increase the tumor selective activity of 5'-DFUR, we selected 5'-deoxy-5-fluorocytidine (5'-DFCR)[1] as the lead compound from many 5'-DFUR derivatives synthesized. 5'-DFCR is metabolized to 5'-DFUR by cytidine (Cyd) deaminase, the enzyme responsible for the metabolism of cytosine arabinoside (Ara C), a cytotoxic drug for the treatment of leukemia, into the inactive molecule uracil arabinoside. The enzyme is highly expressed in the liver, kidney,[6] and solid tumors[7] of humans, as well as in mature, normal granulocytes.[8] In immature, growing bone marrow cells, however, it is only minimally expressed. The unique localization of the enzyme explains the clinical efficacy of Ara C for leukemia and its dose-limiting adverse effect to bone marrow. We confirmed this unique tissue enzyme localization,[4] and low expression in the granulocyte progenitor cells of both human bone marrow and umbilical cord blood (unpublished). 5'-DFCR was thus selected as a potential lead compound based on the rationale that the specific tissue distribution of Cyd deaminase would result in the generation of higher concentrations of 5'-DFUR in liver and tumors but not in growing bone marrow cells (Fig. 1). Capecitabine, a derivative of 5'-DFCR, demonstrated minimal BM toxicity in clinical studies.[9]

N^4-acyl-5'-DFCR Derivatives[3]

Because certain levels of Cyd deaminase and TP activities have been found in the human intestinal tract,[4] it was hypothesized that 5-FU could be generated from 5'-DFCR to some extent within the intestinal mucosa and thus cause gastrointestinal toxicity (particularly diarrhea) when given orally. In addition, the oral absorbability of 5'-DFCR was poor in mice, possibly due to its high hydrophilicity.

In order to overcome these problems, the N^4-amino group of 5'-CFCR was protected by lipophilic acyl groups with the hope for a selective hydrolysis by an enzyme preferentially localized in the liver. In our first study, we synthesized a series of N^4-acyl-5'-DFCR derivatives, and directly screened them *in vivo*, for identification of a prodrug with wider therapeutic window than furtulon. The antitumor efficacy in mice tumor models was compared with intestinal toxicity in mice (occult blood test and histology). As a consequence, we identified N^4-(3,4,5-trimethoxybenzoyl)-5'-DFCR (galocitabine), as the first clinical candidate that met the selection criteria. However, in the

human phase 1 study, the bioconversion of galocatabine into furtulon was found to be very low. Thus, the development of galocitabine was terminated. The investigation of the *in vitro* bioconversion of galocitabine into 5'-DFCR using crude enzyme extracts from human and mouse liver and colon tissues revealed that there is a big species difference in the substrate specificity of the respective liver enzyme; namely the hydrolysis rate of the amide bond was very high in mouse liver, but very low in human liver (unpublished result; 280 vs 3.7 nmol/mg protein/h).

N^4-alkoxycarbonyl-5'-DFCR Derivatives[4,5]

To cope with this species difference in the prodrug studies, we initiated the second study with new screening strategies: i) use of the crude enzyme extracts from human and monkey liver and colon tissues for an *in vitro* bioconversion assay of the prodrugs; ii) PK and safety studies in monkeys, but not in mice. For this new screening, we synthesized a number of N^4-acyl as well as N^4-alkoxycarbonyl derivatives and tested their enzyme susceptibility towards the crude enzyme extracts from human liver and colon.[5] All N^4-acyl derivatives failed to show high selectivity for liver enzymes vs colon, whereas all the N^4-alkoxycarbonyl derivatives showed extremely high selectivity for the liver enzymes. There was a clear relationship between the alkyl chain length of the alkoxycarbonyl group and enzyme susceptibility liver vs colon in humans and monkeys. C-5 and C-6 alkyl chains showed highest susceptibility for human liver enzymes, whereas C-8 alkyl chains showed high susceptibility for monkey enzymes.

We isolated the enzyme responsible, and characterized it as an isozyme of the 60-kD carboxylesterase family (identified by sequence analysis of the N-terminal fragment). This enzyme preferentially exists in the liver and hepatoma but not in the intestine in humans.[4,5]

Among a series of N^4-alkoxycarbonyl-5'-DFCR derivatives synthesized, those that were chemically stable at acidic pH, susceptible to human hepatic carboxylesterase, effective in human cancer xenografts in mice, and orally available in monkeys were further investigated.[5] Capecitabine was moderately susceptible to carboxylesterase (20-fold more susceptible than galocitabine), yielded the largest AUC for 5'-DFUR in plasma (4.2-fold higher than that of galocitabine), and was efficiently metabolized (66%) to 5-FU and its further metabolites when given orally to monkeys.[5] It was highly effective in human cancer xenograft models and demonstrated much less intestinal toxicity and myelotoxicity than 5'-DFUR in monkeys.[4,5] Finally, we confirmed the improved pharmacokinetic profile of capecitabine in an exploratory human pharmacokinetic study before starting phase 1 studies in 1994 simultaneously in the US, EU, and Japan.

Tumor-selective Delivery of the Active 5-FU

Capecitabine selectively generates 5-FU in tumors as rationally intended. Therefore, it can be safely given at higher doses, which leads to higher 5-FU concentrations in tumors than is possible with either 5-FU or 5'-DFUR.[10] When 5-FU (*i.p.*) was given at the maximum tolerated dose (in long-term treatment) to mice bearing the HCT116 human colon cancer xenograft, it yielded generally uniform concentrations of 5-FU in plasma, muscle, and tumors (Fig. 2). In contrast, after oral administration of capecitabine at equitoxic doses, the amount of 5-FU in tumor tissue was considerably higher relative to the concentrations in plasma or muscle: the intratumor AUCs for 5-FU were 114 and 209 times greater than the plasma and muscle AUCs, respectively.[10] The administration of capecitabine (*p.o.*) also resulted in a 5.5- to 36-fold and 2.8- to 4.3-fold higher AUC for 5-FU within tumors compared to 5-FU (*i.p.*) and 5'-DFUR (*p.o.*), respectively, in four human cancer colon xenograft models studied (HCT116, COLO205, CXF280, and WiDr). The higher concentrations of 5-FU generated in tumors explain well why capecitabine was more effective than 5-FU in these and other tumor models despite the fact that the active principal, 5-FU, is the same. The tumor selective 5-FU delivery was later demonstrated in a pharmacodynamic study in colorectal cancer patients.[11]

Antitumor Activities

We demonstrated that capecitabine is more effective and has a wider spectrum of antitumor activity than 5-FU, 5'-DFUR, or UFT (a fixed combination of tegafur and uracil, 1:4) against 24 randomly selected human cancer xenograft models, which included colon, breast, gastric, cervical, bladder, ovarian, and prostate cancers.[4,12] In these experiments, capecitabine administered orally at MTD was effective (defined as >50% growth inhibition) in 18 of 24 models (75%) and inhibited tumor growth by more than 90% in seven models.[12] In contrast, 5'-DFUR was effective in 15 models (63%) and inhibited tumor growth by >90% in only one model. 5-FU and UFT were effective in one (4.1%) and five (21%) models, respectively. Neither of them inhibited the growth in any of the tumor models tested by more than 90%. Capecitabine thus showed activity against tumors that are resistant to 5-FU and UFT *in vivo*.

Safety

The four-week toxicity study of capecitabine in monkeys as compared with that of furtulon revealed that oral capecitabine showed clearly less intestinal and BM toxicities even at higher plasma AUC level of 5'-DFUR than oral furtulon.

Thus, preclinical proof of concept was achieved: namely when capecitabine is given orally, it passes thorough the intestine

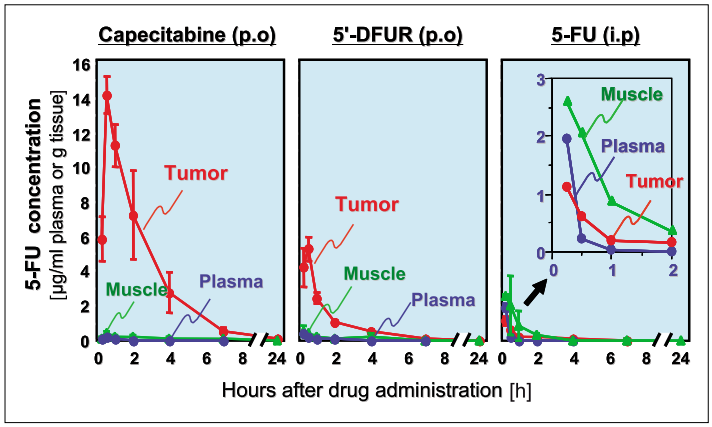


Fig. 2. Tumor selective delivery of 5-FU at MTD (HCT116 human colon cancer xenograft model)

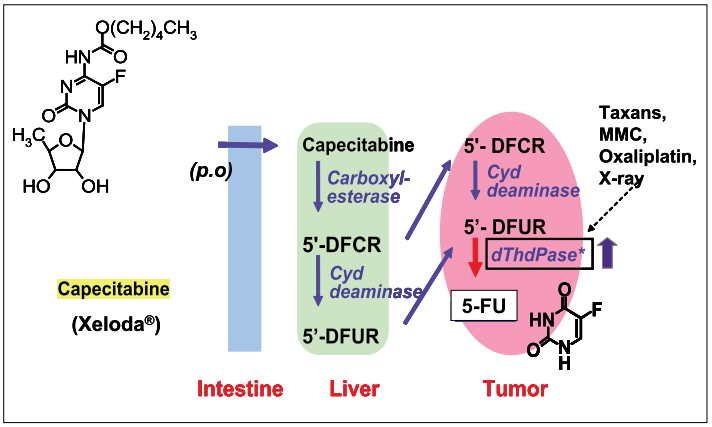


Fig. 3. Capecitabine, a tumor-activated prodrug of 5-FU

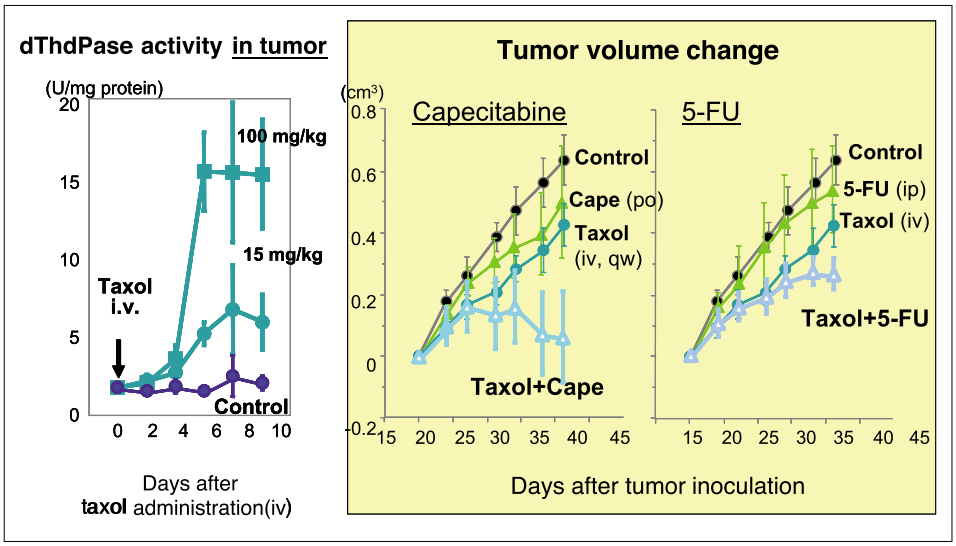


Fig. 4. dThdPase induction by taxol and enhancement of antitumor activity of capecitabine; human colorectal cancer xenograft, WiDr (refractory to capecitabine due to low TP/DPD ratio)

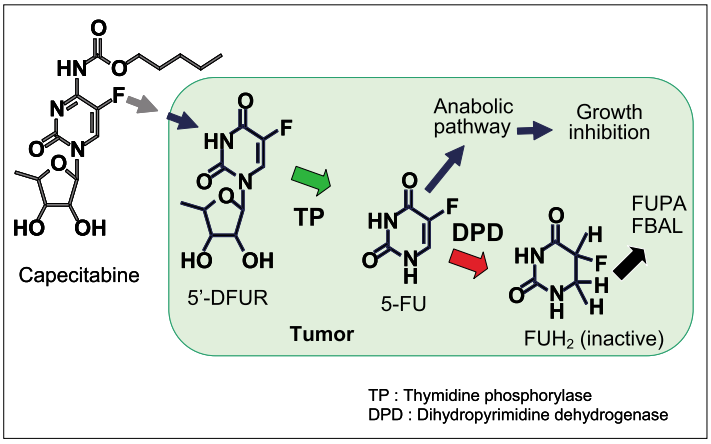


Fig. 5. Rationale for combination with DPD inhibitor: Efficacy of capecitabine correlates with TP/DPD ratio

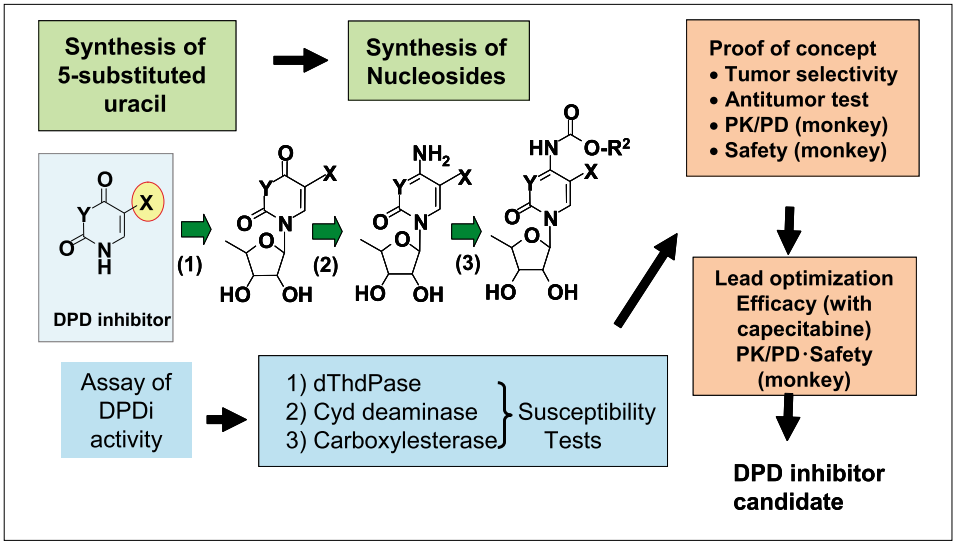


Fig. 6. Strategy for the discovery of tumor-activated prodrug of DPD inhibitor

as an intact molecule, and is then efficiently converted to 5'-DFCR by carboxyl esterase (in the liver) and to 5'-DFUR by Cyd deaminase (in the liver and in tumors), and finally selectively into 5-FU in tumors by TP that is overexpressed in many tumors (Fig. 3)

Combination Therapy with dThydPase (TP) Up-regulators

One approach to optimize capecitabine therapy is to up-regulate TP in tumor tissues. We found that TP can be upregulated by taxans, mytomicin C, oxaliplatin or X-ray radiation. One single *i.v.* injection of paclitaxel resulted in sustained induction of TP in a human colon cancer WiDr xenograft model, that is resistant to capecitabine.[13] When capecitabine (*p.o.; q.d.* × *14*) and paclitaxel (*i.v.; q.w.* × *2*) were combined, significant synergy was observed in tumor growth inhibition in the same tumor model. Such a synergy was not seen with 5-FU (Fig. 4). Based on these preclinical data, various rational combination studies have been conducted in clinical studies.[14,15]

In various clinical trials, oral capecitabine showed at least equal clinical efficacy as the 5-FU infusion regimen. Capecitabine showed a significantly lower incidence of BM toxicity and alopecia than 5-FU, though a higher incidence of the hand-foot syndrome (redness, swelling, and pain on the palms of the hands and/or the soles of the feet) was observed.

These results strongly suggest a possible replacement of 5-FU infusions by oral capecitabine, enabling home-based therapy and providing better quality of life.

Discovery of RO0094889, a Tumor-activated Prodrug of the DPD Inhibitor 5-Vinyluracil for Enhancement of Capecitabine Efficacy

Dihydropyrimidine dehydrogenase, DPD, is an enzyme that inactivates 5-FU by reduction of the 5,6-double bond. It is known that high levels of DPD activity exist in various types of human cancers.[16] The efficacy of capecitabine correlates with the TP/DPD ratio.[17] In humans, DPD activity also exists mainly in the liver.[16] Therefore, when combined with capecitabine, only tumor-selective and/or preferential DPD inhibitors should enhance capecitabine efficacy, whereas non-tumor-selective DPD inhibitors should enhance levels of 5-FU in the liver and consequently distribute it into the whole body, resulting in increased toxicity.

We therefore intended to develop a tumor-activated prodrug of a DPD inhibitor aiming for an enhancement of the efficacy of capecitabine without increase of toxicity[18,19] (Fig. 5, 6).

It is known that some 5-substituted uracil derivatives show potent DPD inhibitory activity.[20]

By use of a similar prodrug strategy to that used to develop capecitabine, we synthesized a series of uridine and cytidine derivatives and the respective N^4-alkoxylcarbonyl analogues, and tested their *in vitro* bioconversion by the same three key enzymes used for the activation of capecitabine. The further selection was done in a similar way to the capecitabine case. As a consequence, we identified a capecitabine-type prodrug N^4-pentyloxycarbonyl-5-vinyl-5'-deoxycytidine, RO0094246. However, this

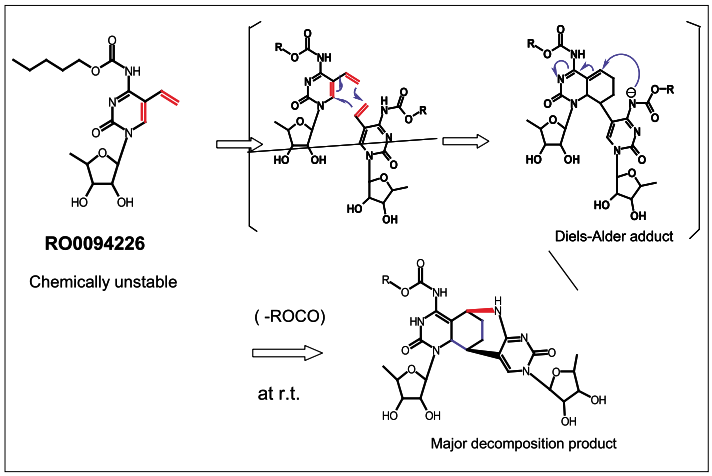


Fig. 7. Possible degradation mechanism of RO0094226

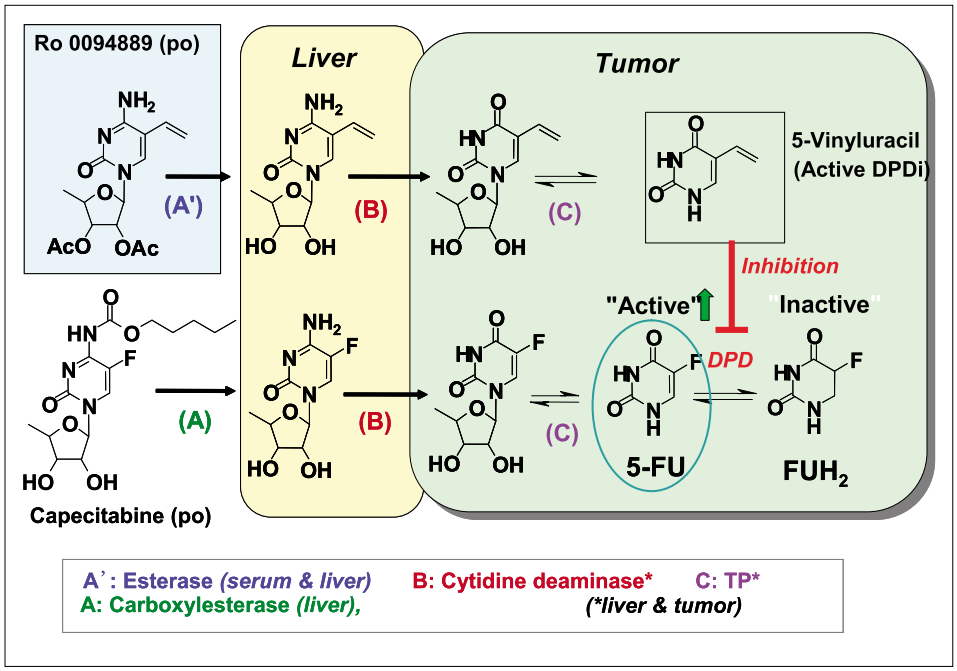


Fig. 8. Concept of double tumor targeting

compound was chemically unstable even at room temperature and gradually generated the dimeric product by 4+2 cyclo-addition, followed by intra-molecular cyclization as shown in Fig. 7.

Fortunately, it was found that a N^4-nonprotected derivative, 5-vinyl-5'-deoxycytidine (5'-DVCR) was chemically stable, though its oral bioavailability was very low due to its high hydrophilicity. The oral bioavailability was improved by acetylation of 2',3'-OH groups. The diester prodrug, RO0094889, was chemically stable under acidic pH, and showed favorable esterase selectivity liver over plasma and colon. Thus, it was selected as a clinical candidate. The prodrug itself is inactive, but its active form, 5-vinyluracil (5-VU) irreversibly inhibits DPD with IC_{50} of 89 nM. The sequential conversion of RO0094889 into the active 5-VU by three key enzymes was confirmed to be similar to that of capecitabine. Fig. 8 outlines the concept of double tumor targeting with an oral DPD inhibitor prodrug and capecitabine.

When this prodrug is administered orally together with capecitabine, synchronized conversions could occur because of their structural similarity and the use of essentially the same prodrug activation enzymes: first conversion by an esterase or carboxylesterase in the liver, second conversion by Cyd deaminase in the liver and in the tumor, and finally conversion to active drugs (5-VU and 5-FU) by TP selectively in tumors. As a consequence, the DPD inhibitor, 5-VU, inhibits degradation of 5-FU selectively in tumors, resulting in significant increase of the 5-FU concentration in tumor tissues. Thereby, the antitumor efficacy of capecitabine could be enhanced without increasing toxicity.

The tumor selective delivery of 5-VU was confirmed in a HT-3 human cervical cancer xenograft model. Fig. 9a shows tissue distribution of 5-VU after oral administration of the prodrug vs 5-VU administration. Except for the intestine (where high drug exposure occurs after *p.o.* administration), high tumor selective delivery of 5-VU was achieved by prodrug application, but not by administration of 5-VU. Especially, higher tumor/plasma ratio of 5-VU was achieved by use of the prodrug.

Furthermore, the ratio of 5-FU concentration in tumors vs plasma after co-administration of RO0094889 (*p.o.*) and capecitabine (*p.o.*) was significantly increased compared to that seen with capecitabine alone; 13 vs 5.8 respectively. On the other hand, combination of capecitabine with 5-VU itself abrogated the tumor selectivity of capecitabine (Fig. 9b). In HT-3 human cervical cancer xenografts (where DPD is over-expressed), RO0094889 significantly enhanced the efficacy of capecitabine in a dose-dependent manner (Fig. 10).

Thus, the concept of our double tumor-targeting strategy was confirmed. The combination with a DPD inhibitor not only enhances efficacy and spectrum of capecitabine, but also reduces the incidence of the hand-foot syndrome as a consequence of a dose reduction of capecitabine. The result of a human pilot PK study with RO0094889 as a single agent showed that the PK profile of RO0094889 was similar to that of capecitabine.

These data suggest that combination of capecitabine with this DPD inhibitor can further enhance the capecitabine efficacy and broaden its antitumor spectrum without increase of toxicity.

Search for Prodrug Activation Enzymes in Human Tumor Tissues by DNA Microarray, and its Application[21]

As an extension of our tumor targeting strategy, we investigated possible prodrug-activation enzymes by gene expression profiling of human tissues. We aimed for identification of enzymes that are over-expressed in tumors, but low in adjacent normal tissues as well as BM, liver and intestine (Fig. 11).

We used 2'-deoxy-2'-methylidenecytidine (DMDC) as a parent drug for this study. DMDC is a strong DNA polymerase inhibitor originally synthesized by A. Matsuda *et al.*[22] Oral DMDC showed extremely potent antitumor activity in various human cancer xenograft models. However, development of DMDC has failed due to severe BM toxicity in human. Thus, we tried to reduce the BM toxicity by using a tumor-activated prodrug approach.

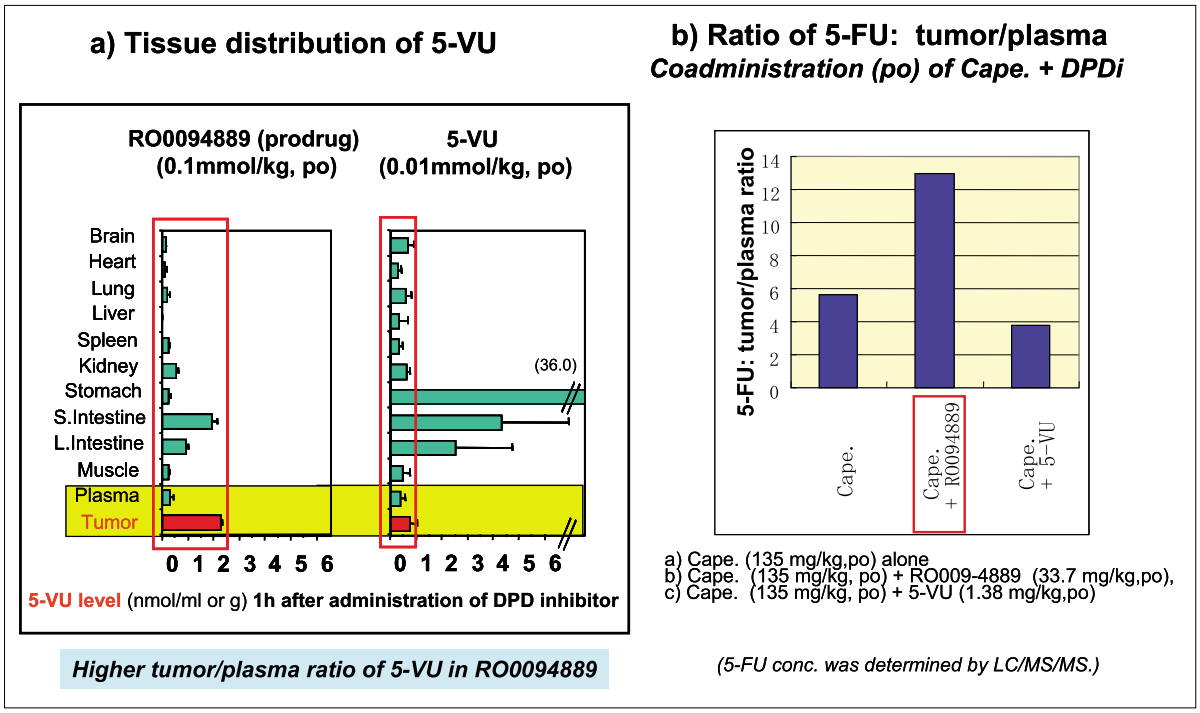


Fig. 9. Tumor selective delivery of 5-vinyluracil (5-VU) by oral administration of RO0094889 (HT-3 human cervical cancer xenograft)

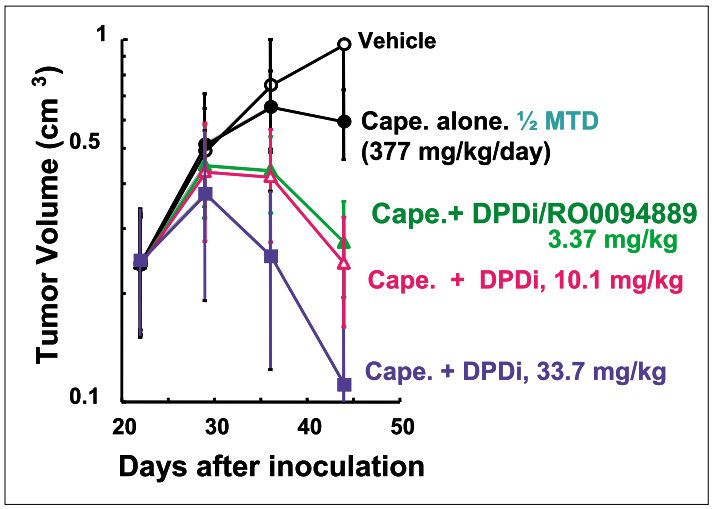


Fig. 10. RO0094889: Enhanced efficacy of capecitabine in HT-3 human cervical cancer xenograft in which DPD over-expressed

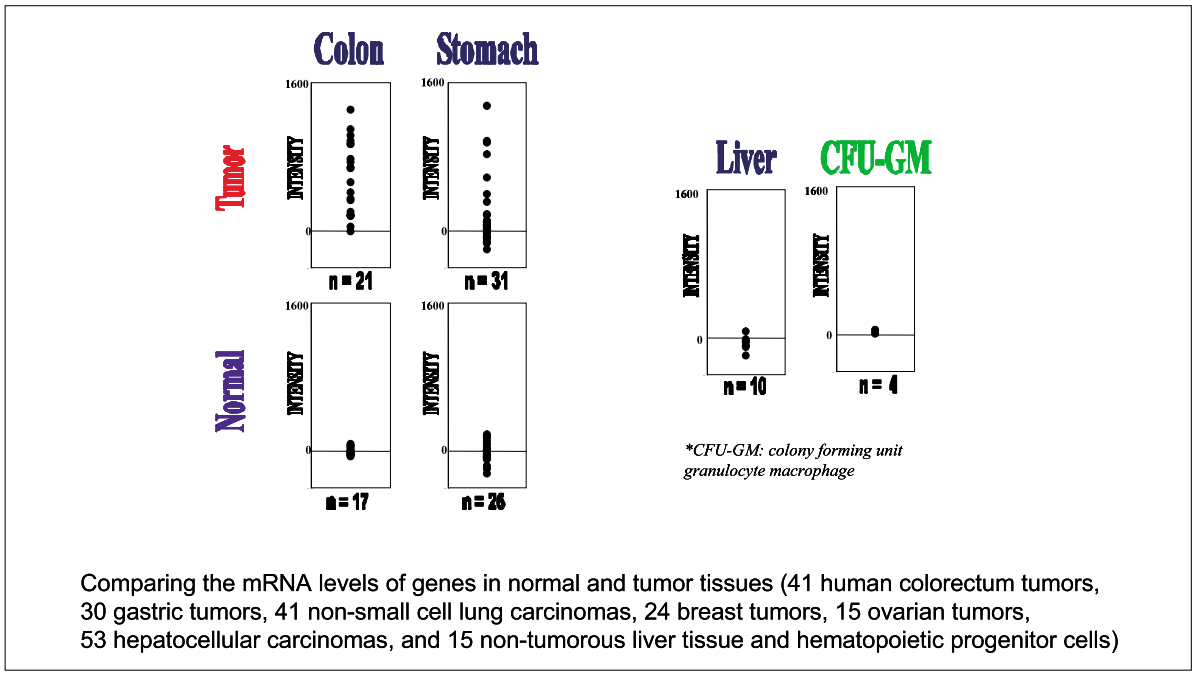


Fig. 11. Identification of enzyme (MDP) for human tumor-activated prodrugs by DNA micro-array (204 human tumor tissues, 15 normal liver and 1 bone marrow)

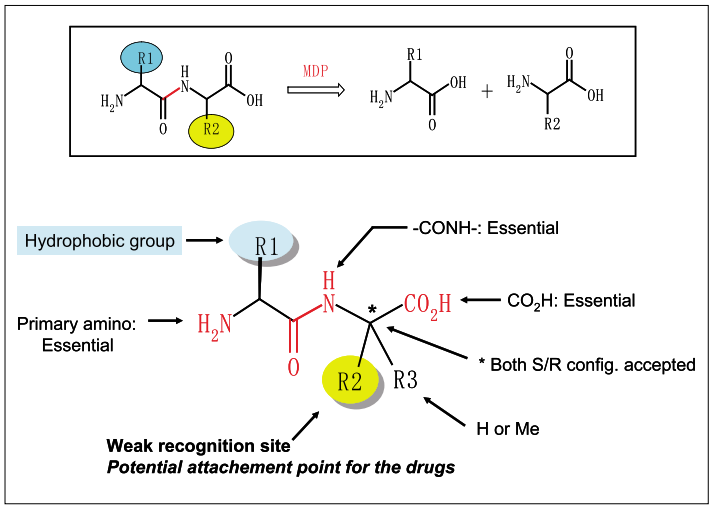


Fig. 12. Substrate specificity of MDP

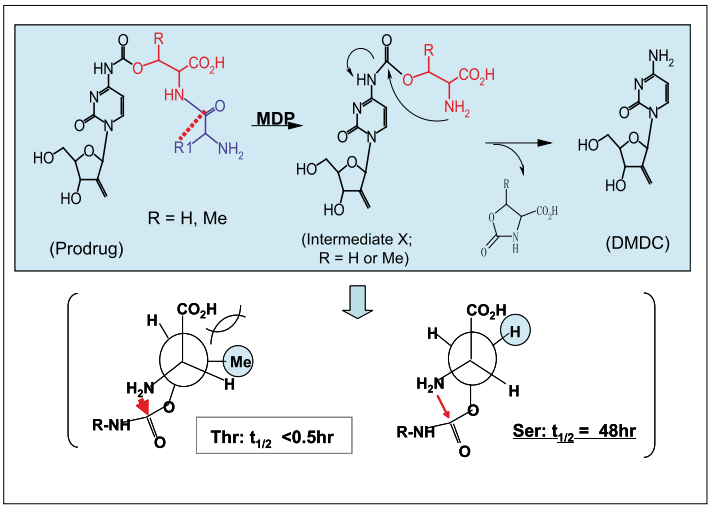


Fig. 13. Prodrug design: Activation by membrane dipeptidase (MDP)

DNA microarrays were used to identify enzymes to design tumor-activated prodrugs of DMDC by comparing the mRNA levels of genes in normal and tumor tissues (Fig. 11) (41 human colorectal tumors, 30 gastric tumors, 41 non-small cell lung carcinomas, 24 breast tumors, 15 ovarian tumors, 53 hepatocellular carcinomas, and 15 non-tumorous liver tissues and hematopoietic progenitor cells). DNA micro-array analysis of the human tissue samples provided several interesting enzymes with the desired expression profiles; *e.g.* membrane dipeptidase (MDP), DT-diaphorase, arylsulfatase.[23] MDP was found to be overexpressed in human colorectal and stomach tumor tissues but not in adjacent normal tissues, in the liver, or in hematopoietic progenitor cells such as colony-forming unit granulocyte-macrophage (CFU-GM). Fig. 11 shows the expression profile of MDP.

Therefore, we tried to design tumor-selective prodrugs of DMDC that can be activated by MDP. MDP is an enzyme that catalyzes the hydrolysis of dipeptide bonds. In order to know which part of the dipeptide molecule can be incorporated into the active drug, we first investigated the substrate specificity of MDP. The results are summarized in Fig. 12.

The amino, amide and carboxyl groups are essential for enzyme recognition. MDP prefers hydrophobic residues for R1. R2 was found to be the position for the introduction of the active drug since MDP does not recognize R2. Both configurations at C-terminal amino acid are accepted. Introduction of a methyl group at R3 is tolerable.

Based on these findings, we designed and synthesized DMDC prodrugs, in which DMDC links to the dipeptide moiety through the oxygen atom of a Ser or Thr moiety as shown in Fig. 13.

We expected that hydrolysis of the dipeptide bond by MDP followed by spontaneous intra-molecular cyclization would generate the active drug DMDC. In the model experiment for this cyclization using Ser or Thr intermediates, the cyclization speed was much faster in the Thr intermediate than the Ser analogue.[21] A possible reason is that the steric repulsion between methyl and CO_2H in the Thr intermediate could force the compounds into a conformation more favorable for the cyclization (Fig. 13).

As a consequence of a screening of Thr containing prodrugs, we selected a dipeptide derivative having (D)-Thr-(L)-cyclohexylalanine, RO0794111, for further studies.

The *in vitro* proof of concept (using a cell-free enzyme assay system) was achieved as shown in Fig. 14.

This prodrug was rapidly converted to DMDC in a HCT 116 human colon cancer cell line transfected with MDP cDNA, but not in the respective cell line with vector only, and not in BM cells. Thus, we could demonstrate the possibility to reduce BM toxicity of DMDC without losing antitumor activity by this prodrug approach. Further optimization studies are required to identify a clinical candidate with favorable pharmacokinetic profile suitable for oral administration.

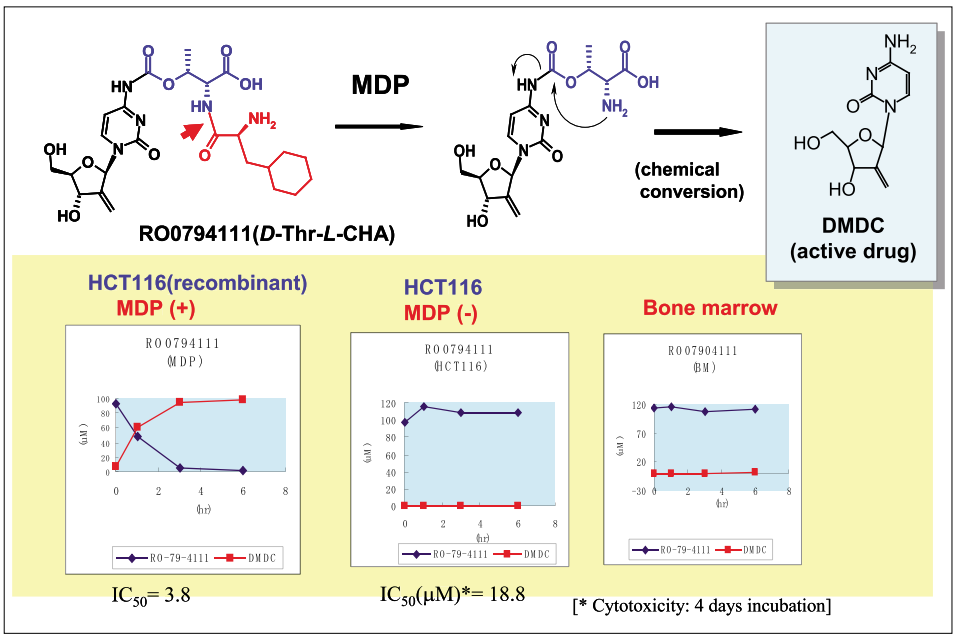


Fig. 14. Activation of RO0794111 by membrane dipeptidase (MDP)

Conclusion

The tumor-activated prodrug strategies based on the difference in expression levels of activation enzymes among different tissues can provide safer and more efficacious antitumor agents, similar to other molecular targeting concepts.

[1] A. F. Cook, M. J. Holman, M. J. Kramer, P. W. Trown, *J. Med. Chem.* **1979**, *22*, 1330.

[2] H. Ishitsuka, M. Miwa, K. Takemoto, K. Fukuoka, A. Itoga, H. B. Maruyama, *Jpn. J. Cancer Res.* **1980**, *71*, 112.
[3] Y. Ninomiya, M. Miwa, H. Eda, H. Sahara, K. Fujimoto, M. Ishida, I. Umeda, K. Yokose, H. Ishitsuka, *Jpn. J. Cancer Res.* **1990**, *81*, 188.
[4] M. Miwa, M. Ura, M. Nishida, N. Sawada, T. Ishikawa, K. Mori, N. Shimma, I. Umeda, H. Ishitsuka, *Eur. J. Cancer* **1998**, *34*, 1274.
[5] N. Shimma, I. Umeda, M. Arasaki, C. Murasaki, K. Masubuchi, Y. Kohchi, M. Miwa, M. Ura, N. Sawada, H. Tahara, I. Kuruma, I. Horii, H. Ishitsuka, *Bioorg. Med. Chem.* **2000**, *8*, 1697.
[6] G. W. Camiener, C. G. Smith, *Biochem. Pharm.* **1965**, *14*, 1405.
[7] G. Giusti, C. Mangoni, B. De Petrocellis, E. Scarano, *Enzym. Biol. Clin.* **1970**, *11*, 375.
[8] B. A. Chabner, D. G. Johns, C. N. Coleman, J. C. Drake, W. H. Evans, *J. Clin. Invest*. **1974**, *53*, 922.
[9] W. Scheithauer, J. McKendrick, S. Begbie, M. Borner, W. I. Burns, H. A. Burris, J. Cassidy, D. Jodrell, P. Koralewski, E. L. Levine, N. Marschner, J. Maroun, P. Garcia-Alfonso, J. Tujakowski, G. Van Hazel, A. Wong, J. Zaluski, C. Twelves, *Ann. Onc.* **2003**, *14*, 1735.
[10] T. Ishikawa, M. Utoh, N. Sawada, M. Nishida, Y. Fukase, F. Sekiguchi, H. Ishitsuka, *Biochem. Pharmacol.* **1998**, *55*, 1091.
[11] J. Schuller, J. Cassidy, E. Dumont, B. Roos, S. Durston, L. Banken, M. Utoh, K. Mori, E. Weidekamm, B. Reigner, *Cancer Chemother. Pharmacol.* **2000**, *45*, 291.
[12] T. Ishikawa, F. Sekiguchi, Y. Fukase, N. Sawada, H. Ishitsuka, *Cancer Res*. **1998**, *58*, 685.
[13] N. Sawada, T. Ishikawa, Y. Fukase, M. Nishida, T. Yoshikubo, H. Ishitsuka, *Clin. Cancer Res*. **1998**, *4*, 1013.
[14] J. O'Shaughnessy, D. Miles, S. Vukelja, V. Moiseyenko, J. P. Ayoub, G. Cervantes, P. Fumoleau, S. Jones, W.-Y. Lui, L. Mauriac, C. Twelves, G. V. Hazel, S. Verma, R. Leonard, *J. Clin. Oncol.* **2002**, *20*, 2812.
[15] R. Glynne-Jones, J. Dunst, D. Sebag-Montefiore, *Ann. Onc*. **2006**, *17*, 361.
[16] M. Mori, M. Hasegawa, M. Nishida, H. Toma, M. Fukuda, T. Kubota, N. Nagasue, H. Yamane, *Int. J. Oncol*. **2000**, *17*, 33.
[17] T. Ishikawa, F. Fukase, T. Yamamoto, F. Sekiguchi, H. Ishitsuka, *Biol. Pharm. Bull.* **1998**, *21*, 713.
[18] M. Endoh, M. Miwa, H. Eda, M. Ura, H. Tanimura, T. Ishikawa, T. Miyazaki-Nose, K. Hattori, N. Shimma, H. Yamada-Okabe, H. Ishitsuka, *Int. J. Cancer* **2003**, *106*, 799.
[19] K. Hattori, Y. Kohchi, N. Oikawa, H. Suda, M. Ura, T. Ishikawa, M. Miwa, M. Endoh, H. Eda, H. Tanimura, A. Kawashima, I. Horii, H. Ishitsuka, N. Shimma, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 867.
[20] a) F. N. M. Naguib, M. H. El Kouni, S. Cha, *Biochem. Pharmacol.* **1989**, *38*, 1471; b) D. P. Baccanani, S. T. Davis, V. C. Knick, T. Spector, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11064; c) T. Shirasaka, Y. Shimamato, H. Ohshimo, M. Yamaguchi, T. Kato, K. Yonekura, M. Fukushima, *Anti-Cancer Drugs* **1996**, *7*, 548.
[21] Y. Kohchi, K. Hattori, N. Oikawa, E. Mizuguchi, Y. Isshiki, K. Aso, K. Yoshinari, H. Shirai, M. Miwa, Y. Inagaki, M. Ura, K. Ogawa, H. Okabe, H. Ishitsuka, N. Shimma, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2241.
[22] a) K. Takenuki, A. Matsuda, T. Ueda, T. Sasaki, A. Fujii, K. Yamagami, *J. Med. Chem.* **1988**, *31*, 1063; b) T. Ueda, A. Matsuda, Y. Yoshimura, K. Takenuki, *Nucleosides Nucleotides* **1989**, *8*, 743; c) A. Matsuda, K. Takenuki, M. Tanaka, T. Sasaki, T. Ueda, *J. Med. Chem.* **1991**, *34*, 812; d) K. Yamagami, A. Fujii, M. Arita, T. Okumoto, S. Sakata, A. Matsuda, T. Ueda, T. Sasaki, *Cancer Res.* **1991**, *51*, 2319.
[23] H. Ishitsuka, H. Okabe, N. Shimma, T. Tsukuda, I. Umeda, PCT Int. Appl. WO2003043631A2, **2003**.