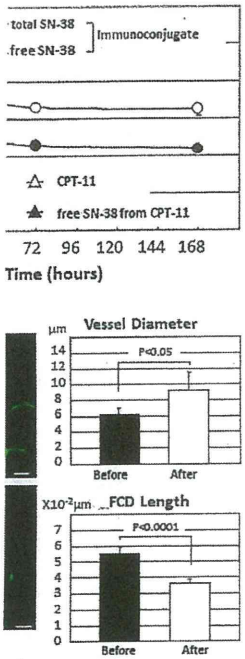

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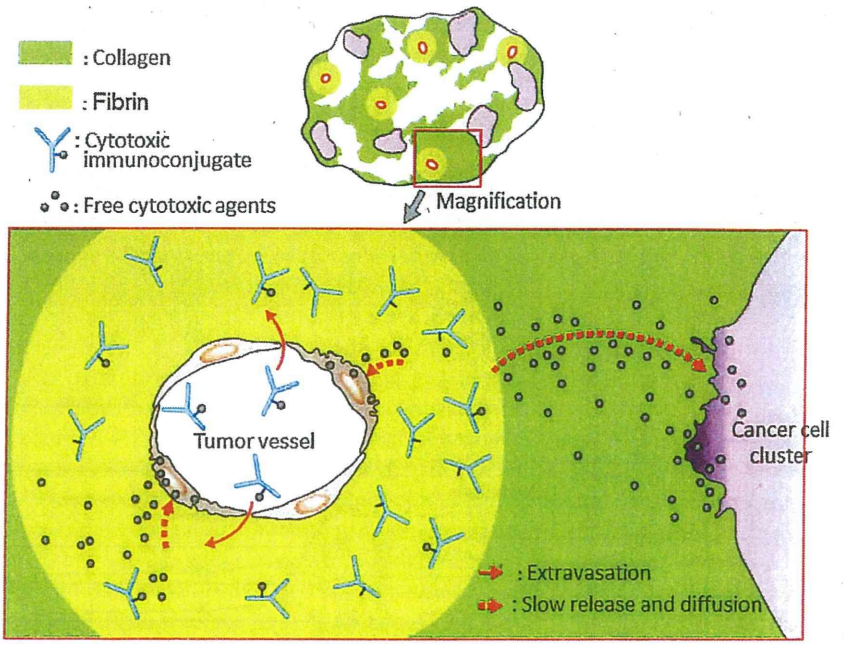

Fig. 6.10 Diagram of new concept of drug delivery using tumor stroma as a ligand. Newly developed anti-fibrin mAb conjugated with SN-38 extravasate selectively from leaky tumor vessels, bind specifically to the fibrin network around the tumor vessels to create a scaffold, and then allow the effective sustained release of SN-38, a time-dependent anti-cancer agent, from the scaffold. Since this released anti-cancer agent is LMW, it is subsequently distributed over the entire tumor-stroma barrier and induces damage not only to tumor cells but also to tumor vessels

Tailored ADC Depending on Quantity of Tumor Stroma

Difference of Tumor Stromal Component Between Malignant Lymphoma and Pancreatic Cancer

Anti-collagen 4 mAb was prepared to evaluate the stromal component. Human malignant lymphoma, RL tumor, consisted of CD20-positive tumor cells and collagen-4-positive blood vessels, which was stained fine linearly but not interspersed fibrously like the intercellular stroma. On the other hand, human pancreatic tumor, SUIT2 tumor reported as the histopathology relatively resembling original human pancreatic cancer [29, 46], consisted of EpCAM-positive cancer cells and collagen-4-positive extracellular component, the latter was composed of both CD31-positive blood vessel wall and high amount of CD31-negative stroma.

Preparation and Characterization of Cell-Targeting or Stroma-Targeting Immunoconjugate-PEG-SN-38 via a Carbamate Bond or Ester Bond

To specify the appropriate immunoconjugate therapy against malignant lymphoma or pancreatic cancer, we prepared two types of the conjugates, one being mAb-PEG-SN-38 via a carbamate bond [47] (Fig. 6.11a) and another being mAb-PEG-SN-38 via an ester bond [29, 30] (Fig. 6.11b). Consequently, six types of immunoconjugates, anti-CD20, anti-EpCAM, anti-collagen 4, or mAb-SN-38 via a carbamate bond or an ester bond, were obtained. The average number of conjugated SN-38 per one mAb (drugs/mAb), the range from 7 to 8.5, was shown in Fig. 6.11c. There was no clear loss of antigen-binding activity of each mAb after the conjugation (Fig. 6.11d). In in vitro release experiment, both bonds can be cut by a carboxylesterase localized in the cytoplasm to release SN-38 inside various cells (Fig. 6.11e). However, in physiological condition (non-enzymatically hydrolysis), the immunoconjugate prepared via an ester bond can release SN-38 gradually and effectively. In contrast, the immunoconjugate via a carbamate bond cannot release SN-38 effectively in the conditions outside the cells (Fig. 6.11e). We then evaluated the release profiles of SN-38 from both type of immunoconjugate in mouse blood, which contained high amounts of carboxylesterase [48]. In in vivo analysis of the mouse plasma, the concentration of unbound SN-38 or bound and unbound of SN-38 from the immunoconjugate via an ester bond or a carbamate bond at 72 h after the mice tail vein injection were shown. Most of the immunoconjugates in the mouse blood were protected from the enzymatic cleavage (Fig. 6.11f). Next, we examined the difference between carbamate bond and ester bond in the combination with cell-targeting or stromal-targeting antibody by the cytotoxicity assay. In RL cells, anti-CD20 immunoconjugate via carbamate bond showed strong cytotoxicity compared to anti-CD20 immunoconjugate via ester bond significantly (anti-CD20 mAb is known to possess high internalization ability). In SUIT2 cells, although no significant difference, anti-EpCAM immunoconjugate via carbamate bond had a lower tendency in the cytotoxic effect compared to anti-EpCAM immunoconjugate via ester bond (anti-EPCAM mAb is known to possess low internalization ability). Anti-collagen 4 immunoconjugate via ester bond showed higher cytotoxic activity than anti-collagen 4 immunoconjugate via carbamate bond in both cells significantly (Table 6.1). These results indicated that a carbamate bond was useful for the immunoconjugate linker to work inside of the cells and an ester bond to work outside the cells.

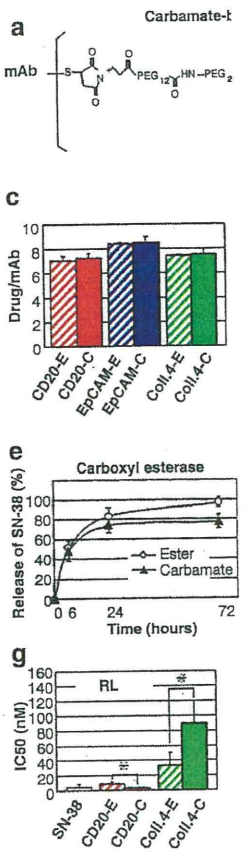

Fig. 6.11 Preparation and carbamate-bond and eser-b SN-38 via carbamate-bond molecules of SN-38. The a average number of conjug binding activity of the mAb mAb were examined by FA 4 mAb was examined by types of immunoconjugate (right) (n=3). Bar=SD, *P unbound form of SN-38 fr after the mice tail vein inje HPLC. Bar=SD. (g) In vitr (right) was shown (n=3). B

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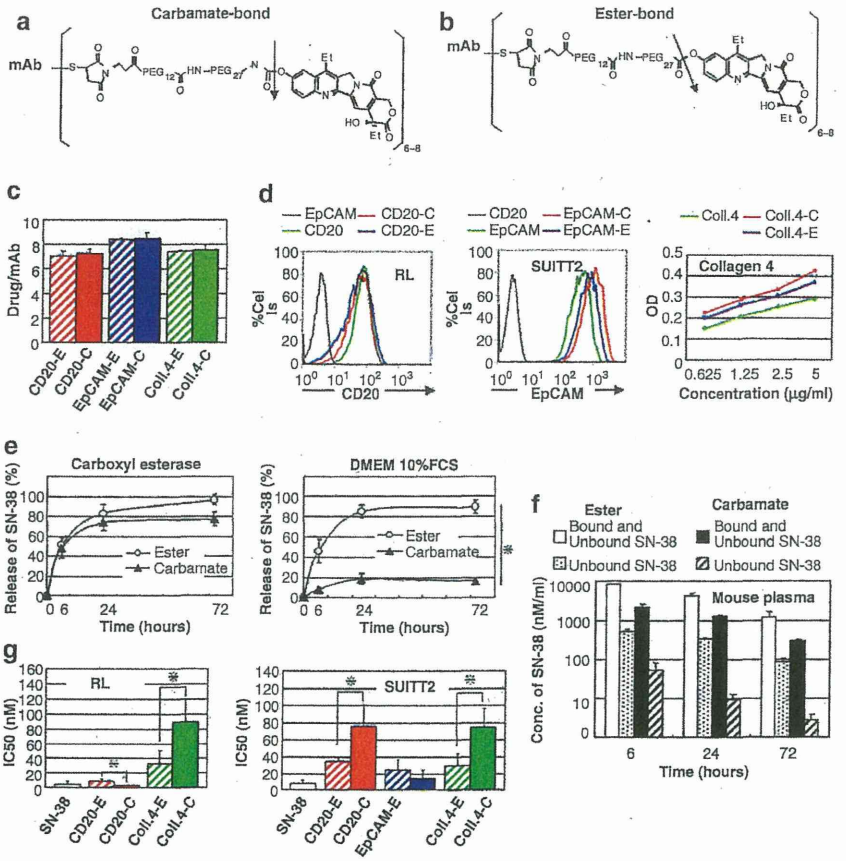

Fig. 6.11 Preparation and characterization of 2 types of immunoconjugqates-PEG-SN-38 via carbamate-bond and eser-bond. **(a) (b)** Drug design of 2 types of immunoconjugates; mAb-PEG-SN-38 via carbamate-bond **(a)** and mAb-PEG-SN-38 via ester-bond **(b)**. One antibody bears 6-8 molecules of SN-38. The arrow indicates the cleavage site for releasing free active SN-38. **(c)** The average number of conjugated SN-38 per one mAb was shown (n=3). Bar=SD. **(d)** Antigen-binding activity of the mAb before and after the conjugation was shown. Anti-CD 20 and EpCAM mAb were examined by FACS analysis using RL cells and SUIT2 cells respectively. Anti-collagen 4 mAb was examined by ELISA using purified protein. **(e)** In vitro release of SN-38 from two types of immunoconjugates in carboxylesterase-contained solution (left) and DMEM 10%FCS (right) (n=3). Bar=SD, *P<0.05. **(f)** Concentration of bound and unbound form of SN-38, and unbound form of SN-38 from two types of immunoconjugates in the moue plasma at 6, 24, 72 h after the mice tail vein injection, were shown (n=3). Concentrations of SN-38 were determined by HPLC. Bar=SD. **(g)** In vitro cytotoxicity with immunoconugates in RL cells (left) or SUIT2 cells (right) was shown (n=3). Bar=SD, *P<0.05

Table 6.1 IC50 of free SN-38 and SN-38 conjugated to mAb (immunoconjugate) for malignant lymphoma and pancreatic cancer cell line

		SN-38 conjugated to mAb					
Malignant lymphoma cell lines	Free SN-38	CD20				Collagen 4	
		Ester	Carbamate			Ester	Carbamate
RL	4.6±3.7	8.7±2.9 vs. 2.1±1.0*				34±17 vs. 90±30*	
		SN-38 conjugated to mAb					
Pancreatic cancer cell lines	Free SN38	CD20		EpCAM		Collagen 4	
		Ester	Carbamate	Ester	Carbamate	Ester	Carbamate
SUIT2	7.8±3.6	35±5 vs. 77±25*		24±13 vs. 15±9		29±15 vs. 75±22*	

IC50 (50% cell survival) (nM), Mean±standard deviation (n=3), *P<0.05

Cell-Targeting or Stroma-Targeting Immunoconjugate-PEG-SN-38 via Carbamate Bond or Ester Bond Differs Drastically in Their Antitumor Effects Depending on Tumor Stromal Component in Mice

Three mAbs conjugated with SN-38 via carbamate bond or ester bond (administered once, at an equivalent SN-38 dose of 3 mg/kg) were evaluated in order to know their antitumor effects in RL (CD20-positive stroma-poor human malignant lymphoma), SUIT2 (EpCAM-positive stroma-rich human pancreatic tumor). In RL lymphomas, cell-targeting anti-CD20 mAb-SN-38 via carbamate bond showed superior antitumor activity compared to anti-CD20 mAb-SN-38 via ester bond after the treatment (Fig. 6.12a). Stroma-targeting anti-collagen 4 mAb-SN-38 via ester bond showed significant superior antitumor activity as compared to saline as control, but inferior to anti-CD20 mAb-SN-38 via carbamate bond (Fig. 6.12a). On the contrary to RL tumor, in SUIT2 tumor, the most potent antitumor activity was obtained by the stroma-targeting anti-collagen 4 mAb-SN-38 via ester bond (Fig. 6.12b). However, there was no significant difference of antitumor activity between anti-EpCAM mAb-SN-38 via carbamate bond and via ester bond, whereas the antitumor activity of anti-collagen 4 mAb-SN-38 via carbamate bond was inferior to that of anti-collagen 4 mAb-SN-38 via ester bond (Fig. 6.12b). These results clearly indicated that in stroma-poor solid tumors like malignant lymphoma, cytotoxic immunoconjugate should target to the tumor cell surface and ACA should be conjugated to mAb through carbamate bond which can be specifically cut by a carboxylesterase inside the tumor cell after the internalization. On the other hand, in stroma-rich tumors, the immunoconjugate should target to the stroma within tumor tissue and ACA should be attached to the mAb via ester bond which can be cut gradually outside the tumor cell following the accumulation of the cytotoxic immunoconjugate in

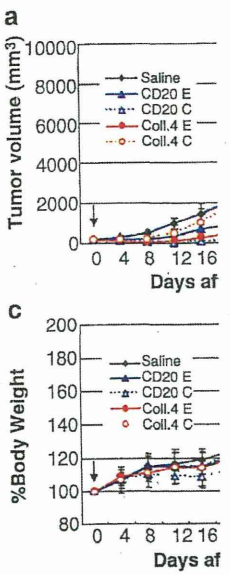

Fig. 6.12 Antitumor effe anti-stroma targeting, carl cent changes of body wei the 6 types of immunocon or anti-collagen 4 mAb=C administered once at an e intravenous bolus injectic curves illustrate the effec CD20-C vs. CD20-E, C Coll.4-E, Coll.4-E vs. Ep (Saline vs. Coll.4-E in R Bar=SD

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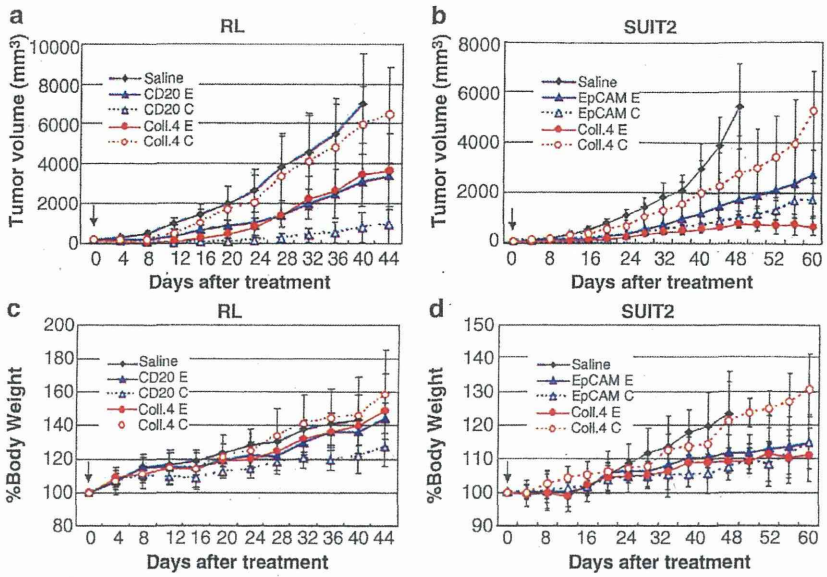

Fig. 6.12 Antitumor effects of immunoconjugates-PEG-SN-38 in the combinations of anti-cell or anti-stroma targeting, carbonate-bond or ester-bond. (**a**) (**b**) Anti-tumor activities and (**c**) (**d**) percent changes of body weight were examined. In animal models of RL (A)(C) and SUIT2 (B)(D), the 6 types of immunoconjugates (combined anti-CD20 mAb=CD20, anti-EpCAM mAb=EpCAM or anti-collagen 4 mAb=Coll.4 and ester-bond=E or carbamate-bond=C), or saline as control, were administered once at an equivalent SN-38 dose of 3 mg/kg to separate groups of mice (n=5) by intravenous bolus injection to the mice on day 0. Arrows indicate day of administration and the curves illustrate the effect of treatment on tumor size. $P < 0.0001$ (Saline vs. CD20-E or CD20-C, CD20-C vs. CD20-E, Coll.4-E or Coll.4-C in RL tumor; saline vs EpCAM-E, EpCAM-C or Coll.4-E, Coll.4-E vs. EpCAM-C or Coll.4-C, EpCAM-C vs Coll.4-C in SUIT2 tumor), $P < 0.001$ (Saline vs. Coll.4-E in RL tumor; saline vs. Coll.4-C, Coll.4-E vs. EpCAM-E in SUIT2 tumor). Bar=SD

the tumor stroma. It is remarkable that the feature of tumor stromal component influence the outcome of the two types of immunoconjugation drugs, cell-targeting mAb-PEG-SN-38 via carbamate bond, or stroma-targeting mAb-PEG-SN-38 via ester bond.

Regarding normal tissue distribution and elimination of antibodies and SN-38, there was no difference among immunoconjugates on day 7 after the administration. The dose in this study did not cause significant toxicity as shown by the change of mouse body weight (Fig. 6.12c, d). Moreover, there was no hepatotoxicity, nephrotoxicity, or bone marrow toxicity in mice treated with all three immunoconjugates as compared to controls (Fig. 6.12e). In addition, no autoimmune disease-like adverse effects such as arthritis and nephritis were observed in the administration of anti-collagen 4 mAb, whereas anti-collagen 2 mAb combined with lipopolysaccharide caused severe arthritis [49](Fig. 6.12f).

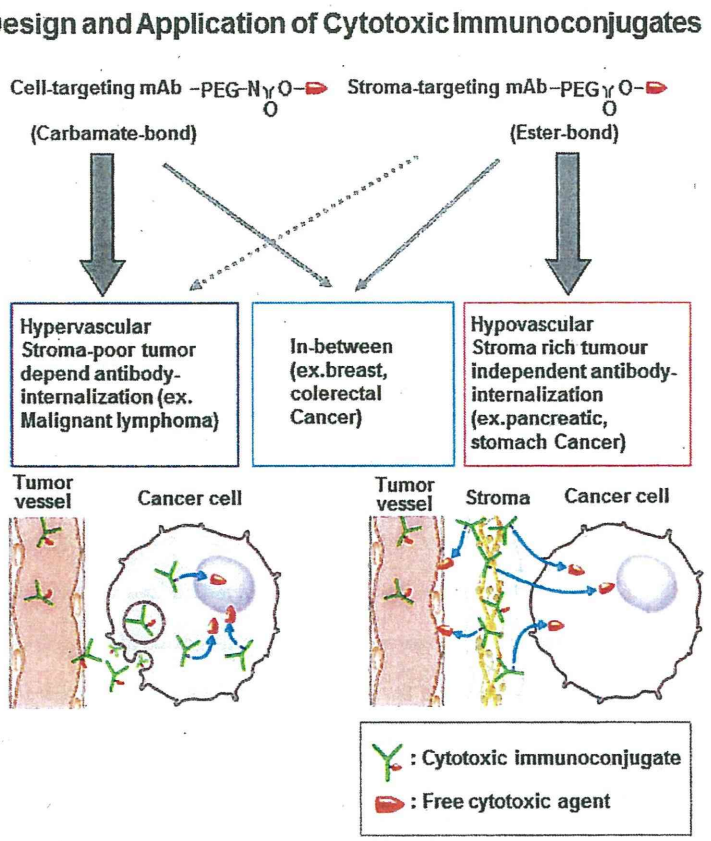

Fig. 6.13 Diagram of Immunoconjugate strategy to tumor tissue component and characteristic of cancer-cells. Design and application of cytotoxic immunoconjugates. SN-38 conjugated cell-targeting monoclonal antibody (mAb) via carbamate-bond is suitable for hypervascular, stroma-poor tumor dependent antibody-internalization. SN-38 conjugated stroma-targeting mAb via ester-bond is suitable for hypovascular, stroma-rich tumor independent antibody-internalization

In general, human cancer is classified into three types according to the tissue component. One is hypervascular stroma-poor tumor such as malignant lymphoma, the second is hypovascular stroma-rich tumor such as pancreatic cancer and stomach cancer, and the third is intermediated tumor between the two types such as breast cancer and colorectal cancer. We thus propose the new therapeutic strategy of immunoconjugates to the feature of individual tumor as tissue stromal component: (1) cell-targeting mAb conjugated with ACAs via carbamate bond for hypervascular and stroma-poor tumor and (2) stroma-targeting mAb conjugated with ACAs via ester bond for hypovascular and stroma-rich tumor, both cell-targeting immunoconjugate via carbamate bond and stroma targeting via ester bond for intermediated type of tumor [49] (Fig. 6.13).

Conclusion

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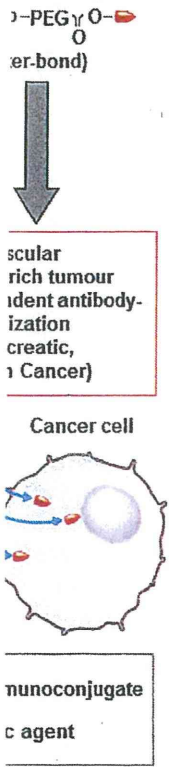

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Conclusion

Although there have been numerous reports of genetic and phenotype changes in tumors, a large body of pathological and clinical evidence indicates that there are no pivotal changes in tumor cells that distinguish them from normal dividing cells. Unlike in the case of using antibiotics against bacterial infection, therefore, ACAs need to be delivered selectively to tumor tissues and should be kept there long enough to reproduce the concentrations they reach in the Petri dish, which is a closed space where the cytocidal effects of any ACAs including molecular targeting agents are very strong. In the body, however, administered ACAs are cleared with the passage of time. Furthermore, as described in the main part of this topic, most human cancers possess abundant stroma that hinders the penetration of DDS including ACA-conjugated antibodies specific to surface antigens on cancer cells. We are now concerning that current studies mainly based on molecular and cellular biology while ignoring pathophysiology and pharmacology may be leading the development of antitumor drugs in the wrong direction. The present discovery by a hybrid of stromal biology with organic chemistry may open a new field of science and produce many and useful treatment modalities in the area of oncology, cardiovascular diseases, and inflammation.

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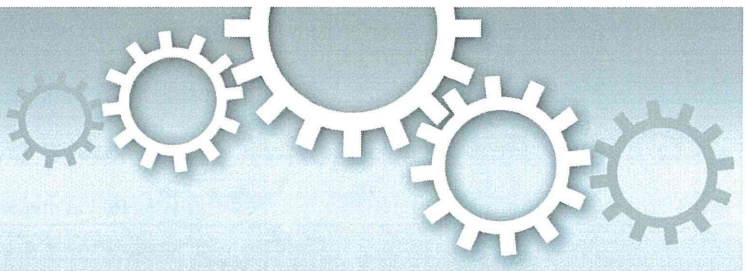

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The significance of microscopic mass spectrometry with high resolution in the visualisation of drug distribution

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The visualisation and quantitative analysis of the native drug distribution in a pre-clinical or clinical setting are desirable for evaluating drug effects and optimising drug design. Here, using matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) with enhanced resolution and sensitivity, we compared the distribution of a paclitaxel (PTX)-incorporating micelle (NK105) with that of PTX alone after injection into tumour-bearing mice. We demonstrated optically and quantitatively that NK105 delivered more PTX to the tumour, including the centre of the tumour, while delivering less PTX to normal neural tissue, compared with injection with PTX alone. NK105 treatment yielded a greater antitumour effect and less neural toxicity in mice than did PTX treatment. The use of high-resolution MALDI-IMS may be an innovative approach for pharmacological evaluation and drug design support.

Advances in our understanding of cancer at the cellular and molecular levels have promoted the development of new drugs[1,2]. Pharmacokinetic (PK) and pharmacodynamic (PD) studies are very important to evaluate the efficacy and toxicity of new drugs as well as to optimise drug design. For these purposes, tissue homogenate samples are generally analysed by high-performance liquid chromatography (HPLC) or liquid chromatography mass spectrometry (LC-MS)[3]. For the development of anticancer drugs (ACAs), including molecular targeting agents, precise chemical modulation is needed because the small differences between cancer cells and their host cells creates a narrow therapeutic window. In addition, clinical human cancer tissues generally exhibit abundant and versatile stroma, which is the result of the process of tumour cell invasion into tumour vessels, haemorrhage, fibrin clot formation, and replacement with collagen tissues and non-malignant stromal cells. Therefore, it is very important to consider the delivery of ACAs to cancer tissues and their distribution to target cancer cells within this heterogeneous tumour microenvironment. Furthermore, the drug distribution within normal tissues, particularly vital organs, should also be evaluated because ACAs frequently cause adverse effects[4]. A large body of clinical evidence has revealed that neoadjuvant chemotherapy is useful for a variety of solid tumours. The tissue excised during surgery or endoscopic biopsy can be used to investigate drug distribution[5,6]. Thus, a convenient method for evaluating the distribution of clinically used native (non-radiolabeled or non-chemically modified) drugs is urgently required.

Matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) has been developed for the investigation of the distribution of molecules such as small peptides, drugs, and their metabolites[7–12]. Moreover, MALDI-IMS can be used to evaluate numerous molecules in a single measurement without a specialised probe[7–12]. Therefore, this method enables the observation of a drug directly within tissue with the distinction between the original compound and its metabolites.

We have developed a mass microscopy method in which a microscope is coupled with a high-resolution atmospheric pressure-laser desorption/ionisation and quadruple ion trap time-of-flight (TOF) analyser. In this study, we investigated the ability of our mass microscopy technique to visualise the tissue distribution of unlabelled ACA and its micellar formulation and obtain precise regional information about the drug distribution in a specific anatomical area.