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Cytostatic evaluations of nucleoside analogs related to unnatural base pairs for a genetic expansion system

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Abstract—The introduction of an unnatural base pair into DNA enables the expansion of genetic information. To apply unnatural base pairs to in vivo systems, we evaluated the cytostatic toxicity of several nucleoside analogs by an MTT assay. Several nucleoside analogs based on two types of unnatural base pairs were tested. One is a hydrogen-bonded base pair between 2-amino-6-(2-thienyl)purine (**s**) and pyridin-2-one (**y**), and the other is a hydrophobic base pair between 7-(2-thienyl)imidazo[4,5-*b*]pyridine (**Ds**) and pyrrole-2-carbaldehyde (**Pa**). Among the nucleoside analogs, the ribonucleoside of 6-(2-thienyl)purine possessed the highest cytostatic activity against CCRF-CEM and especially HT-1080, as well as the normal fibroblast cell line, WI-38. The other analogs, including its 2′-deoxy, 2-amino, and 1-deazapurine nucleoside derivatives, were less active against CCRF-CEM and HT-1080, and the toxicity of these nucleosides toward WI-38 was low. The nucleosides of **y** and **Pa** were inactive against CCRF-CEM, HT-1080, and WI-38. In addition, no cytostatic synergism was observed with the combination of the pairing nucleosides of **s** and **y** or **Ds** and **Pa**.

The development of unnatural base pairs that work with the natural A–T (U) and G–C base pairs in nucleic acids enables the expansion of the genetic alphabet and the creation of DNA and RNA molecules with increased functionality. So far, many unnatural base pairs have been reported, and several of them are good substrates for DNA and RNA polymerases.[1–3] We previously described various unnatural base pairs, such as 2-amino-6-(2-thienyl)purine (denoted by **s**) and pyridin-2-one (denoted by **y**),[4,5] and 7-(2-thienyl)imidazo[4,5-*b*]pyridine (denoted by **Ds**) and pyrrole-2-carbaldehyde (denoted by **Pa**)[6] (Fig. 1a). In in vitro experiments, the **s**–**y** pair functions in transcription and translation, and the **Ds**–**Pa** pair functions in replication and transcription with high selectivity and efficiency. Accordingly, the functionality of the unnatural base pairs in the cell and the biological activity of these unnatural bases are the next topics of interest. For both replication and transcription involving unnatural base pairs in a cell, the nucleoside derivatives of the unnatural bases must be added to the cell culture medium, because the cell itself cannot make the unnatural base nucleosides for the substrates of replication and transcription. Thus, the cytostatic toxicity of the nucleoside derivatives related to the unnatural base pairs is critical.

Recently, Hocek and coworkers reported that ribonucleoside derivatives of 6-aryl- and 6-hetarylpurines, including the 6-(2-thienyl)purine ribonucleoside (**2R** in Fig. 1b), exert significant in vitro inhibitory effects on cell growth.[7–12] They synthesized an extended series of 6-hetarylpurine ribonucleosides, and revealed that the ribonucleosides of 6-(2-thienyl)- and 6-(2-furyl)purines have considerable cytostatic activity against leukemia cell lines, as well as anti-HCV activity.[9,11] Although the 2-amino-6-phenylpurine ribonucleoside and the 6-phenylpurine deoxyribonucleoside were inactive in their previous reports,[7,8] the biological

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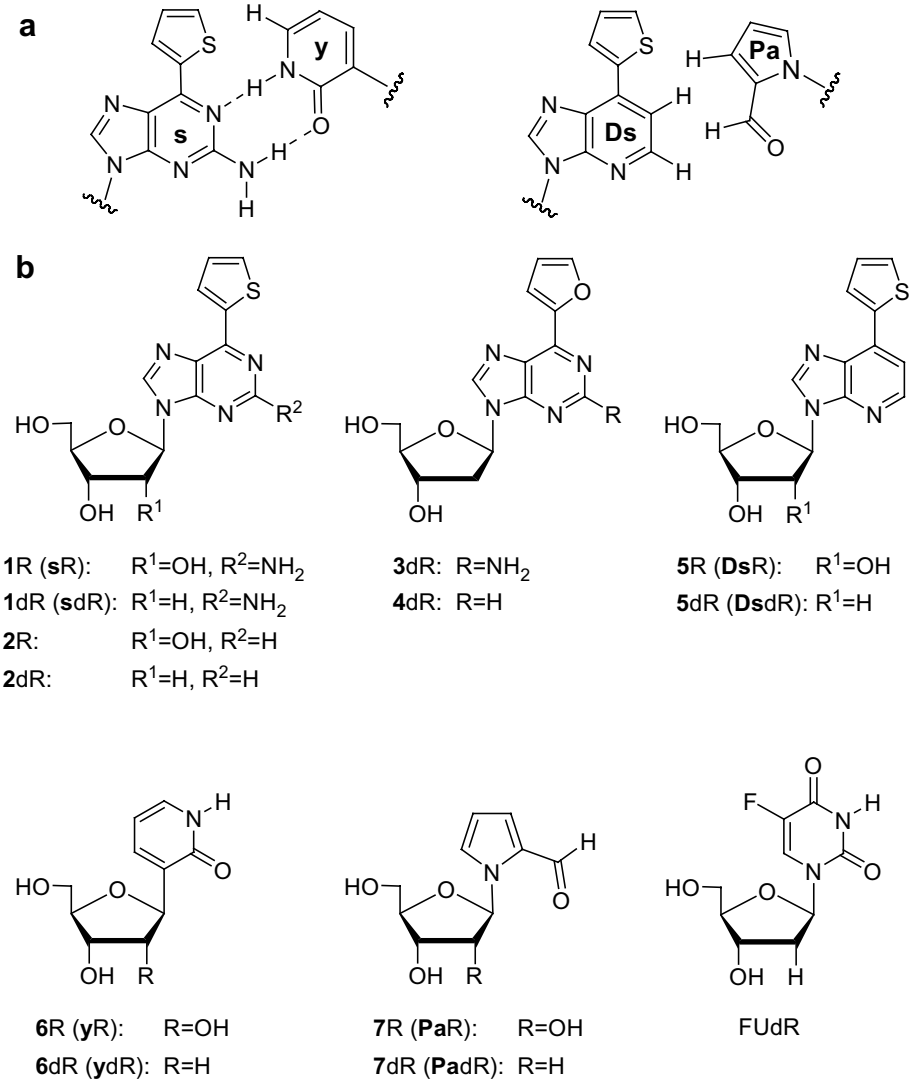

Figure 1. The unnatural **s**–**y** and **Ds**–**Pa** pairs for expansion of the genetic alphabet (a). The 6-hetarylpurine, 2-amino-6-hetarylpurine, 1-deaza-6-(2-thienyl)purine, pyridin-2-one, and pyrrole-2-carbaldehyde nucleoside derivatives, as well as the 5-fluoro-2′-deoxyuridine, assayed in this study (b).

effects of the nucleosides of 2-amino-6-hetarylpurine and its 1-deaza-6-hetarylpurine derivatives were still unclear.

Here, we examined the cytostatic activity of the nucleoside derivatives of **s** (**1**–**4** in Fig. 1b) and the nucleosides of **Ds** (**5**), **y** (**6**), and **Pa** (**7**) against human T acute lymphoblastic leukemia CCRF-CEM cells, human fibrosarcoma HT-1080 cells, and normal human fetal lung fibroblast WI-38 cells, for further application of unnatural base pairs to in vivo systems.

The nucleoside analogs assayed in this study (Fig. 1b) were chemically synthesized, as we previously reported. The deoxyribonucleosides of **1**dR–**4**dR were prepared by the palladium catalyzed coupling of 6-iodopurine deoxyribonucleoside derivatives with tributylstannylthiophene or tributylstannylfurane.[4,13] The ribonucleosides **1**R and **2**R were also prepared by the same coupling method, but with 6-chloropurine ribonucleoside derivatives.[14] The **Ds** nucleosides (**5**R and **5**dR) were synthesized by the coupling reaction of 1-chloro-2-deoxy-3,5-di-*O*-toluoyl-α-D-*erythro*-pentofuranose with the sodium salt of 7-(2-thienyl)-3*H*-imidazo[4,5-*b*]pyridine.[6] The nucleosides of **y** (**6**dR and **6**R) and **Pa** (**7**dR and **7**R) were synthesized by previously reported procedures.[6,15] All of the nucleosides were purified by HPLC.

The cytostatic activity of these nucleosides was determined by an MTT assay, using human T lymphoblastoid CCRF-CEM cells (from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University), human HT-1080 cells (JCRB9113), and human WI-38 cells (IFO50075), cultured in medium (RPMI1640, EMEM with non-essential amino acids, and BME, respectively) containing 10% FBS.[16] We chose 5-fluoro-2′-deoxyuridine (FUdR) as a control, since its inhibitory effects on the growth of the CCRF-CEM and HT-1080 cell lines were reported previously (IC_{50} = 0.017–0.5 μM and 0.12 μM, respectively).[17–19] The

inhibitory potency (IC_{50}) values of the nucleosides are listed in Table 1.

As reported by Hocek et al.,[9,11] the ribonucleoside **2**R displayed significant cytostatic activity, especially against HT-1080, for which the activity was as high as that of FUdR. However, the cytotoxic activity of **2**R against the normal fibroblast WI-38 cells was also high. The 2-aminopurine ribonucleoside and its deoxyribonucleoside derivatives (**1**R, **1**dR, and **2**dR) decreased the activity. The 6-(2-furyl)purine deoxyribonucleosides (**3**dR and **4**dR) were much less active, relative to the 6-(2-thienyl)purine nucleosides, and **3**dR and **4**dR marginally inhibited only the HT-1080 cell growth, with IC_{50} values of 290–330 μM. In contrast, the previously reported data showed that the activity against CCRF-CEM of the ribonucleoside of 6-(2-furyl)purine was as high as that of **2**R.[9,11] These results suggest that the 2′-hydroxy group in **2**R is important for robust cytostatic activity, and that the amino group at position 2 of 6-hetarylpurine decreases the activity.

The 1-deazapurine derivatives (**5**R and **5**dR) related to the unnatural **Ds** base possessed moderate cytostatic activity against these cell lines. However, the activities of **5**R and **5**dR were significantly lower than that of 2**R** (Table 1). This suggests that the 1-nitrogen in 6-(2-thienyl)purine is critical for the robust cytostatic activity. As for the unnatural base pairs, the exclusive selectivity of the **Ds**–**Pa** pair in replication is higher than that of the **s**–**y** pair, and the misincorporation of the deoxyribonucleoside triphosphate of **Ds** into native DNA fragments is much lower than that of **s**.[6] Although the mechanism of the cytostatic action of these nucleosides is still unknown, a comparison of the cytostatic and mutagenic activities of the nucleoside derivatives might provide clues toward understanding the mechanism. In contrast to the **s** and **Ds** derivatives, the pairing partners, the nucleosides of **y** (**6**R and **6**dR) and **Pa** (**7**R and **7**dR) were inactive toward all of the cell lines, CCRF-CEM, HT-1080, and WI-38.

Table 1. Cytostatic activity of unnatural base nucleosides

Compound	IC_{50} (μM)[a]		
	CCRF-CEM	HT-1080	WI-38
1dR (**s**dR)	340 ± 20	38 ± 11	~500
1R (**s**R)	NA[b]	88 ± 23	NA
2dR	420 ± 20	95 ± 14	NA
2R	2.3 ± 0.4	0.33 ± 0.08	5.7 ± 1.9
3dR	NA	290 ± 50	NA
4dR	NA	330 ± 70	NA
5dR (**Ds**dR)	120 ± 30	56 ± 39	190 ± 140
5R (**Ds**R)	230 ± 30	190 ± 120	310 ± 190
6dR (**y**dR)	—	NA	NA
6R (**y**R)	—	NA	NA
7dR (**Pa**dR)	NA	NA	NA
7R (**Pa**R)	NA	NA	NA
FUdR	0.29 ± 0.15	0.18 ± 0.14	~500

[a] The data are presented as means with standard deviations.
[b] NA, not active (inhibition of cell growth at a 500 μM concentration was lower than 50%).

Next, we examined the inhibitory effect of the combinations of the pairing bases, **1**dR and **6**dR, and **5**dR and **7**dR. However, neither the mixture of **6**dR (40 μM) and **1**dR (40 μM) nor that of **7**dR (40 μM) and **5**dR (40 μM) generated cytostatic synergism in HT-1080 and WI-38. In another respect, since **1**dR has cytostatic potency toward HT-1080, but less cytotoxicity against WI-38, we also tested the inhibitory effect of the combination of **1**dR with a prodrug, FUdR, toward HT-1080 and WI-38.[20] These two deoxyribonucleosides, as well as 10 nM or 100 nM FUdR and 5 μM or 25 μM **1**dR, were each added to the cultured HT-1080 and WI-38 cells at the same time, and the cell survival (%) was determined by the MTT assay (Table 2). As for HT-1080, cytostatic synergy was observed in the presence of 25 μM **1**dR when combined with FUdR, especially at 10 nM ($P = 0.014$), although the presence of 5 μM **1**dR did not cause any cytostatic synergism when combined with either 10 nM or 100 nM FUdR. In the normal WI-38 cells, the cytostatic effect was much smaller as compared with that against HT-1080. Thus, FUdR and **1**dR might be a useful anticancer drug combination for decreasing the dosage of both nucleosides, to reduce the adverse effects.

In this study, we evaluated the cytostatic activity of nucleoside analogs related to unnatural **s**–**y** and **Ds**–**Pa** pairs, using malignant and normal cells. The normal cells, WI-38, tolerated all of the nucleoside analogs, except **2**R. The ribonucleoside **2**R exhibited the highest cytostatic activity among the nucleoside analogs we examined. The other related analogs, **1**dR, **2**dR, and **1**R, were about 100 times less effective than **2**R. The 6-(2-furyl)purine derivatives, **3**dR and **4**dR, were even less active toward HT-1080, and were inactive toward CCRF-CEM and WI-38. These nucleoside analogs showed relatively higher efficiency toward HT-1080, as compared to those toward CCRF-CEM and WI-38. The 1-deazapurine derivatives, **5**dR and **5**R, displayed moderate activity toward HT-1080, and lower activity toward CCRF-CEM and WI-38. In addition, no cytostatic synergism of the pairing nucleotides between **1**dR and **6**dR or **5**dR and **7**dR was observed in HT-1080 and WI-38. Thus, these unnatural base pairs could be candidates for in vivo application. These data also provide additional information for the further development of unnatural base pairs, as well as for enhanced

Table 2. Relative percent survival of HT-1080 and WI-38 cells after treatment with various combinations of **1**dR and FUdR[a]

Compound	None	**1**dR (5 μM)	**1**dR (25 μM)
HT-1080			
None	100	97 ± 4	69 ± 10
FUdR 10 nM	104 ± 8	96 ± 8	49 ± 8*[b]
FUdR 100 nM	52 ± 3	53 ± 3	29 ± 3
WI-38			
None	100	95 ± 2	91 ± 4
FUdR 10 nM	99 ± 2	97 ± 1	86 ± 3
FUdR 100 nM	99 ± 2	93 ± 1	83 ± 2*

[a] The data are presented as means with standard deviations.
[b] An asterisk indicates a significant difference ($P < 0.05$) in cell survival between a particular combination and the FUdR control alone.

analyses of the activity mechanism of this type of nucleoside analogs.

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16. Aliquots (90 μl) of cell solutions (1×10^5 cells/ml for CCRF-CEM and 1×10^4 cells/ml for HT-1080 and WI-38) were plated into 96-well microplates and were incubated for 24 h (for CCRF-CEM) or 4 h (for HT-1080 and WI-38). Various concentrations of nucleoside solutions (10 μl) in PBS containing 5% DMSO were then added into each well. The presence of 5% DMSO, which was used to dissolve the nucleosides, did not affect the cell growth (data not shown). After an incubation for 72 h, the MTT solution (25 μl) in PBS (5 mg/ml) was added to each well, and the plates were incubated for an additional 2 h. Finally, a 100 μl aliquot of a lysis solution (50% DMF/20% SDS) was added to each well, in order to dissolve the resulting MTT-formazan, and after an incubation for 16 h, the absorbance of the formazan product obtained by MTT reduction was measured at 570 nm, using a microplate reader.
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