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Design and synthesis of novel prodrugs of 2′-deoxy-2′-methylidenecytidine activated by membrane dipeptidase overexpressed in tumor tissues

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Abstract—DNA microarray analysis comparing human tumor tissues with normal tissues including hematopoietic progenitor cells resulted in identification of membrane dipeptidase as a prodrug activation enzyme. Novel prodrugs of 2′-deoxy-2′-methylidenecytidine (DMDC) including compound **23** that are activated by membrane dipeptidase (MDP) preferentially in tumor tissue were designed and synthesized to generate the active drug, DMDC, after hydrolysis of the dipeptide bond followed by spontaneous cyclization of the promoiety.

In recent years, several molecular targeting agents such as signal transduction inhibitors have been developed. These agents have better safety profiles than conventional cytotoxic agents, but their efficacy is limited to certain populations of tumors. The conventional cytotoxic agents, on the other hand, usually have a wide antitumor spectrum, but their narrow therapeutic window due to severe myelosuppression and intestinal toxicity often limits the clinical efficacy. Development of tumor-activated prodrugs of cytotoxic agents is an alternative option to provide safer and more efficacious antitumor agents. We previously developed an oral fluoropyrimidine, capecitabine (Fig. 1), which is sequentially converted to 5-fluorouracil selectively in tumors[1,2] by the enzymes overexpressed in the liver and tumor tissues but not in bone marrow. Capecitabine is now widely used for treatment of metastatic breast and colorectal cancers.

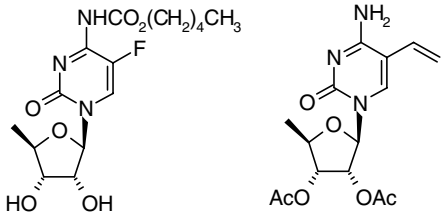

Figure 1. Tumor targeting prodrugs.

We also reported the tumor-activated prodrug of 5-vinyluracil **1**, a dihydropyrimidine dehydrogenase inhibitor, as a potentiator of capecitabine.[3]

To further expand this strategy, we investigated enzymes useful for prodrug activation that are preferentially overexpressed in human tumors. DNA microarray analysis of human tumor tissues versus normal tissues including hematopoietic progenitor cells yielded mem-

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brane dipeptidase (MDP) as a prodrug activation enzyme. 2′-Deoxy-2′-methylidenecytidine (DMDC) is a DNA polymerase inhibitor showing potent antitumor activity against a wide variety of tumors.[4] However, because of severe hematological toxicity in human, clinical development of DMDC has failed. Here, we report the design and synthesis of novel prodrugs of DMDC that are activated by MDP preferentially in tumor tissues.

Identification of membrane dipeptidase (MDP) as a prodrug activation enzyme. DNA microarrays were used to identify enzymes for designing tumor-activated prodrugs of DMDC by comparing the mRNA levels of genes in normal and tumor tissues (41 human colorectum tumors, 30 gastric tumors, 41 non-small cell lung carcinomas, 24 breast tumors, 15 ovarian tumors, 53 hepatocellular carcinomas, and 15 non-tumorous liver tissue and hematopoietic progenitor cells). We found MDP is overexpressed in human colorectal and stomach tumor tissues but not in adjacent normal tissues, liver, or hematopoietic progenitor cells such as colony-forming units granulocyte-macrophage (CFU-GM) (Fig. 2).[4]

Design and synthesis of tumor activated DMDC prodrugs. Membrane dipeptidase is an enzyme that hydrolyzes dipeptides. We designed prodrugs of DMDC **2** that are activated by MDP, preferentially in tumor tissues after hydrolysis of the dipeptide bond followed by spontaneous cyclization of the promoiety as shown in Figure 3.

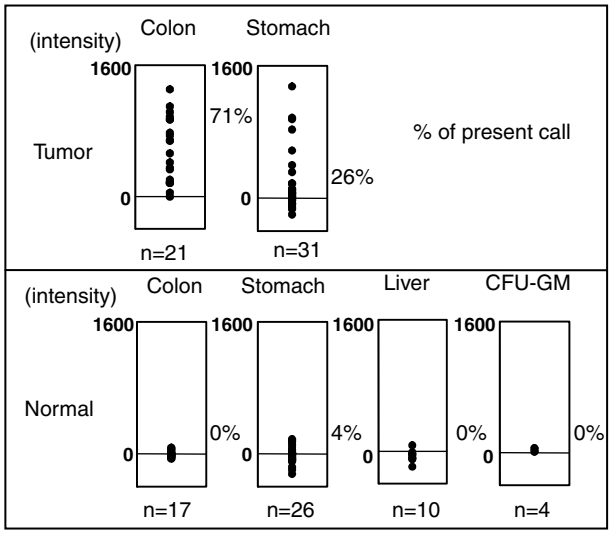


Figure 2. mRNA levels of genes of MDP in normal and tumor tissues.

In this design, the N^4-amino group of DMDC was linked to serine or threonine derivatives of a dipeptide. We first confirmed the concept of this spontaneous cyclization leading to the generation of DMDC by synthesizing two model compounds (**5** and **9)** as shown in Figure 4.

When the benzyl protecting groups were removed by catalytic hydrogenolysis, both **5** and **9** generated cytidine derivative **8** with simultaneous formation of cyclized compounds (**7** and **11**). Release of compound **8** was much faster in the threonine prodrug **5** ($T_{1/2}$ of <30 min) than the corresponding serine prodrug **9** ($T_{1/2}$ of 48 h). The structures of **7** and **11** were confirmed by LC–MS and $^{1}H/^{13}C$ NMR.

We then synthesized a series of dipeptide prodrugs and examined whether or not this large side chain in dipeptides including DMDC is recognized by MDP. Figure 5 shows the synthetic route of representative compound **23**. Compound **16** was prepared from BOC-(D)-Thr(OBn)-OH **12** and Fmoc-cyclohexylalanine **14** in 5 steps. Compound **23** was synthesized by coupling with activated *p*-nitrophenylcarbonate derivative **17** and 3′,5′-bis-O-TBS-DMDC **18**, followed by removal of the FMOC, TBS, and TMSE groups with TBAF.[5]

The results of the clarification of the substrate specificity of MDP are summarized in Figure 6.

Regarding a substituent at R^1, both threonine and serine are recognized by MDP, but isopropyl serine **27** is not a substrate. As a substituent at R^2, α-methyl-(L)-serine **34** is recognized by MDP. Replacement of the terminal carboxyl group with hydroxyl methyl **30**, an amide **28**, and an ester group **29** abolished the recognition by MDP. Therefore, we found the terminal carboxyl group is essential for the recognition by MDP.

Regarding the stereochemistry of the C-terminal amino acid, both L- and D-forms are recognized by MDP (**26** and **23**).

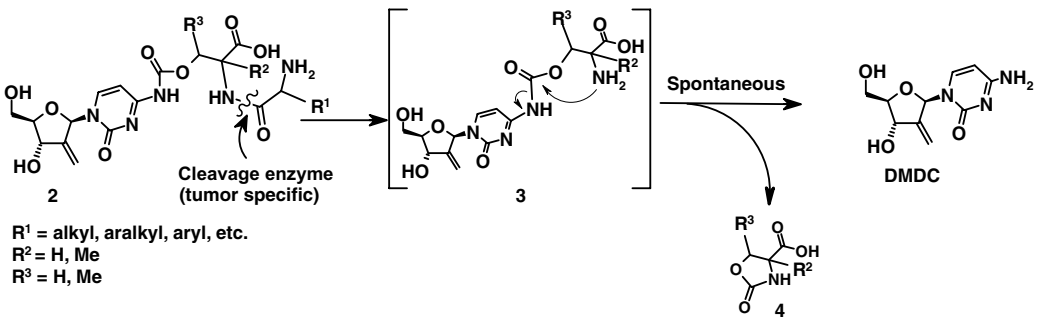


Figure 3. Design of tumor-activated DMDC prodrugs.

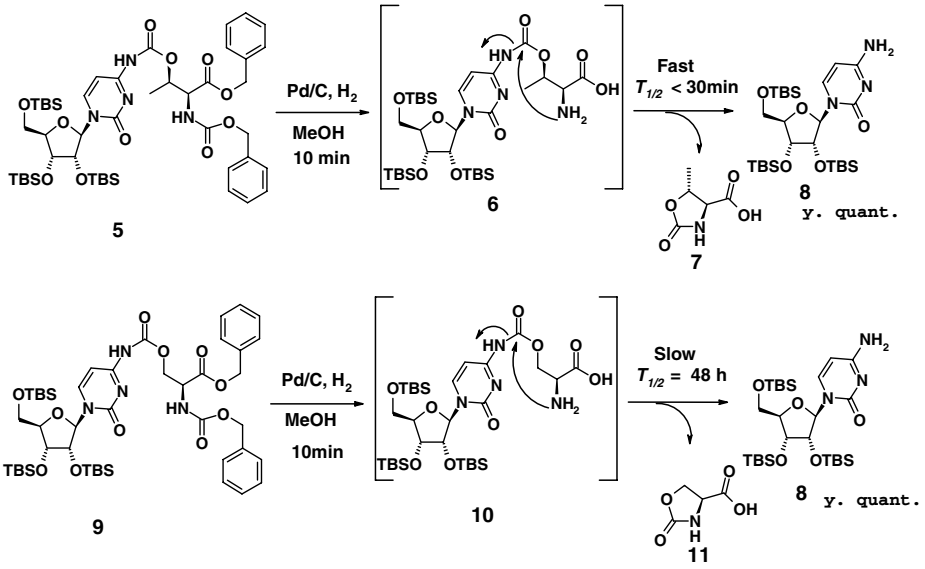


Figure 4. Proof of concept study using model compounds.

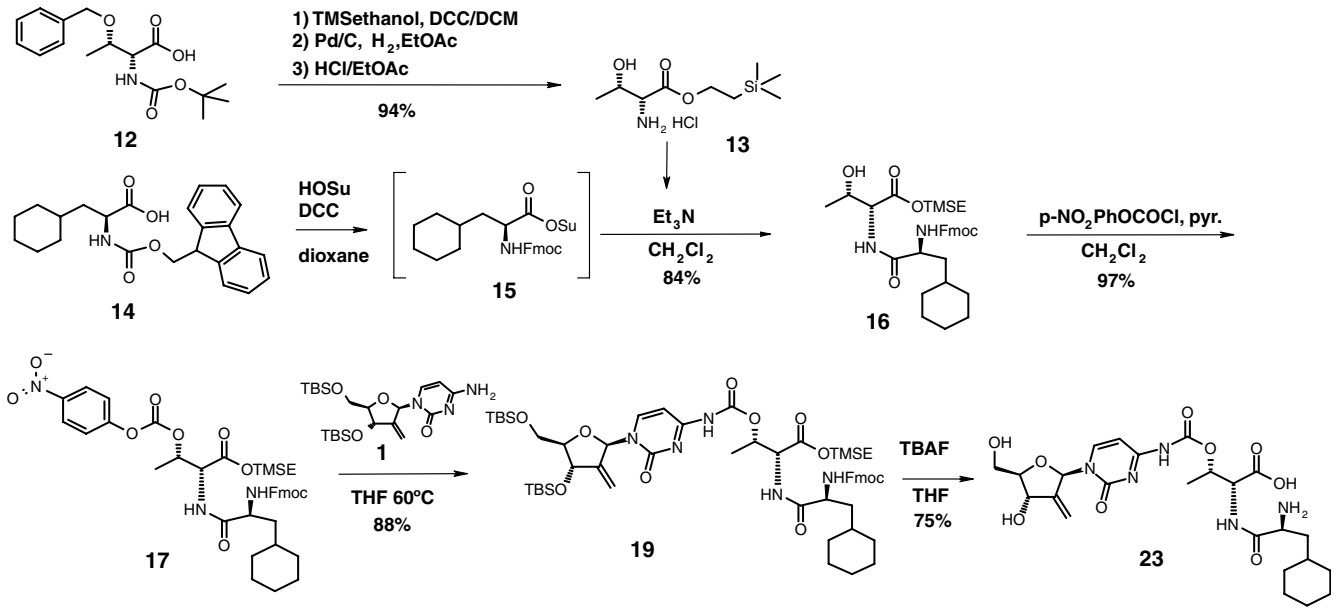


Figure 5. Synthesis of compound **23**.

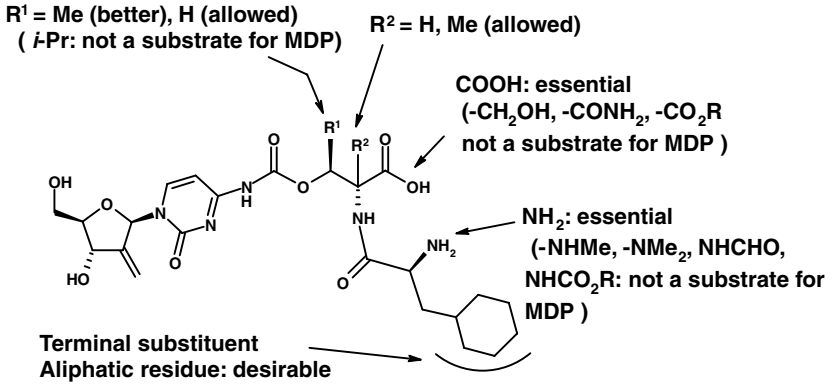
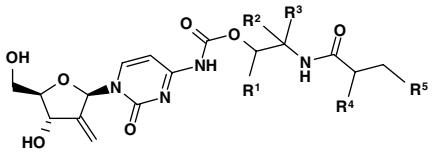


Figure 6. Summary of the substrate specificity of MDP.

Table 1. Results of cytotoxicity and plasma stability of prodrugs



						Cytotoxicity[a] IC_{50} (μM)		MDP selectivity	Plasma stability residual % after 2 h	
Compound	R^1	R^2	R^3	R^4	R^5	HCT116 MDP(−)	HCT116 MDP(+)	Relative IC_{50} ratio	Mouse	Human
DMDC	—	—	—	—	—	0.3–0.7	0.3–0.7		106.7	102.2
20	(*S*)Me	H	(*S*)COOH	(*R*)NH_2	Phenyl	8.7	15.7	0.6	ND[b]	84.3
21	(*S*)Me	H	(*S*)COOH	(*S*)NH_2	Phenyl	4.1	5.1	0.8	ND	90.0
22	H	H	(*R*)COOH	(*S*)NH_2	Cyclohexyl	17.0	2.8	6.1	52.3	87.4
23	(*S*)Me	H	(*R*)COOH	(*S*)NH_2	Cyclohexyl	18.8	3.1	6.1	100.5	97.8
24	(*S*)Me	H	(*R*)COOH	(*S*)NH_2	Biphenyl	67.2	15.4	4.4	86.7	86.0
25	(*S*)Me	H	(*R*)COOH	(*S*)NH_2	Naphtyl	26.3	8.3	3.2	97.7	85.5
26	(*S*)Me	H	(*S*)COOH	(*S*)NH_2	*i*-Propyl	4.4	0.8	5.5	ND	55.6
27	(*S*)*i*-Pr	H	(*R*)COOH	(*S*)NH_2	Cyclohexyl	20.3	20.3	1.0	88.5	89.3
28	(*S*)Me	H	(*R*)$CONH_2$	(*S*)NH_2	Cyclohexyl	8.1	7.3	1.1	95.1	103.9
29	(*S*)Me	H	(*R*)COO-*i*-Pr	(*S*)NH_2	Cyclohexyl	7.4	8.2	1.0	0	87.2
30	(*S*)Me	H	(*R*)CH_2OH	(*S*)NH_2	Cyclohexyl	10.8	10.5	1.0	ND	85.4
31	(*S*)Me	H	(*R*)COOH	(*S*)NHMe	Cyclohexyl	47.8	48.9	1.0	91.4	85.4
32	(*S*)Me	H	(*R*)COOH	(*S*)NMe_2	Cyclohexyl	56.6	44.9	1.3	101.3	86.8
33	(*S*)Me	H	(*R*)COOH	(*S*)NHCHO	Cyclohexyl	>50.0	>50.0	—	ND	ND
34	H	Me	(*S*)COOH	(*S*)NH_2	Cyclohexyl	72.3	5.9	12.3	19.4	95.9
35	H	Me	(*R*)COOH	(*S*)NH_2	Cyclohexyl	51.9	21.8	2.4	101.5	89.1

[a] Assay condition; 24 h pulse treatment and 72 h incubation.
[b] ND, not done

The terminal amino group is essential for recognition by MDP. Mono-methylamino **31**, di-methylamino **32**, *N*-formyl **33**, and the carbamate derivatives (not listed) are not recognized by MDP. For the N-terminal substituent, lipophilic and aliphatic residue is preferable for enzyme recognition (**22**, **23**, **24**, **25**, **26**, and **34**).

We also examined the ability of the prodrugs to inhibit the growth of human colon cancer HCT116 cells that does not overexpress MDP (MDP-negative HCT116) and those transfected with MDP cDNA (MDP-positive HCT116) for proof-of-concept in vitro and stability under different pH conditions and in plasma (mice and human). The results are summarized in Table 1.

Among the (L)-threonine derivatives, **20** and **21** having L- and D-Phe, respectively, as a trigger moiety showed no selectivity in cytotoxic activity in MDP-positive and MDP-negative HCT116 cells, whereas compound **23** with (D)-Thr-(L)-cyclohexylalanine (L-Cha) showed selectively inhibited the growth of the MDP-positive HCT116 (IC_{50} = 3.1 μM vs IC_{50} = 18.8 μM in MDP(negative) HCT116). In an attempt to improve the selectivity to other peptidases, we synthesized (L)-α-methylserine-(L)-Cha derivative **34** and found further improvement of selectivity to MDP-positive HCT116 (IC_{50} = 5.9 μM vs IC_{50} = 72.3 μM in MDP-negative HCT116). Regarding plasma stability, both compound **23** and compound **34** were stable in human plasma, but the latter was rather unstable in mouse plasma (only 19.4% remained after 2 h incubation).

The highly selective conversion of compound **23** to DMDC in the cell free enzyme assay with MDP-positive HCT116 versus MDP-negative HCT 116 and hematopoietic progenitor cells was further confirmed by direct measurement of the prodrug and DMDC levels by LC–MS (Fig. 7). In the MDP-negative cell line and

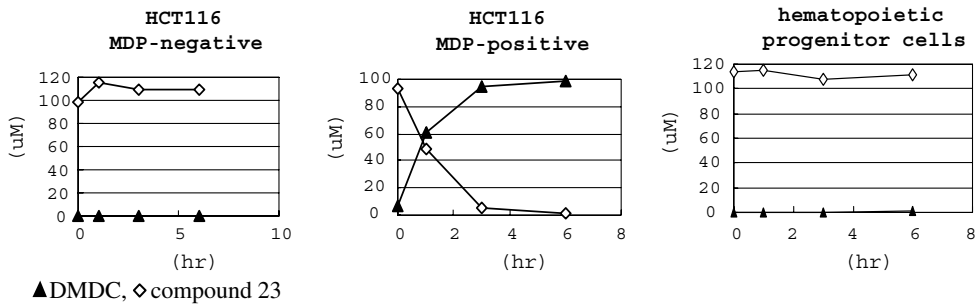


Figure 7. The in vitro conversion of compound **23** to DMDC in MDP-negative HCT116, MDP-positive HCT116, and hematopoietic progenitor cells.

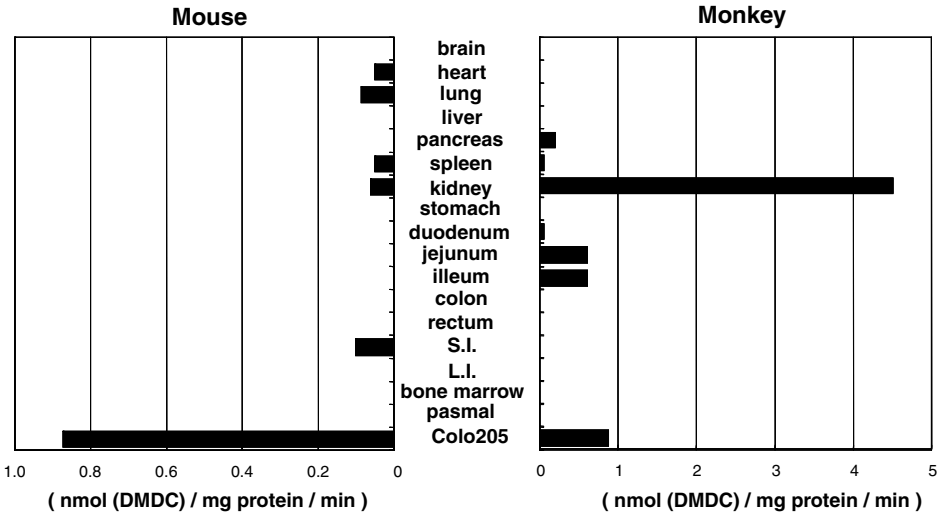


Figure 8. The results of level of DMDC by crude enzyme extracts from mouse and monkey tissues.

hematopoietic progenitor cells, compound **23** was quite stable. Thus, in vitro proof-of-concept of the tumor-activated prodrugs was achieved.

The tumor selective conversion of compound **23** (50 μM) to DMDC was further examined with extracts of COLO 205 human colon cancer xenograft that expressed MDP and was compared with extracts of various normal tissues from mice and monkeys (Fig. 8). Compound **23** was efficiently converted to DMDC in COLO 205, whereas its conversion was very low in other normal tissues from mice. On the other hand, among the monkey tissues examined, the level of DMDC was much higher in kidney than, and nearly equal in jejunum and ileum to, that in COLO 205.

Species difference in tissue distribution of MDP is of concern for development of oral compound **23** for human. Further investigation is required to identify prodrugs with less species difference in enzymatic activation.

In conclusion, this study demonstrates the gene expression profiling of tumor and normal tissues including hematopoietic progenitor cells by DNA microarray is an effective approach in identifying enzymes for the design of tumor-activated prodrugs of cytotoxic agents. We designed compound **23** which was selectively converted to an active drug, DMDC, by MDP-positive tumor tissues. The desired tumor selective conversion was clearly demonstrated in vitro from the cytotoxicity assays (tumor cells with high vs low MDP), cell free enzyme assays, and tissue conversion assays (tumor vs normal tissue including hematopoietic progenitor cells) performed. A general concern with the prodrug approach is species difference in prodrug activation since the absolute enzyme levels in all human tissues are unknown. In the case of compound **23**, some species difference was observed between mice and monkeys. Further investigation is needed to assure product safety and efficacy for clinical development of this type of prodrug.

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