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Synthesis of a Novel Duocarmycin Derivative DU-257 and its Application to Immunoconjugate Using Poly(ethylene glycol)-dipeptidyl Linker Capable of Tumor Specific Activation

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Abstract—Novel anti-tumor agent, duocarmycin derivative DU-257, was designed and synthesized to prepare immunoconjugate in order to confirm the feasibility of enzymatically cleavable linker consisting of poly(ethylene glycol) (PEG) and dipeptide, L-alanyl-L-valine. Oxyethylamine arm was introduced at 4-methoxy position of segment B of DU-86 to form DU-257 and evaluated its property. DU-257 retained similar stability and potency with DU-86 while enhanced hydrophilicity suggested. DU-257 was condensed to the PEG-dipeptidyl linker through carboxyl terminal of dipeptide, and enzymatic release of DU-257 using a model enzyme, thermolysin, similar enzyme of which was shown to be overexpressed at various tumor sites, was evaluated by HPLC analysis. Cleavage between the linker amino acids by the model protease and release of DU-257 as valine conjugated form was confirmed. The enzymatically released form of DU-257 expressed its cytotoxicity without loss of the potency for $HeLaS_3$ and SW1116 tumor cell lines, although the efficacy was different in individual cells. DU-257 was then conjugated through the linker to KM231 monoclonal antibody specifically reactive to GD3 antigen which was shown to be expressed on the surface of many malignant tumors such as SW1116. The conjugate retained its binding specificity for SW1116 cell with a similar activity with KM231. Furthermore, the conjugate showed significant growth inhibition on SW1116 cell at a concentration of 75 μg/mL while no effect on antigen negative cell, $HeLaS_3$. These results suggest that the conjugate retained its anti-tumor effect only when it bound on and was activated at the target cell, simultaneously. DU-257 will be one of the candidate of anti-tumor agent for application to immunoconjugate and its conjugate with KM231 via PEG-dipeptidyl linker will be a useful entity for cancer therapy related to sLe^a expression.

Introduction

Duocarmycins are unique anti-tumor antibiotics isolated from the culture broth of *Streptmyces sp.*[1,2] and a series of duocarmycin derivatives has been synthesized.[3,4] One of their potent active metabolites is DU-86 which possesses two segments represented as segment A for DNA alkylating ability and segment B for noncovalent binding to DNA (Scheme 1).[5,6] Based on its potency and efficacy, DU-86 derivative has been applied for clinical investigations. However, in the most duocarmycin derivatives their marginal activity against human solid tumors and their insolubility in an aqueous solution dissuaded us from considering them for further evaluations. In cancer chemotherapy using anti-tumor agents including duocarmycins, side effects due to toxicity on normal tissues, rapid elimination from the body due to lower molecular size and poor distribution to target sites have been major problems to be overcome.

In order to enhance efficacy and reduce side effects of these anti-tumor agents, monoclonal antibody (mAb) has been demonstrated to be a promising carrier to deliver them to specific tumor tissues.[7–9] Anti-tumor agents,[10–12] toxin[13] and toxic protein[14]-conjugated mAbs have been extensively studied. Although such efforts have been made for many years, clear treatment effect and clinical application were not still gained. The reasons considered were toxicity of conjugated agents and immunoreaction against conjugate as well as poor specificity and affinity of mAb. Furthermore, even though conjugate is prepared to maintain its specificity and affinity, cytotoxic potency may be affected because covalently linked drugs are structurally altered by the coupling chemistry, and their original state may not be restored even after cleavage from the antibody. Therefore, one of the most important points for the preparation of desired

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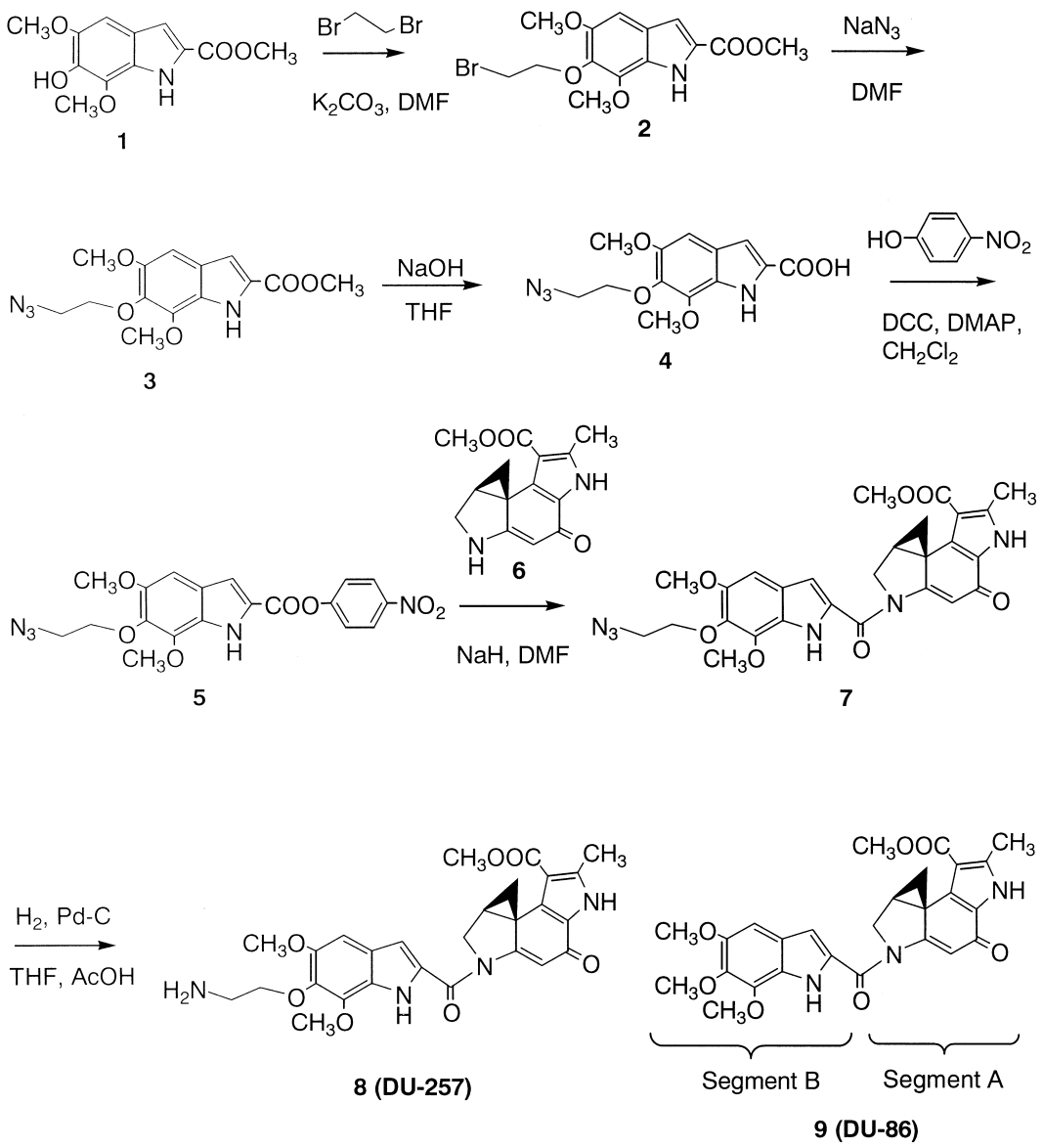


Scheme 1. Synthetic route to DU-257.

immunoconjugate seems to control the release of the agents at desired tumor sites.[15,16] On the basis of linker technology, such conjugate is being prepared in recent years. The linker which can be cleaved to release toxin when conjugate was internalized into acidic compartment in tumor cells,[11] and peptidyl linker which can be recognized by tumor specific proteases to release anti-tumor agent from polymer prodrugs[17] have been reported.

On the other hand, poly(ethylene glycol) (PEG), non-toxic, amphiphilic and non-immunogenic polymer, has been intensively studied as a modification agent of proteins for the last two decades.[18–20] Modification of proteins with PEG results in prolonged half-life in blood stream, reduced antigenicity and immunogenicity, increased protease resistance, and enhanced solubility in both aqueous and organic solutions. Furthermore, PEG has not only been used as such modification agent but also applied to a hydrophilic linker among bioactive substances such as hydrophobic antibiotics[21] and different proteins.[22]

Therefore, combination of PEG with peptide as a linker between anti-tumor agent and mAb may be desirable for the preparation of immunoconjugate. From this point of view, we have designed PEG linker combined with dipeptide and showed enzymatic release of segment B of duocarmycin as a model agent.[23] It has been suggested that tumor specific digestion of the linker and release of bioactive agents may be carried out only when the conjugate is delivered to the desired sites, while stable and non-toxic during circulation in the body. One of the purposes of this report is to provide a novel potent duocarmycin derivative carrying an amino residue, to which mAb can be conjugated via the linker, since preparation of immunoconjugate with anti-tumor agent has been restricted to particular compounds such as adriamycin[10–12,17] and mitomycin.[24] We describe here the synthesis of a novel duocarmycin derivative DU-257 and the preparation of its immunoconjugate with KM231 mAb[25] to evaluate their biological activities based on the concept of the PEG-dipeptidyl linker system. KM231 is a

mouse monoclonal antibody specifically reactive to sialyl lewis a (sLe[a]) carbohydrate antigen, which has been shown to be overexpressed on the cell surface of various tumor cells[26] including adenocarcinoma such as gastric cancer, pancreatic cancer and hepatocarcinoma, and therefore this mAb seems to be a suitable model as a tumor specific carrier of a novel duocarmycin derivative.

Results

Synthesis of DU-257

Synthetic procedure of DU-257 was illustrated in Scheme 1. Azido group was introduced into 4-hydroxyl position of segment B via oxyethylene spacer as designed before[23] and segment A of DU-86 was condensed to the resulting segment B, followed by hydrogenation reaction to form compound **8** with 6 steps.

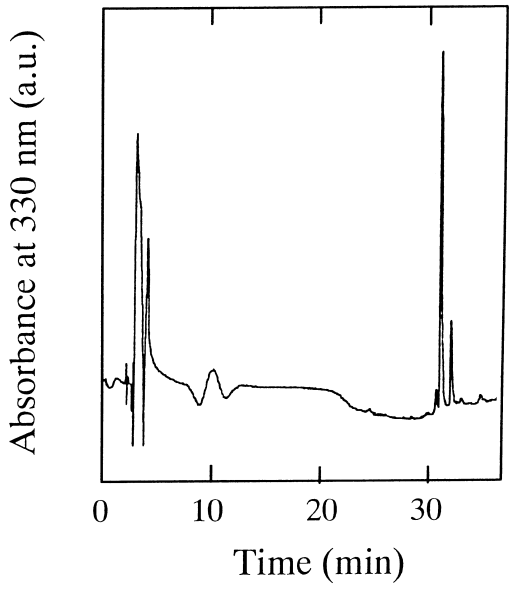


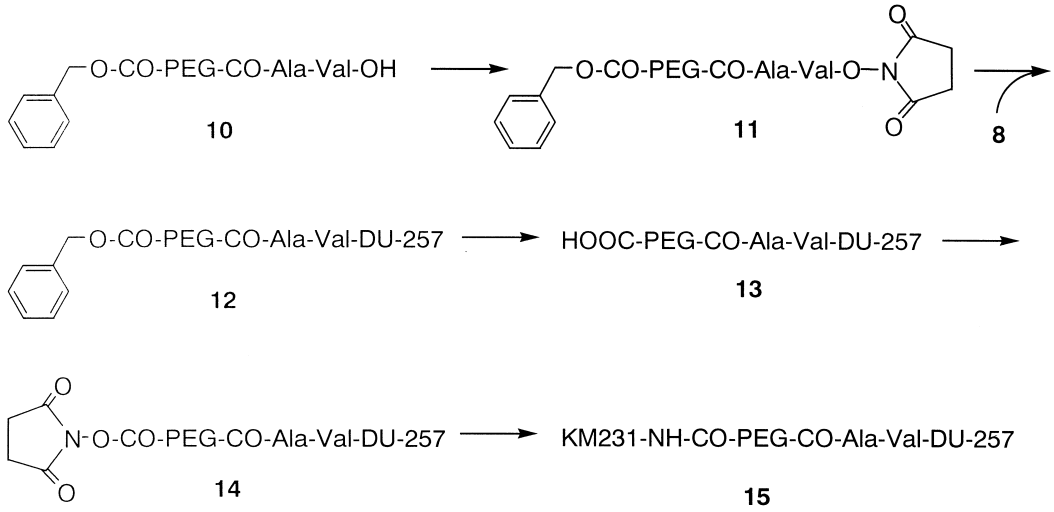
Figure 1. Typical elution profile of DU-257 determined by reversed-phase HPLC analysis. An aliquot of compound 8 diluted with a mobile phase (50mM phosphate buffer, pH 5.9) was injected to the reversed-phase column.

Yields of the compounds in each step were almost reasonable except for the final step, by which azido group of compound **7** was converted to amino group. Although compound **7** was unstable under the normal reaction conditions, compound **8** could be prepared as a yellowish powder with approximately 70–90% purity by controlling reaction conditions such as temperature, solvent and reaction time. The stable reaction conditions avoiding generation of side product and the effective purification conditions to gain quantitative and reproducible yield for compound **8** up to 90% purity are still under investigation. Typical HPLC profile of the resulting compound **8** was illustrated in Fig. 1, in which a slight amount of side product, the structure of which was determined as elsewhere, was observed. Retention time of the desired product was at 30.6 min. Condensation of DU-257 to the PEG-dipeptidyl linker followed by conjugation to mAb was carried out using DU-257 of ca. 70% purity without further purification.

Condensation of DU-257 to the linker and preparation of its mAb conjugate

Condensation of DU-257 to the PEG-dipeptidyl linker and following preparation of mAb conjugate were carried out by the procedure shown in Scheme 2. Compound **8** with approximately 70% purity was used for preparation of DU-257-linker moiety without further purification. Condensation reaction of DU-257 to compound **11**, *N*-hydroxysuccinimide (NHS) activated form prepared using *N*, *N*′-dicyclohexylcarbodiimide (DCC) from compound **10**, underwent quantitatively to yield compound **12**. Benzyl protecting group of PEG terminal was subjected to deprotection just before conjugation to mAb.

Conjugation of compound **13** to mAb was performed via NHS active ester as described before[23] and 30 equivalent of compound **14** relative to one mAb molecule



Scheme 2. Synthetic route to the conjugate.

was used for the reaction. There were no significant aggregation and polymerization fractions when purified by gel filtration chromatography, although the yield (45.1%) was slightly low. This may be probably because KM231 antibody used for conjugation reaction contained a slight amount of aggregate and the preparation scale was somewhat smaller than that performed before.[23] The resulting conjugate was prepared as a colorless clear solution. Change in binding affinity and precipitate formation were not observed for the conjugate during storage for more than one month at 4 °C. The substitution ratio of conjugated DU-257 per single mAb was determined to be approximately 2 using the data of Fig. 3(d) based on the method described before.[23]

Comparison of DU-257 with DU-86 in their physicochemical properties

Physicochemical property of DU-257 was evaluated using DU-257 which was further purified by silica gel chromatography up to 95% purity. Purified DU-257 and DU-86 were analyzed by reversed-phase HPLC as shown in Fig. 2(a). Purity of DU-257 was ca. 98%. Retention time of DU-257 shifted to 30.6 min relative to that of DU-86 (34.0 min), indicating that introduction of amino group into segment B increased hydrophilicity of the compound.

Stability of DU-257 in phosphate buffer (pH 7.0) was examined by HPLC. In order to prevent nonspecific adsorption and precipitate formation of DU-86, stability test was carried out in the presence of 20% acetonitrile.[27] As shown in Fig. 2(b), change in peak height was

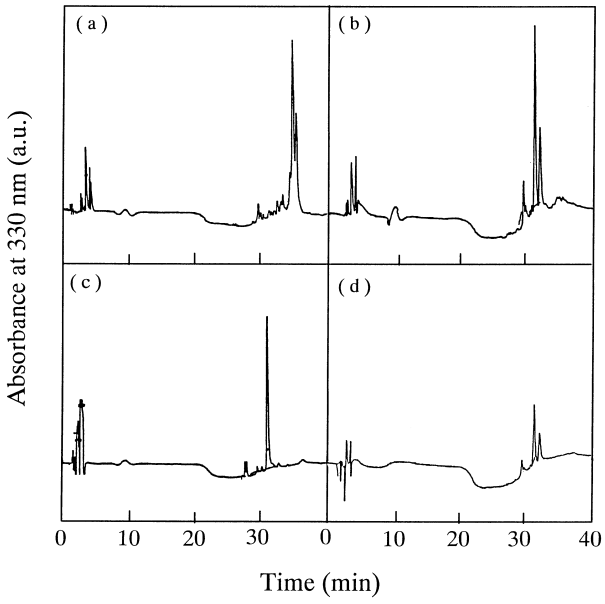


Figure 3. Release of DU-257 from PEG-dipeptidyl linker. A portion of the solution containing the compound **12** (0.1 mg/mL, 200 μL) was analyzed by reversed-phase HPLC before (a) and after (b) incubation with 100 μL of thermolysin for overnight at 37 °C. For complete digestion, the enzyme/substrate ratio of 1/10 was employed. The resulting main compound after thermolysin treatment was separated and re-analyzed by reversed-phase HPLC to confirm the purity and elution profile of valine-conjugated DU-257 moiety (0.20 μg) (c). Compound **15** (15 μg of the conjugate) was subjected to thermolysin treatment in the same conditions for overnight and the portion of the resulting mixture containing 12 μg of the conjugate was injected to the column (d). In contrast to the other figure, disodium hydrogenphosphate-citric acid buffer (50 mM, pH 4.8) was used as mobile phase.

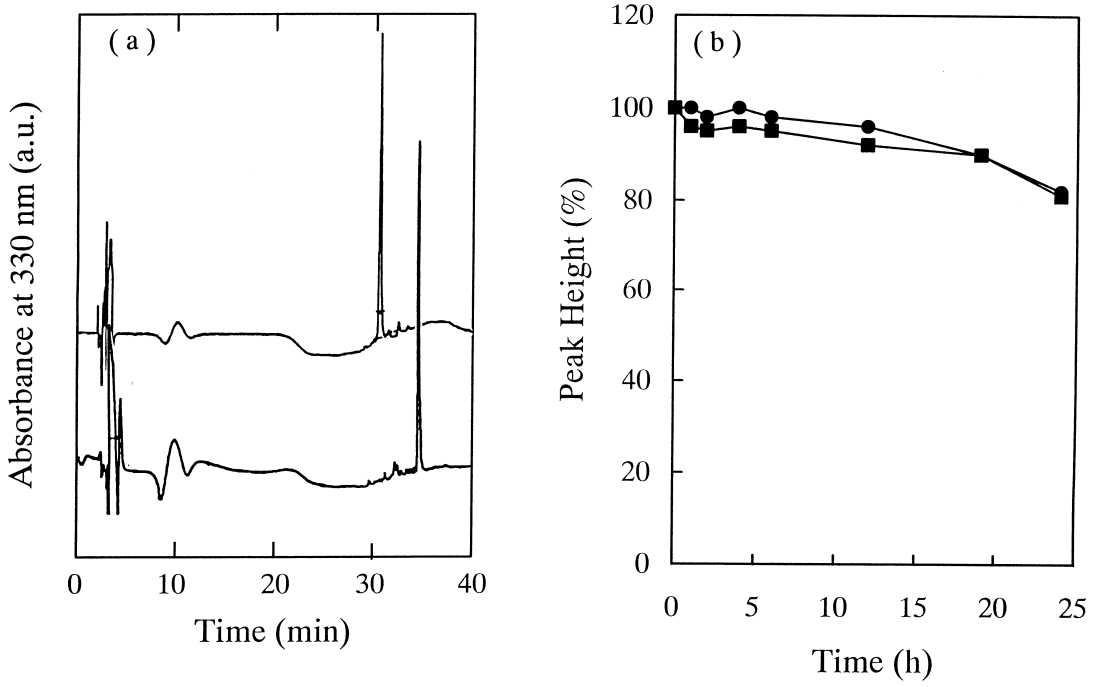


Figure 2. Comparison of DU-257 with DU-86 in terms of reversed-phase HPLC profile (a) and stability in aqueous solution (b). Both DU-257 (■) and DU-86 (●) were dissolved in PBS (pH 7.0) containing 20% acetonitrile and the solutions were incubated at 37 °C. Both solutions containing 0.125 μg of the individual compounds were injected to the column. Elution profiles of the compounds before incubation (a) and the change in relative peak height during 24 h incubation (b) were plotted.

almost the same with that observed for DU-86 throughout the experimental period. Approximately 80% of both compounds remained even after 24 h incubation. These results indicate that the stability of DU-257 was comparable to that of DU-86 when incubated in aqueous solutions.

Enzymatic digestion of PEG-dipetidyl linker

HPLC profile for compound **12** was shown in Fig. 3(a). As expected from the data shown in Fig. 1, compound **12** generated double peaks, because the presence of approximately 30% of impurity was suggested in compound **8** before condensation with the linker moiety. When the compound was treated with thermolysin overnight to complete the digesting reaction, both peaks almost disappeared and new peaks generated at retention times of 31.2 min and 32.2 min, respectively (Fig. 3 (b)). These newly generated peaks were isolated as shown in Fig. 3(c) and determined by mass analysis. Indicated molecular masses were $m/z = 620$ $(M+H)^+$ for the compound with former retention time and $m/z = 622$ $(M+H)^+$ for that of the latter one. From these results, the main peak generated by thermolysin treatment was identified to be DU-257-valine moiety, and the minor peak seemed to be hyperhydrated form of this compound. Results of amino acid composition analysis also suggested these peaks as valine-conjugated forms.

When the mAb conjugate was subjected to thermolysin treatment under the same conditions, similar HPLC profile with that shown in Fig. 3(b) was observed although signal intensity was not necessarily high (Fig. 3(d)). No detectable peaks were observed for the conjugate solution when the enzyme was not added to the reaction mixture (data not shown). These data indicate that the conjugate was also cleaved by the enzyme to release DU-257-valine moiety even if DU-257 was conjugated to mAb through the linker.

Cytotoxic activity of DU-257 and its released form from the linker

Cytotoxic activity of DU-257 and enzymatically digested compound purified by HPLC (H-Val-DU-257, Fig. 3(c)) was examined using $HeLaS_3$ cell. As shown in Fig. 4, DU-257 significantly inhibited growth of the cell with dose dependent fashion. Substitution of oxyethylamine arm at 4-methoxy position of DU-86 did not affect the cytotoxicity of the compound. IC_{50}s were summarized in Table 1. Interestingly, the released compound from the linker, L-valine-conjugated form with DU-257, also exhibited significant cytotoxicity. This means that, even if the linker was digested specifically between amino acids at tumor sites, the conjugate possibly kill tumor cells with almost the same efficacy as free DU-257. The side product generated during preparation of compound **8** and the isolated compound after the treatment with the enzyme, which is identified to be valine-conjugated side product, were quite low cytotoxicity for the cells investigated (data not shown). Consequently, the conjugate composed of the side product does not seem to be responsible for growth inhibition of the target cell. IC_{50}s

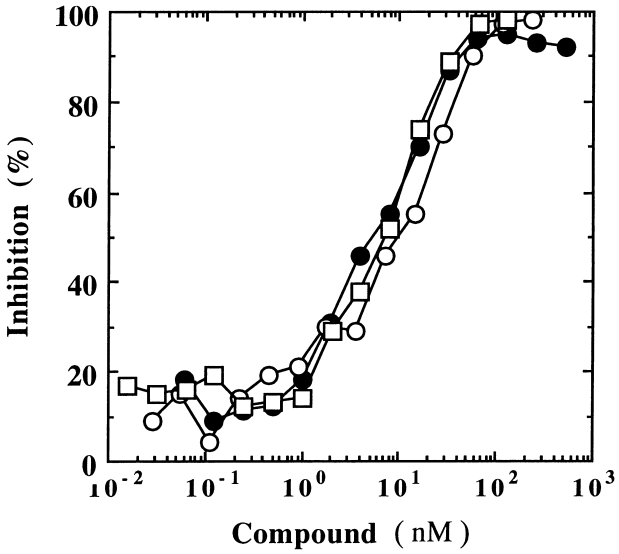


Figure 4. Concentration dependency of DU-257 and its related compound on growth inhibition of $HeLaS_3$ cell. DU-86 (●) and purified DU-257 (○) as well as DU-257 enzymatically generated as valine-conjugated form (□) were subjected to growth inhibition assay using HeLa S_3 cell described as in experimental.

Table 1. Cytotoxic effect of DU-257 and its related compounds

Compound	IC_{50}(nM)	
	$HeLaS_3$	SW1116
DU-86	5.2	300
DU-257	6.3	280
H-Val-DU-257	6.9	300

against SW1116 cell was also examined in a similar way to obtain the data shown in Table 1. Cytotoxic activities of DU-86 and DU-257 for SW 1116 cell were 40 to 60 times weak as compared to those for $HeLaS_3$ cell.

Affinity and cell specificity of the conjugate

We have designed the conjugation reactions so that the substitution ratio is to be approximately 2 based on the fact shown previously[23] that excessive modification with the linker resulted in significant loss of binding affinity of the conjugate. As expected, the conjugate retained similar binding affinity with KM231 mAb for SW1116 cell expressing GD3 antigen (Fig. 5(a) and (b)). Consequently, the loss of binding affinity was not caused under the conditions of our conjugate preparation. In contrast, both conjugate and mAb showed no binding affinity for $HeLaS_3$ cell (Fig. 5(d) and (e)). Thus, affinity and specificity of the mAb were completely maintained in the conjugate.

Cell growth inhibition of the conjugate

Growth inhibitiory effect of the conjugate on tumor cells were summarized in Fig. 6. The conjugate exhibited significant inhibitory effect on the growth of SW1116 human colorectal carcinoma cell and the growth of

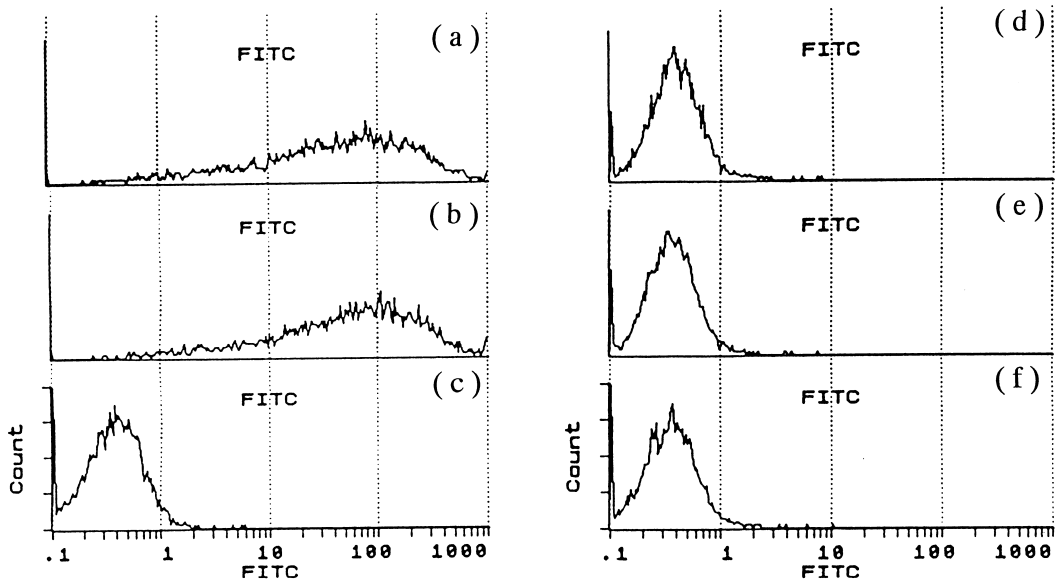


Figure 5. Binding of the conjugate to the SW1116 (a–c) and HeLa S_3 (d–f) cell lines. Conjugate (a, d) and KM231 mAb (b, e) were reacted to the individual cells at a concentration of 20 μg/mL and the resulting cells were incubated with FITC-labelled anti-mouse IgG antibody followed by FACS analysis. The cells in the absence of both conjugate and KM231 were also analyzed as a control (c, f).

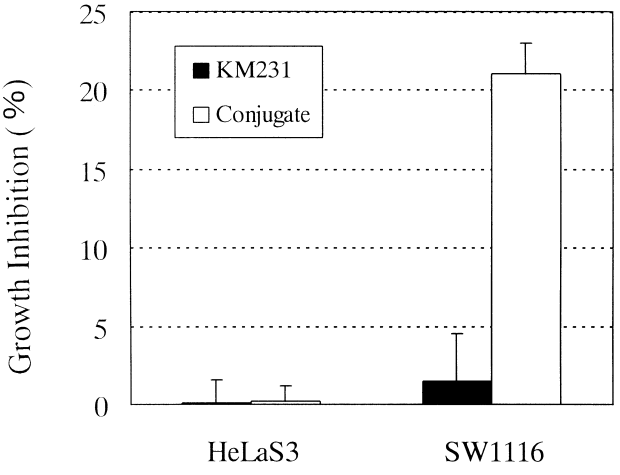


Figure 6. Cell growth inhibition effect of the conjugate on HeLa S_3 and SW1116 cell lines. The conjugate (□) and KM231 mAb alone (■) were reacted to the individual cells at a concentration of 75 μg/mL and the growth of these resulting cells was compared to that of untreated cells. Each point was measured in triplicate.

more than 20% of the cell were inhibited at 75 μg/mL of the conjugate, the concentration of which corresponds to approximately 1 μM of DU-257 calculated from the substitution ratio. However, no effect of the conjugate was observed for the growth of $HeLaS_3$ cell. When the mAb alone was added, an inhibitory effect was slightly observed only on the growth of SW1116 cells, but this effect seemed to be nonspecific. Enhancement of SW1116 cell specific growth inhibitory effect of the conjugate was thus confirmed in this experiment.

Discussion

We have proposed a novel immunoconjugate system using PEG-dipeptidyl linker for the conjugation of anti-tumor agent with tumor-specific antibody, the linker of which is cleaved by tumor expressing protease only after the conjugate is reached to the target site and then activated form of anti-tumor agent is released to kill tumor cell.[23] Previously, we used segment B of duocarmycin derivatives as a model compound of anti-tumor agent to show the preparation of the conjugate and enzyme-specific digestion of the linker.[23] In this report, in order to demonstrate the principle of the linker system and tumor-specific efficacy of the immunoconjugate, a novel duocarmycin derivative DU-257 was designed to possess an amino residue capable of its conjugation to the linker. The mechanism of duocarmycin derivatives on killing tumor cells has been recently investigated[5] and cancer chemotherapy using duocarmycins has been suggested to overcome multi-drug resistance,[28] which most hydrophobic anti-tumor agents encountered in tumor chemotherapy. This report is the first approach using a biologically active duocarmycin derivative for the development of immunoconjugate.

Various duocarmycin derivatives have been reported,[1–4] and their cytotoxicities depend largely upon the structure of the individual segments.[3,4] Considering the level of potency, stability and material supply, DU-86 was selected to design DU-257, and synthetic route of DU-257 was established with a reasonable yield as shown in Scheme 1. Introduction of the oxyethylamine arm into DU-86 to form DU-257 retained its cytotoxicity without loss of potency (Fig. 4, Table 1) and the stability in aqueous solution was maintained (Fig. 2). Additionally, DU-257 enhanced its hydrophilicity estimated from the profile of reversed-phase HPLC analysis, probably due to the introduction of amino residue within the molecule. One of the problems to be solved for the development of duocarmycin derivatives as preferable anti-tumor agents has been their hydrophobic properties. Increased hydrophilicity of DU-257 will be preferable like DU-86 analogue, KW-2189[6,29] and these properties of DU-257

seem to be enough for further evaluation as an anti-tumor agent.

DU-257 was then condensed to the linker moiety to form compound **12** and evaluated its release using thermolysin as a model enzyme to confirm whether the digested compound is biologically active or not. Only if the linker was cleaved around the tumor site by tumor-specific proteases to release biologically active form of DU-257, anti-tumor effect will be anticipated. The results suggest that DU-257 was released almost completely as a conjugated form with valine from the linker consisting of alanyl-valiyl sequence after the treatment of thermolysin. This finding was agreed with the data shown previously.[23] Digestion efficacy by thermolysin seemed to be very high in the case of alanyl-valyl linker. It should be noted that this digested compound had a similar cytotoxic activity with unconjugated DU-257 (Fig. 4, Table 1). Consequently, DU-257 moiety seems to be released as an active form whenever the target tumor cells express thermolysin-like enzyme. In fact, elevated expression of thermolysin-like enzymes has been reported in many tumors[30–32] and existence of digesting activity of the alanyl-valyl linker in cell homogenate from melanoma cell line was also reported.[23] It is well known that many tumor cells overexpress specific proteases such as cathepsins[33] and matrix metalloproteases.[34] Such enzymes may also have an ability to digest the linker peptide and must be available as candidate activators of the conjugate, although further analysis including cell specificity, digestion efficacy and cleavage site within the linker should be required.

In the preparation steps of immunoconjugate, denaturation and precipitation of mAb should be generally avoided.[35] Both in the preparation steps and during storage, loss of specificity and affinity of the conjugate (Fig. 5) as well as generation of precipitates were not observed. PEG-based linker seems to stabilize the conjugate. Hydrophobic property of anti-tumor agent sometimes results in such undesired phenomenon and has been required to be overcome for many years. Improvement of the solubility is important for the preparation of effective and stable immunoconjugate. Furthermore, PEG-linker seems to be responsible for exposing the dipeptide portion to the target enzyme since release of DU-257 was retained effectively even if the linker was conjugated to the antibody. DU-257 moiety was released when the conjugate was subjected to the reaction with thermolysin (Fig. 3(d)), and at least 2 molecules of DU-257 moiety could be released from single conjugate as an active form. These indications suggest some advantages of the PEG-dipeptidyl linker system for application to immunoconjugate.

Cytotoxic effect of the conjugate was evaluated using SW1116 cell from human colorectal adenocarcinoma origin, which is known to be invasive malignant cancer. Most of the known hydrophobic anti-tumor agents can not be available due to multi-drug resistance[36] and effective therapeutics still have not been established for the treatment of such cancers. One can easily recognize the malignancy of SW1116 cell, because the potency of DU-257-related compounds for SW1116 cell was 40 to 60 times lower than that for $HeLaS_3$ cell in our conditions, even though circumvention of multi-drug resistance has been suggested for duocarmycin derivatives.[28] As sLe^a antigen expressed on SW1116 cell was estimated to be approximately 1.9×10^6,[37] the cell seems to be one of the feasible targets for confirming the principle of the linker system and evaluating the ability of the conjugate. The conjugate almost completely abolished its cytotoxicity against $HeLaS_3$ cell (Fig. 6), indicating that DU-257 was bound stably to the conjugate and none of the effects were expressed to antigen negative cells; i.e. the linker was not digested at non-target cells and undesired side effects were supposed to be diminished. However, on the basis of the fact that inhibition of SW1116 cell growth was specifically enhanced at the concentration of 75 μg/mL, it is concluded that cytotoxic activity was expressed only when i) the conjugate bound specifically to SW1116 cell, and simultaneously, ii) the linker was specifically digested at the cell. Our results suggest that SW1116 cell possesses linker-digesting activity to release active form of DU-257 moiety. When the cell is treated with DU-257 or H-Val-DU-257 at a concentration of 1 μM as free drug, ca. 80% of cell growth inhibition should be anticipated (Table 1). Considering the potency of conjugated DU-257 shown in Fig. 6, at least 10–20% of the conjugate was estimated to be digested under the experimental conditions. The efficacy of the conjugate seems to depend greatly upon digestion efficacy of the linker peptide as well as sensitivity of target cell against anti-tumor agent. The amount of the conjugate prepared in this study was not enough for complete evaluation of the efficacy especially at higher concentrations. In order to enhance anti-tumor effect, further study should be required to increase the concentration of the conjugate and/or to prepare the conjugate in which substitution ratio of DU-257 in one mAb molecule is increased.[38] Additionally, selection of linker peptide must be essential for increasing the potency of the conjugate.

Conclusion

We demonstrated here the preparation of a novel anti-tumor agent DU-257 capable of conjugation to antibody via a PEG-dipeptidyl linker. DU-257 seemed to be one of the promising candidates for the preparation of immunoconjugate because similar potency and stability with DU-86, and increased hydrophilic property have been retained. The basic concept of our linker system has been confirmed by demonstrating enzymatic release of DU-257 moiety and cytotoxic activity of digested compound using DU-257 conjugated to PEG-dipeptidyl linker. Finally, we tried to apply the conjugate for the therapy of malignant colorectal cancer using SW1116 cell as a model in vitro and demonstrated the conjugate to be activated specifically at target cell, although some improvements including peptide sequence and the extent of substitution will be suggested in future study. In summary, DU-257 described here will be one of the candidates for novel anti-tumor agent and its conjugated form with KM231 antibody via PEG-dipeptidyl linker will also contribute to increase the treatment efficacy for cancer patient bearing sLe^a positive tumor.

Experimental

Materials

PEG-dicarboxylic acid (average molecular weight 600) and protected amino acid were purchased from Fluka Biochemicals and Kokusan Chemicals, Co., respectively. Thermolysin (EC 3.4.24.4, 9390 pU/mg) was the product of Daiwa Kasei K.K. (Osaka, Japan). DU-86[39] and segment A of duocarmycin (compound **6**)[3] were prepared as described previously. KM231 mouse IgG1 mAb was supplied in our laboratory.[25,26] Other compounds were of the highest pure grade commercially available.

Tumor cell line

$HeLaS_3$ (human cervix epitheloid cancer) and SW1116 (human colorectal carcinoma cell) were obtained as previously indicated.[3,25]

General procedures

TLC was run on glass-backed silica gel plates (Kieselgel 60 F254, Merck & Co., Inc.) using various solvents as indicated. Protein concentration was determined by absorbance at 280 nm (1 mg/mL = 1.4 absorbance units).[40] ^{1}H NMR spectra were recorded on JEOL FX100 or Bruker AM-500. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. Mass spectra were measured with a Hitachi B-80 or a Shimadzu QP-1000 spectrometer. Amino acid analysis was performed by acid degradation of the compound followed by HPLC analysis of phenylisothiocyanate derivatization of resulting amino acids.[37]

HPLC

HPLC was performed at room temperature on TRI-ROTAR SR HPLC system (Japan Spectroscopic Co.) equipped with a UNISIL PACK 5C18-150Å reversed-phase column (GL Sciences, Inc.). Absorbance was monitored at 330 nm. Solvent was 50 mM phosphate buffer (pH 5.9) with an acetonitrile linear gradient from 10% to 70% concentration at a flow rate of 0.7 mL/min. Alternatively, 50 mM disodium hydrogenphosphate-citric acid buffer (pH 4.8) was also used as a mobile phase.

Compound 2. In 5 mL of DMF was dissolved compound **1** (100 mg, 0.4 mmol) prepared as described previously,[23] and anhydrous potassium carbonate (275 mg, 2 mmol) was added. To the resulting solution was added dropwise 1,2-dibromoethane (173 μL, 2.0 mmol), followed by stirring under nitrogen atmosphere at room temperature for 19 h. After addition of a phosphate buffer (pH 7), the mixture was extracted with ethyl acetate. The organic layer was washed with brine, and then dried over anhydrous sodium sulfate. After the solvent was removed under reduced pressure, the residue was purified using 20 mL of silica gel using a hexane-ethyl acetate mixture (2:1). The solvent was evaporated off to give 94 mg of compound **2** (yield: 65%). SIMS *m/z*: 359 $(M+H)^+$; ^{1}H NMR (100 MHz, $CDCl_3$): δ 9.23 (brs, 1H, 1-NH), 7.10 (s, 1H, H-3), 6.82 (s, 1H, H-4), 4.38 (t, J = 5.2 Hz, 2H, OCH_2), 4.00 (s, 3H, 7-OCH_3), 3.91 (s, 3H, 5-OCH_3), 3.80 (s, 3H, 2-$COOCH_3$), 3.32 (t, J = 5.2 Hz, 2H, $BrCH_2$).

Compound 3. Compound **2** (94 mg, 0.26 mmol) was dissolved in 9.5 mL of DMF, and sodium azide (85 mg, 1.3 mmol) was then added, followed by stirring at room temperature for 25 h. Appropriate amounts of ethyl acetate and a phosphate buffer (pH 7) were added to the resulting solution to extract the ethyl acetate layer. After the organic layer was dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure to give 79 mg of compound **3** (yield 95%). SIMS *m/z*: 321 $(M+H)^+$; ^{1}H NMR (100 MHz, $CDCl_3$): δ 9.27 (brs, 1H, 1-NH), 7.10 (s, 1H, H-3), 6.80 (s, 1H, H-4), 4.24 (t, J = 5.2 Hz, 2H, OCH_2), 4.00 (s, 3H, 7-OCH_3), 3.91 (s, 3H, 5-OCH_3), 3.82 (s, 3H, $COOCH_3$), 3.43 (t, J = 5.2 Hz, 2H, N_3CH_2).

Compound 4. Compound **3** (79 mg, 0.25 mmol) was dissolved in a mixture of tetrahydrofurane (THF) (8 mL) and water (10 mL), and then 1N sodium hydroxide (2.5 mL) was added, followed by stirring at room temperature for 3.5 h. The reaction mixture was made acidic by addition of 1N HCl, and extracted using chloroform. The chloroform layer was washed with brine, dried over anhydrous sodium sulfate, and the solvent was removed to give 75 mg of compound **4** (yield 98%). SIMS *m/z*: 307 $(M+H)^+$; ^{1}H NMR (100 MHz, $CDCl_3$): δ 9.40 (brs, 1H, 1-NH), 7.20 (s, 1H, H-3), 6.81 (s, 1H, H-4), 4.25 (t, J = 5.2 Hz, 2H, OCH_2), 3.98 (s, 3H, 7-OCH_3), 3.90 (s, 3H, 5-OCH_3), 3.42 (t, J = 5.2 Hz, 2H, N_3CH_2).

Compound 5. Compound **4** (75 mg, 0.25 mmol) was dissolved in 7 mL of methylene chloride and DCC (103 mg, 0.5 mmol) was added, followed by stirring under ice cooling for 1 h. To the resulting solution were added *p*-nitrophenol (70 mg, 0.5 mmol) and dimethylaminopyridine (61 mg, 0.5 mmol), followed by stirring for 80 min. The insoluble matter was filtered off, and 0.5N HCl was added to the filtrate, followed by extraction with chloroform. The organic layer was washed with a saturated sodium hydrogencarbonate solution and brine, and then dried over anhydrous sodium sulfate. After the solvent was evaporated off, compound **5** was recrystallyzed from ethanol to give 72 mg of compound **5** (yield 69%). SIMS *m/z*: 428 $(M+H)^+$; ^{1}H NMR (100 MHz, $CDCl_3$): δ 9.08 (brs, 1H, 1-NH), 8.26 (d, J = 8.98 Hz, 2H, $C_6H_4NO_2$), 7.40 (d, J = 8.98 Hz, 2H, $C_6H_4NO_2$), 7.21 (s, 1H, H-3), 6.81 (s, 1H, H-4), 4.10 (t, J = 5.4 Hz, 2H, OCH_2), 3.96 (s, 3H, H-7), 3.81 (s, 3H, H-5), 3.46 (t, J = 5.4 Hz, 2H, N_3CH_2).

Compound 7. In a stream of argon, 50% sodium hydride (3.7 mg, 90 μmol) was dissolved in 1.5 mL of DMF, and a solution prepared by dissolving compound **6** (18.5 mg, 70 μmol) obtained according to the method described previously[3] in 2.9 mL of DMF was added at −20 °C, followed by stirring for 3 h. To the resulting solution was added a solution prepared by dissolving compound **5** (37 mg, 87 μmol) in 6 mL of DMF, and the temperature of the mixture was allowed to rise from −20 °C to room temperature, followed by stirring for 24 h. Ethyl acetate and a phosphate buffer (pH 7.0) were added to the

resulting mixture, the organic layer was washed with brine, and dried over anhydrous sodium sulfate. After the solvent was evaporated off, the residue was purified by 10 mL of silica gel using a chloroform:methanol (100:1) to give 28 mg of compound **7** (yield 57%). SIMS *m/z*: 548 $(M+H)^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.32 (brs, 1H, 1-NH), 9.37 (brs, 1H, 1′-NH), 7.10 (s, 1H, H-7), 7.06 (s, 1H, H-3′), 6.80 (s, 1H, H-4′), 4.42 (m, 2H, H-5), 4.10 (t, $J=5.4$ Hz, 2H, OCH_2), 4.02 (s, 3H, 7′-OCH_3), 3.88 (s, 3H, 5′-OCH_3), 3.82 (s, 3H, 3-$COOCH_3$), 3.67 (m, 1H, H-4a), 3.47 (t, $J=5.4$ Hz, 2H, N_3CH_2), 2.60 (s, 3H, 2-CH_3), 2.37 (dd, $J=7.5$, 3.4 Hz, 1H, H-4), 1.37 (t, $J=4.2$ Hz, 1H, H-4).

Compound 8 (DU-257). Compound **7** (20 mg, 36.6 μmol) was dissolved in 1.1 mL of a mixture of acetic acid (0.2 mL) and THF (9.8 mL), and 10% palladium carbon catalyst (7.3 mg) was added at 10–15 °C, followed by vigorous stirring in a stream of hydrogen for 80 min while maintaining the temperature at 10–15 °C. After the mixture was cooled below −20 °C and the catalyst was removed by filtration, the solvent was evaporated off to give 9 mg (17.3 μmol) of compound **8** (yield 47%). SIMS *m/z*: 521 $(M+H)^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.58 (brs, 1H, 1-NH), 9.40 (brs, 1H, 1′-NH), 7.12 (s, 1H, H-7), 6.95 (d, $J=2.3$ Hz, 1H, H-3′), 6.81 (s, 1H, H-4′), 4.45 (m, 2H, H-5), 4.08 (s, 3H, 7′-OCH_3), 3.92 (t, $J=5.2$ Hz, 2H, OCH_2), 3.90 (s, 3H, 5′-OCH_3), 3.82 (s, 3H, 3-$COOCH_3$), 3.67 (m, 1H, H-4a), 3.20 (q, $J=6.4$ Hz, 2H, CH_2), 2.63 (s, 3H, 2-CH_3), 2.38 (dd, $J=7.5$, 3.4 Hz, 1H, H-4), 1.37 (t, $J=4.2$ Hz, 1H, H-4).

BzlO-CO-PEG-CO-Ala-Val-OH (10). Benzylated PEG-dipeptidyl linker (compound **10**) was prepared as described elsewhere.[23] The procedure is stated briefly; *tert*-butyl ester of dipeptides, H-Ala-Val-O^{t}Bu, was condensed to mono-benzyl ester of PEG-diacid using NHS and DCC, followed by deprotection of *tert*-butyl protecting group to form compound **10**. TLC ($CHCl_3$/CH_3OH = 10:1) $Rf=0.2$, ^{1}H NMR (100 MHz, $CDCl_3$): δ 7.36 (5H, m, C_6H_5), 5.19 (2H, s, CH_2), 4.12 (4H, s, OCH_2), 3.64 (4nH, brs, $(OCH_2CH_2)_n$), 3.23(1H, s, CH(Ala)), 2.23 (1H, brq, $J=6.0$ Hz, CH(Val)), 1.26 (1H, s, CH(Val)), 1.17 (3H, d, $J=2.8$ Hz, CH_3(Ala)), 0.89 (6H, brd, $J=2.5$ Hz, CH_3(Val)).

BzlO-CO-PEG-CO-Ala-Val-DU-257 (12). Compound **10** (27 mg, 30 μmol) was dissolved in 2.4 mL of methylene chloride, and then NHS (4.2 mg) and DCC (7.5 mg) were successively added under ice cooling, followed by stirring for 2 h. The insoluble matter was filtered off, and the solvent was removed from the filtrate under reduced pressure. Then the residue was dissolved in 2 mL of pyridine, followed by addition of compound **8** (7.8 mg, 15 μmol) in 1.5 mL of pyridine at 0 °C. The resulting mixture was stirred for 3 h. After the solvent was removed under reduced pressure, the residue was purified using 5 mL of silica gel using 5 mL each of chloroform–methanol mixture (100:1, 80:1, 60:1, 40:1, 20:1, 10:1, 5:1) to give 18 mg (13 μmol) of desired compound **12** (yield 86.7%). TLC($CHCl_3$/CH_3OH = 10:1) $Rf=0.5$, ^{1}H NMR (500 MHz, $CDCl_3$): δ linker moiety: 7.36 (m, 5H, C_6H_5), 5.19 (s, 2H, CH_2), 4.12 (s, 4H, OCH_2), 3.64 (brs, 4nH, $(OCH_2CH_2)_n$), 3.23 (s, 1H, CH(Ala)), 2.23 (brq, $J=6.0$ Hz, 1H, CH(Val)), 1.26 (s, 1H, CH(Val)), 1.17 (d, $J=2.8$ Hz, 3H, CH_3(Ala)), 0.89 (q, $J=2.5$ Hz, 6H, CH_3(Val)), DU-257 moiety; 11.58 (brs, 1H, 1-NH), 9.40 (brs, 1H, 1′-NH), 7.12 (s, 1H, H-7), 6.95 (d, $J=2.3$ Hz, 1H, H-3′), 6.81 (s, 1H, H-4′), 4.45 (m, 2H, H-5), 4.08 (s, 3H, 7′-OCH_3), 3.90 (s, 3H, 5′-OCH_3), 3.88 (t, $J=5.2$ Hz, 2H, OCH_2), 3.82 (s, 3H, 3-$COOCH_3$), 3.67 (m, 1H, H-4a), 3.20 (q, $J=6.4$ Hz, 2H, CH_2), 2.63 (s, 3H, 2-CH_3), 2.38 (dd, $J=7.5$, 3.4 Hz, 1H, H-4), 1.37 (t, $J=4.2$ Hz, 1H, H-4).

Conjugation of linker-DU-257 moiety to antibody. Compound **12** (0.45 mg, 0.33 μmol) was dissolved in 0.4 mL of methanol, and 10% palladium carbon catalyst (1 mg) was added, followed by vigorous stirring in a hydrogen stream at −15 °C for 5 h. After removal of the catalyst by filtration, the solvent was removed from the filtrate under reduced pressure at a temperature below 0 °C to obtain 0.14 mg (0.11 μmol) of compound **13**. The resulting compound **13** (0.14 mg) was dissolved in 250 μL of NHS in methylene chloride (0.076 mg/mL), and 250 μL of DCC in methylene chloride (0.14 mg/mL) was added, followed by stirring for 2.5 h at 0 °C. The insoluble matter was filtered off, and the solvent was removed under reduced pressure to form compound **14**. The residue was dissolved in 36 μL of dimethylsulfoxide, and 204 μL of phosphate buffer was added. To the resulting mixture was added 0.56 mL of KM231 antibody (0.99 mg/mL) under ice cooling, followed by gentle stirring at 4 °C for 24 h. The reaction mixture was then purified by gel filtration chromatography using Superose 12 column (10×300 mm, Amersham-Pharmacia Biotech) equilibrated with phosphate buffered saline (PBS). Antibody fractions were concentrated to give 250 μL of compound **15** (0.5 mg/mL) (yield 45.1%).

Digestion of linker with model enzyme. Thermolysin (0.1 mL) was added to the compound **12** (0.1–0.2 mL, 0.2 mg/mL in PBS) to make a solution, in which final enzyme-substrate ratio was to be 1/10 so that the linker could be digested completely. The resulting solution was incubated at 37 °C and an aliquot of reaction mixture was analyzed by reversed-phase HPLC. In the case of the conjugate, an aliquot of the solution containing 15 μg of the conjugate was digested in a similar way and the portion of the reaction mixture corresponding to 12 μg of the conjugate was injected to the column.

Flow Cytometry. Binding activities of the conjugates and uncoupled antibody to target cells were measured as follows; Samples (10 μg/mL) were added to SW1116 (1×10^6 cells), and the mixture was subjected to reaction for 30 min under ice cooling. The cells were centrifuged and washed with PBS for three times, followed by removal of unreactive conjugate. To the resulting mixture was added 20 μL of fluorescein isothiocyanate-labelled anti-mouse IgG antibody (Wako Pure Chemical Industries, Ltd., 30 times dilution) and the resulting mixture was subjected to reaction for 30 min. After centrifugation and washing with PBS were repeated three times, measurement was carried out by fluorescence-activated cell sorter (FACS) analysis using EPICS Elite flow cytometer (Coulter Corporation, Hialeah, Fla.).

Cell growth inhibition assay. Inhibitory effect of duocarmycin derivatives and the conjugate on cell growth was examined by using $HeLaS_3$ and SW1116 cell lines. Each of the cell suspensions was put into a 96-well flat plate in an amount of 50 μL (1×10^3 cells/well), and cultured in a CO_2 incubator at 37 °C for 2 h. Then the cells in the plate were mixed with 50 μL of a solution containing each compound, followed by further incubation for 68 h. After washing the plate, 20 μL of ^{3}H-thymidine (463 kBq/mL) was added to each well and the cells were incubated for 4 h. Then the cells were harvested to determine the radioactivity of ^{3}H-thymidine incorporated into the cells using Matrix 96 (Packard Japan). The cell growth inhibiting activity was calculated according to the following equations;

$$Cell\ Growth\ Inhibiting\ Activity\ (\%) = [1 - (Radioactivity\ of\ Treated\ Cells)/(Radioactivity\ of\ Control\ Cells)] \times 100$$

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