Antitumor Activity of Sugar-modified Cytosine Nucleosides

Akira Matsuda[1,*] and Takuma Sasaki[2]

[1]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan and [2]Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

*To whom correspondence should be addressed.
E-mail: matuda@pharm.hokudai.ac.jp

Abstract

Nucleoside analogues, which show antimetabolic activity in cells, have been used in the treatment of various tumors. Nucleosides such as cytosine arabinoside, 6-mercaptopurine, fludarabine and cladribine play an important role in the treatemnt of leukemias. On the other hand, gemcitabine, 5-fluorouracil and its prodrugs are used extensively in the treatment of many types of solid tumors. All these nucleosides are metabolized similar to endogenous nucleosides and nucleotides. Active metabolites interfere with the *de novo* synthesis of nucleosides and nucleotides or inhibit the DNA chain elongation after incorporated into DNA strand as a terminator. Furthermore, nucleoside antimetabolite incorporated into DNA strand induces strand-break and finally causes apoptosis. Nucleoside antimetabolite targets one or more specific enzyme(s). Mode of inhibitory action on target enzyme is not always similar even in nucleoside antimetabolites which have the same nucleoside base such as cytosine arabinoside and gemcitabine. Although both nucleosides are phosphorylated by deoxycytidine kinase and also are good substrate of cytidine deaminase, only gemcitabine shows antitumor activity against solid tumors. This fact suggests that differences in pharmacological activity of these nucleoside antimetabolites may show a great influence on different mode of action on target molecule. Design, *in vitro* cytotoxicity, *in vivo* antitumor activity, metabolism, and mechanism of action of sugar-modified cytosine nucleosides, such as (2'*S*)-2'-deoxy-2'-*C*-methylcytidine (SMDC), 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC), 1-(2-*C*-cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC) and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd), which have been developed by our groups, have been described.

Introduction

Nucleoside antimetabolites, such as 1-β-D-arabinofuranosylcytosine (araC), 2-chloro-2'-deoxyadenosine (CldA), 9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate (fludarabine, FaraAMP) and 2'-deoxy-2',2'-difluorocytidine (gemcitabine, dFdC) (Fig. 1), have been widely used as not only antileukemic agents but also antitumor agents against solid tumors.[37] Similar to other nucleoside antimetabolites, such as antiviral agents, these nucleosides themselves are not active components. They have to be metabolically activated by phosphorylations. Therefore, not only the substrate specificities of nucleoside kinases and nucleotide kinases but also their expression at tumor tissues in patients are the most important

factors to be effective in clinic. Target enzymes of each metabolized nucleotide are slightly different. AraC 5'-triphosphate (araCTP) is incorporated into DNA, which become a trigger for apoptosis. 2-Chloro-2'-deoxyadenosine 5'-triphosphate (CldATP) is also incorporated into DNA by DNA polymerase and also inhibits ribonucleotide reductase (RDR) potently instead of dATP.[50] Fludarabine 5'-triphosphate (FaraATP) is incorporated into both DNA and RNA, which causes to inhibit both enzyme activities of DNA and RNA polymerases. Moreover, FaraATP inhibits RDR as well as DNA ligase 1 and DNA primase.[50] Gemcitabine 5'-diphosphate (dFdCDP) is very potent inhibitor of RDR, and its corresponding 5'-triphosphate (dFdCTP) inhibits DNA polymerase.[22] Such differences in target enzymes for nucleotide antimetabolites would reflect the target diseases. Therefore, further developments of new types of nucleoside antimetabolites are very important to fight with tumors. In this review, we describe design, *in vitro* cytotoxicity, *in vivo* antitumor activity, metabolism, and mechanism of action of new sugar-modified cytosine nucleosides such as (2'*S*)-2'-deoxy-2'-*C*-methylcytidine (SMDC), 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC), 1-(2-*C*-cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC) and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd), which we have been developing.[32]

1. Deoxycytidine kinase–dependent chemotherapy

It has been known that there are four nucleside kinases, such as deoxycytidine kinase (dCK), thymidine kinase-1 (TK1), adenosine kinase (AK), and uridine/cytidine kinase (UCK) in cytosole and two nucleoside kinases, such as thymidine kinase–2 (TK2) and deoxyguanosine kinase (dGK) in mitochondria in mammalian cells and tissues.[2, 12, 23] Among the above nucleoside kinases, many of the antitumor nucleosides used in clinic, such as araC, CldA, gemcitabine, and araG derivatives are phosphorylated by dCK to afford their corresponding 5'-monophosphates, which are further phosphorylated by nucleoside monophosphate kinases and then nucloeoside diphosphate kinase (NDPK). Among the nucleoside kinases, dCK has been believed to have a broader substrate specificity and has following properties suitable for activation of the above described nucleosides to exhibit proper antitumor efficacies: 1) dCK is constitutively expressed both at the protein and mRNA levels, due to no cell cycle regurated. 2) dCK has the lowest substrate specificity among the nucleoside kinases. 3) Most solid tumor tissues express dCK activity with exception of brain and liver. 4) The average dCK content in most carcinomas is 3- to 5-fold higher than in

normal tissues and about 5-fold lower than in normal or B-CLL lymphoid cells. These properties give the basis for the dCK dependent-chemotherapy.

dCK activity is regulated by a high concentration of dCTP, the end product of dCyd. Therefore, if certain antitumor cytosine nucleoside is metabolized to its triphosphate, whether the triphosphate acts as a feedback inhibitor of dCK or not is very important for its antitumor activity. It is known that araCTP inhibits dCK like dCTP, which is therefore one of the drawbacks of araC. The cell lines resistant to dCyd nucleoside analogues such as araC, dFdC, DMDC, and CNDAC had mutation of the dCK gene.[39)]

2. Degradation of the active component

When a new cytosine nucleoside is selected as a candidate of antitumor nucleosides *in vitro*, the next step will be *in vivo* tests using tumor-bearing model animals such as mice and/or rats. However, these animals are largely different from human in enzyme activities of nucleotide biosynthesis, especially degradation enzymes, such as cytidine deaminase (CDA), that degrades cytosine nucleosides to chemotherapeutically inactive uracil nucleosides. Since the activity is usually low in rodents and very high in human, therefore, before clinical trials will be carried out, model pharmacokinetic studies using monkeys should be studied. AraC and gemcitabine are both very good substrates of CDA, so that large amounts of the agents have to be administered to be effective. dCMP deaminase (dCMPDA)[27)] is also responsible for the degradation of dCMP and its derivatives. However, its influence on the antitumor activity is not well studies.

Enzymatic stability of nucleotide metabolites of a nucleoside in tumor cells is also important to be effective, because more than certain concentration of nucleotide metabolites is required to strongly inhibit a target enzyme for certain periods of time. AraCTP is known to be unstable in tumor cells, while that of ECTP (ECyd triphosphate) is stable as discussed later. However, enzymatic nature of such degradation activity is not elucidated enough.

3. AraC and gemcitabine

When we started to design sugar-modified antitumor cytosine nucleosides, only araC[16)] had been used in clinic for the treatment of adult acute myeloblastic leukemia. AraC is a good substrate of dCK (*Km* = 8.8 μM, CHO cells) and further metabolized to araCTP, which inhibits DNA polymerization after incorporation into DNA by DNA polymerase α. This inhibition could be a trigger for apoptosis. However, araCTP at high concentration acts

as a feedback inhibitor of dCK and has a short half-life in tumor cells. From these properties together with that araC and araCMP are good substrates of CDA and dCMPDA, respectively, araC is not effective against solid tumors. To overcome these drawbacks, a number of prodrug of araC have been prepared.[17] Although some of them are used against leukemias in clinic in Japan, these araC-prodrugs are not effective against solid tumors yet.

On the other hand, among sugar-modified cytosine nucleosides, gemcitabine (dFdCd) was originally designed as an antiviral agent mimicking FIAC.[26] However, it was too cytotoxic to be developed as an antiviral agent.[21] dFdCyd is a good substrate of dCK (*Km* = 3.6 μM, CHO cells), which is further phosphorylated to its diphosphate (dFdCDP) and triphosphate (dFdCTP). dFdCDP is a potent inhibitor of RDR. On the other hand, dFdCTP inhibits DNA synthesis, the potency is lower than that of araCTP. dFdCTP also inhibits dCMPDA and CTP synthase and is incorporated into RNA.[41]

Although dFdCyd was initially approved as an antitumor agent for pancreatic cancer by FDA to improve quality of life (QOL) but not to prolongate life-spane, combination with other antitumor agents such as cisplatin *etc*. contributes to prolongate life-spane in non-small lung carcinoma.

4. (2'*S*)-2'-Deoxy-2'-*C*-methylcytidine (SMDC)

We have synthesized (2'*S*)-2'-deoxy-2'-*C*-methylcytidine (SMDC), (2'*R*)-2'-deoxy-2'-*C*-methylcytidine (RMDC), and (2'*S*)-2'-deoxy-2'-*C*-ethylcytidine (SEDC) from uridine to elucidate the steric requirement of the substituent at the 2'β-position of 2'-deoxycytidine.[29] Against mouse L1210 cells *in vitro*, SMDC was as potent as araC (Table I) but RMDC was 150-fold less effective than SMDC, and SECD was not effective up to 100 μg/mL. Additionally, 2'-deoxy-2'-(methylthio)-β-D-arabinofuranosylcytosine did not show any cytotoxicity toward L5178Y cells *in vitro* up to 100 μg/mL. Therefore, it appears that increasing bulkiness of the 2'-substituent in 2'-deoxycytidine greatly reduces cytotoxicity.

SMDC is a relatively good substrate of dCK (Table III)[52] and a very poor substrate of CDA. Chemically synthesized 5'-triphosphate (SMDCTP) is potent competitive inhibitors of dCTP against DNA polymerase α, β, and γ with *Ki* values of 0.05, 0.10, and 0.07 μM, respectively (Table IV). These values indicate that SMDCTP binds to DNA polymerase α , β , and γ 25–, 39–, and 11–fold better than the natural substrate dCTP.[32] Although these data were promising SMDC to be effective *in vivo*, SMDC showed only marginal activity against a mouse leukemic P388 model with T/C values of 111% at 30 mg/kg/day and 126% at 100

mg/kg/day treatments, while araC showed good activity with those of 184% at 10 mg/kg/day and 189% at 30 mg/kg/day.

On the basis of these data, SMDCMP (5'-monophosphate) and/or SMDCDP (5'-diphosphate) would not be good substrates for CMP/UMP kinase and/or NDP kinase to produce the active nucleotide, SMDCTP, which is potent inhibitors of several DNA polymerases. Although the structural basis of this drawbacks is not clear enough, the precedent example of this type is 3'-azido-3'-deoxythymidine (AZT) 5'-monophosphate (AZTMP), which is not a good substrate of TMP kinase, although AZT is a good substrate of TK1.[1)] Previously, we believed that nucleoside kinases have most strict substrate specificity among various nucleoside and nucleotide kinases. However, now, we know that a nucleoside 5'-monophosphate kinase might also have rather strict substrate specificity.

5. 1-(2-Deoxy-2-methylene-β -D-*erythro*-pentofuranosyl)cytosine (DMDC)

DMDC has an allylic alcohol system together with the 3'-secondary alcohol in the sugar moiety. This allylic alcohol in DMDC could be stable at a nucleoside level, but if it is phosphorylated to the 5'-polyphosphates by cellular kinases, the following chemical reactivities are expected. (i) At the 5'-diphosphate level (DMDCDP), it could be a mechanism-based inhibitor of ribonucleotide reductase if a 3'-radical is formed by its enzyme action. (ii) When its 5'-triphosphate (DMDCTP), although this could be an inhibitor of DNA polymerases, is incorporated into DNA molecules, the allylic alcohol becomes a more reactive allyl phosphate ester to be cleaved by nucleophiles.

In vitro cytotoxicity of DMDC against various human tumor cell lines including mouse leukemic L1210 cells is shown in Table I.[30, 40)] Cytotoxicity spectrum of DMDC is quite similar to that of 5-FU, but not to those of SMDC and araC. Unlike araC, DMDC was not a substrate of CDA from mouse kidney. Together with these characteristics, chemically synthesized DMDCTP was a potent inhibitor of DNA polymerase α, β, and γ with K_i values of 0.42, 2.52, and 1.00 μM, respectively, in a manner competitive with dCTP, while araCTP inhibited only DNA polymerase α with a K_i of 1.10 μM.[32)] It was also found that DMDCTP was incorporated into DNA molecules at the site complementary to guanine by the action of DNA polymerase α using a synthetic template-primer system. In this experiment, DMDCTP apparently acted as a chain-terminator, but whether it was a real chain-terminator or a result of strand-break after a further elongation of the chain, which we postulated, has not been proved yet. Moreover, DMDCDP was reported to be a time-dependent inhibitor of *E. coli*

RDR,[8] while it did not inhibit RDR in L1210 murine leukemia and LX-1 human lung carcinoma cells.[24]

In vivo antileukemic activity of DMDC was also examined by using female $CD2F_1$ mice bearing ip inoculated L1210 cells. DMDC administered i.p. once a day for 5 days at 250 mg/kg had a T/C (%) of 235.[54] The activity of DMDC was schedule-dependent with much more therapeutic effect obtained by daily treatment than by a single treatment. DMDC was also effective against colon 26 murine carcinoma, M5076 murine reticulum cell sarcoma, LX-1 human lung cancer xenograft, and SK-Mel-28 human melanoma xenograft, which are less sensitive or refractory to araC. The efficacy of DMDC correlates well with tumor the levels of CDA activity in human tumor xenograft models.[11, 36] DMDC was highly effective in tumors with higher levels of CDA activity, whereas lower levels yielded only slight antitumor activity. In contrast, gemcitabine is only slightly effective in tumors with higher levels of the enzyme. The activation of DMDC to DMDCMP by dCK would be competitively inhibited by endogenous dCyd in cells. Therefore, the dCyd concentration in tumor cells, that is regulated by CDA, would be critical for phosphorylation of DMDC. On the other hand, since gemcitabine is a good substrate of CDA, in tumors with higher levels of CDA, both dCyd and gemcitabine are deaminated to be uracil derivatives before phosphorylation.

Phase I clinical studies using DMDC with i.v. administration against solid tumors had already been carried out in Japan.[15] The major dose-limiting toxicity was hematological depression, particularly leukopenia and neutropenia. The maximum tolerated dose for the 5-consecutive-day administration was 40 mg/m^2. Furthermore, phase I clinical trial with p.o. administration was also carried out.[28] The dose-limiting toxicities were anorexia, leukopenia, thrombocytopenia, and anemia. General fatigue was the common nonhematological toxicity. The maximum-tolerated dose was 18 mg/m^2/day. At the 18-mg/m^2/day dose level, the mean terminal half-life, maximum plasma concentration (Cmax), the area under the plasma drug concentration-time curve on day 1 were 1.7496 h, 112.9 ng/mL, and 399.8 ng x h/mL, respectively. Forty to 50% of the administered dose was recovered in the urine, indicating its good bioavailability and resulting significant systemic exposure to the drug, which may enable chronic oral treatment. Details of the clinical pharmacokinetics were also reported.[9, 13, 14]

All-*trans* retinoic acid (ATRA) differentiation therapy against acute promyelocytic leukemia (APL) greatly improved its prognosis. However, although complete remission can be achieved with ATRA alone, disease-free survival is still too short due to relapse. DMDC

was found both to inhibit the growth and to induce differentiation of APL cell lines. ATRA combined treatment with DMDC induced differentiation of leukemia cells that had been freshly isolated from APL patients.[38)]

6. 1-(2-*C*-Cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC)

Introduction of an electron-withdrawing group (EWG) into the 2'β position would increase the acidity of the 2'α proton. If such a nucleoside is incorporated into DNA after its enzymatic phosphorylation, the EWG becomes β to the phosphate diester in the DNA. In this case, β-elimination should produce (i) DNA strand-breaks or (ii) abasic site formation as illustrated in Fig. 2. Since strand-breaks in DNA by radiation therapy have been hypothesized to produce tumor cell death, it is worth examining whether the nucleoside having such chemical reactivities inhibits tumor cell growth or not. As an example of the nucleoside that has an EWG at the 2'β position of 2'-deoxycytidine, we synthesized 1-(2-*C*-cyano-2-deoxy-1-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC) from cytidine.[3, 4, 31, 33, 49)]

In vitro cytotoxicity of CNDAC against various human tumor cell lines was compared with araC and DMDC and is summarized in Table II. Although cytotoxicity spectrum of CNDAC is quite different from that of araC, the potency of CNDAC seemed not to be better than that of DMDC. *In vivo* antileukemic activity of CNDAC was compared with araC against i.p.-implanted P388 in CDF_1 mice. CNDAC administered i.p. 10 consecutive days at a dose of 20 mg/kg had a T/C of more than 600%, and 5 out of 6 mice survived more than 60 days, while araC on the same schedule showed a T/C of only 225%. CNDAC was phosphorylated to CNDACMP by dCK, but its relative activity was just a half of the phosphorylation of 2'-deoxycytidine with about 5-fold larger *Km* value (Table III).[51, 52)] Further phosphorylation of CNDACMP to its 5'-triphosphate (CNDACTP) was essential for its antitumor activity.[18)] Unlike araCTP, CNDACTP would not act as a feedback inhibitor of dCK, because its accumulation in cells was in a concentration-dependent manner over a 1000-fold range of CNDAC concentrations. On the other hand, CNDAC was a poor substrate of CDA from mouse kidney with 4.2% of the relative activity (Table III).

CNDACTP inhibited DNA polymerase α with a *Ki* value of 0.16 μM in a competitive manner with dCTP, the *Ki* value of which is about 7-fold more potent than that of araCTP (Table IV). *In vitro* DNA primer extension experiments demonstrated that CNDACMP was incorporated into the *C* site of the elongating DNA strand, and seemed to cause termination

of the primer DNA elongation at the site of the analog incorporation. CNDACTP was an effective substrate for DNA polymerase α, which exhibited a substrate efficiency that was similar to that for dCTP incorporation. After incorporation of CNDACMP in a DNA strand, further elongation was rather slow process, because of its steric effect. However, once a nucleotide was elongated next to CNDACMP, β-elimination occurred to give 2'-*C*-cyano-2',3'-didehydro-2',3'-dideoxycytidine (ddCNC) at the 3'-terminus as our hypothesis.[6, 18, 33)] This β-elimination reaction was confirmed not only in an enzyme level but also in a tumor cell level. Since DNA replication in intact cells is much more complex than the simple *in vitro* model we have used, it is interesting to examine whether such the chemical reaction actually occurs *in vivo*. Moreover, CNDACTP inhibited T7 RNA polymerase with a *Ki* value of 0.25 mM, while a *Km* value of CTP was 0.45 mM. Using whole cells, it was detected that CNDAC also inhibited RNA synthesis.[7)] However, the mechanism of this action in detail and contribution to its antitumor activity are in future studies.

The ability of CNDAC to induce DNA strand breaks by a β-elimination-mediated mechanism after its incorporation into the DNA strand is a novel mechanism. This unique strand-breaking action seems to be the basis of its ability to induce cell cycle arrest at the G_2 phase, as distinct from the S phase block seen in the cells treated with araC and gemcitabine. Since certain DNA-damaging agents such as ionizing radiation and cisplatin are know to block the cell cycle at the G_2 phase, it is logical to speculate that the β-elimination-mediated DNA strand breaks caused by CNDAC might activate similar signaling pathways.[6, 7)]

Since CNDAC is a weak substrate of CDA, which is a major inactivating enzyme and is abundant in human, further derivatives of CNDAC had been prepared. Among a number of the derivatives, oral administrations of N^4-palmitoyl CNDAC (PCNDAC, CS-682) showed the most potent antitumor activity against s.c. implanted M5076 mouse reticulum cell sarcoma. Oral administration of PCNDAC exhibited more potent activity against human tumor xenografts with low toxicity than CNDAC, 5'-deoxy-5-fluorouridine, 5-FU and gemcitabine. Moreover, PCNDAC was effective against P388 leukemic cells resistant to mitomycin-C, vincristine, 5-FU or cisplatin in syngeneic mice.[18)] Its promising antitumor activity in experimental systems have encouraged investigation of its activities in clinical trials with p.o. administration.[10)]

7. 1-(3-*C*-Ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd)

Tumor cells are well known to be heterogeneous. In this respect, the cell cycle of certain tumor tissues is not synchronized. If certain antitumor agent has a mechanism of action inhibiting only DNA synthesis, such the agent only acts in S phase of the cell cycle. Although solid tumor cells grow more rapidly than normal cells, their growth rate is much slower than blood cells. Therefore, such the antitumor agent can kill only a part of the tumor cells. Inhibition of DNA synthesis is the most prominent activity of CNDAC. However, CNDAC also inhibits RNA synthesis in some extent depending on cells used and this effect on RNA synthesis may also contribute to its cytotoxicity, particularly in more indolent cell populations in which RNA synthesis would take on more importance. Therefore, we have been looking for a nucleoside antimetabolite, which inhibits both DNA and RNA syntheses. We designed 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd), from which we expected that ECyd 5'-diphosphate (ECDP) would inhibit ribonucleotide reductase and ECyd 5'-triphosphate (ECTP) would inhibit RNA polymerase to cause both DNA and RNA synthesis inhibition.[5, 19, 20, 35, 45, 46)]

ECyd showed potent cytotoxicity *in vitro* against various human tumor cell lines with nanomoler to subnanomoler levels of the IC_{50} values. Cytotoxic spectrum of ECyd was somewhat different from those of three DNA synthesis inhibitors, araC, DMDC and CNDAC (Table II). ECyd exhibited strong antitumor activity against various human xenographts implanted into nude mice and nude rats via i.v. administration. Its antitumor potency was rather schedule independent and did not produce severe toxicities such as diarrhea, myelosuppression or loss in body weight.[42, 48)]

UCK is a key enzyme for the first phosphorylation of ECyd to ECyd 5'-monophosphate (ECMP) to show cytotoxicity. Further phosphorylations to its 5'-diphosphate (ECDP) and 5'-triphosphate (ECTP) were concomitantly occurred. ECTP, the dead-end metabolite of ECyd, was accumulated in cells and was rather stable in mouse mammary FM3A cells with a half-life of about 81 h, while that of araCTP was less than 10 min. Therefore, intracellular ECTP was some 500-fold more stable than araCTP.[48)] RNA polymerase was inhibited competitively by ECTP in isolated nuclei of FM3A cells. The a *Ki* value of ECTP was 21 nM, while an apparent *Km* value of RNA polymerase for CTP was 8 μM.[42)] Since an IC_{50} value of ECyd in the growth of FM3A cells in vitro was 30 μM, the target enzyme to be responsible for its cytotoxicity would be RNA polymerase.[43)] There are three RNA polymerases (I, II and III) in eukaryote. ECTP was found no selective inhibitors to these polymerases.[46)] This RNA inhibition could lead the tumor cells to apoptosis.[47)] ECyd

was a very poor substrate of CDA from mouse kidney *in vitro*. However, its deaminated analog, 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd), also exhibited almost similar cytotoxicity to ECyd, which also metabolized to its 5'-triphosphate (EUTP) and inhibited RNA polymerase with a *Ki* value of 84 nM (apparent *Km* value of UTP was 13 μM).[19, 34, 48] Initially, ECDP was anticipated to inhibit ribonucleotide reductase, but it did not inhibt the enzyme from *E. coli* even at mM concentrations.

Since UCK activity in human tumor tissues is well known to be relatively high compared to its activity in normal tissues, tumor selective cytotoxicity of ECyd could therefore be expected clinically. Recently, a UCK family consisting of two members, UCK1 and UCK2, has been reported in human cells.[25, 53] Therefore, to determine which isozyme is responsible to the phosphorylation of ECyd is important for further development. We investigated the relations between expression of UCK1 and UCK2 at both mRNA and protein levels, and ECyd phosphorylation activity in a panel of 10 human tumor cell lines.[44] The UCK activity was found to be well correlated with the cells' sensitivity to ECyd. Furthermore, the mRNA or protein expression level of UCK2 was closely correlated with UCK activity in these cell lines, but neither the level of expression of UCK1 mRNA nor that of protein was correlated with the enzyme activity. Comparison of the protein expression level of UCK2 in several human tumor tissues and the corresponding normal tissues exhibited that the expression of UCK2 protein was barely detectable in 4 out of the 5 human tissues, but tended to be high in the pancreatic tumor tissue. In any of the normal tissues, the expression could not be detected at all. Therefore, expression of UCK2 seemed to be correlated with cellular sensitivity to ECyd, and it may contribute to the tumor-selective cytotoxicity of ECyd. ECyd is now under phase I clinical study against solid tumors in U. S. A.

Conclusion

Since the great success of gemcitabine for the treatment of solid tumors in patients has been achieved with a combination of other antitumor agents or radiation therapy, the concept of a nucleoside antimetabolite, although it is the old idea, has been revived to further developments of this class of compounds. The mechanism of action of gemcitabine is somewhat different from those of DMDC, CNDAC and ECyd. Therefore, these new nucleoside antimetabolites would act as different types of antimetabolites than gemcitabine against different tumors if suitable methods of administration or development of proper prodrugs for each the nucleosides would be found. In particular, ECyd which is a novel

antimetabolite targeting RNA polymerases and is different in metabolic pathway from deoxycytidine analogues may lead to the profit in the combination chemotherapy.

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Table I. Cytotoxicity of araC, SMDC, DMDC, CNDAC and 5-FU against various tumor cells.

	IC_{50} (μg/mL)[a]			
cell line	araC	SMDC	DMDC	5-FU
L1210	0.097	0.26	0.37	0.32
CCRF CEM	0.065	0.15	0.067	40
MOLT4	0.056	0.032	0.041	3.8
HL60	>0.1	0.65	0.040	ND
K562	3.2	2.2	ND	38
U937	0.31	0.38	0.44	3.5
PC10	>100	81.8	>100	>100
PC14	>100	>100	>100	>100
KATO III	>100	>100	8.3	10
SW480	>100	>100	3.8	3.7
TE2	>100	>100	2.9	3.3
T24	>100	1.1	1.1	6.1

[a]Tumor cells (2 x 10^3 cells/well) were incubated in the presence or absence of compounds for 72 h. MTT-reagent was added to each well and the plate was incubated for an additional 4 h. The resulting MTT-formazan was dissolved in DMSO and the OD (540 nm) was measured. Percent inhibition was calculated as follows: % inhibition = [1-OD (540 nm) of sample well/OD (540 nm) of control well] x 100. IC_{50} (g/mL) is the concentration that inhibits cell growth by 50%.

Table II. Inhibitory effects of araC, DMDC, CNDAC and ECyd on the growth of human tumor cell lines in vitro.

	IC_{50} (μg/mL)[a]			
cell line	araC	DMDC	CNDAC	ECyd
PC8	0.28	1.0	4.6	0.024
PC9	1.6	<0.3	40	0.029
QG56	>100	3.4	40	0.012
QG95	50	>100	24	0.055
Lu65	ND	1.3	>100	0.0086
KKLS	>100	0.9	>100	0.0056
NUGC4	>100	0.85	5.2	0.0064
KATO III	>100	1.6	15	0.0082
NAKAJIMA	>100	51	2.8	0.046
ST-KM	>100	1.8	2.8	0.013
MKN45	14	0.46	4.6	0.0024
STSA1	0.34	1.0	1.1	0.039
SW48	>100	5.0	>100	>0.5
SW480	>100	3.8	>100	0.070
MG63	>100	0.45	28	0.040
HT1080	0.13	0.56	0.15	0.020
T24	0.50	1.1	9.4	0.0076

[a]See Table I.

Table III. Relative substrate specificity of dCyd, araC, SMDC, DMDC and CNDAC toward deoxycytidine kinase (dCK) and cytidine deaminase (CDA).

compd	dCK			CDA	
	activity[a] (nmol/min·mL)	relative activity	apparent *Km* (μM)	activity[b] (nmol/min·mL)	relative activity
dCyd	3.58	100	10	24.8	100
araC	6.13	170	17	6.12	25
SMDC	2.77	77	52	0.209	1
DMDC	2.27	63	33	0	0
CNDAC	1.79	50	49	1.03	4.2

[a]The reaction mixture containing 100 mM Tris-HCl buffer (pH 7.5), 50 μM each analog, 0.5 mM UTP, 2.4 mM $MgCl_2$, 1 mg/mL BSA, 0.5 mM DTT, 1 mM NaF, 1 mM tetrahydrouridine, and the enzyme in a total volume of 40 μL was incubated at 37 °C for 15 min, followed by HPLC analysis at 280 nm. Each phosphorylation was confirmed by conversion of the product to the substrate by alkaline phophatase, after incubation overnight. [b]The reaction mixture containing 100 mM Tris-HCl buffer (pH 7.5), 0.05–1.0 mM of the substrate, 2.4 mM $MgCl_2$, 1 mg/mL BSA, 0.5 mM DTT, and the enzyme in a total volume of 40 μL was incubated at 37 °C for 5 min, followed by HPLC analysis at 260 nm.

Table IV. Kinetic data of DNA polymerase inhibition by 5'-triphosphates of deoxycytidine analogues.[a]

inhibitor	DNA pol α		DNA pol β		DNA pol γ	
	Ki (*Km*)	*Ki*/*Km*	*Ki* (*Km*)	*Ki*/*Km*	*Ki* (*Km*)	*Ki*/*Km*
(substrate)	μM		μM		μM	
dCTP	(1.25)		(3.90)		(0.77)	
araCTP	1.10	0.88	No inhibition		No inhibition	
SMDCTP	0.05	0.04	0.10	0.02	0.07	0.08
DMDCTP	0.42	0.34	2.52	0.65	1.00	1.30
CNDACTP	0.16	0.14	ND[b]		ND[b]	

[a] DNA polymerases α and β were purified from calf thymus, and DNA polymerase γ was purified from bovine liver according to the methods described previously. DNA polymerase α activity was measured in a mixture (25 μL) containing 40 mM potassium phosphate (pH 7.5), 100 μg/mL of activated calf thymus DNA, 40 mM each of dCTP, dATP and dGTP, 5 mM of [methyl-^{3}H]TTP (4 Ci/mmol), 1 mM dithiothreitol, 8 mM $MgCl_2$, and an aliquot of the reaction mixture. In the case of DNA polymerase β, potassium phosphate was replaced by 40 mM Tris-HCl (pH 8.5). In the case of DNA polymerase γ, 200 mM KCl was added to the mixture for the assay. For kinetic analysis, the concentration of the inhibitor and dCTP were varied. After incubation, the reaction mixture was chilled in an ice bath and 20 μL was transferred to DEAE-cellulose paper disc. The discs were washed with 5% Na_2HPO_4 (x 6), H_2O (x 1), EtOH (x 2) and ether, and dried. The remaining radioactivity was measured in a scintillation liquid.

Figure 1.

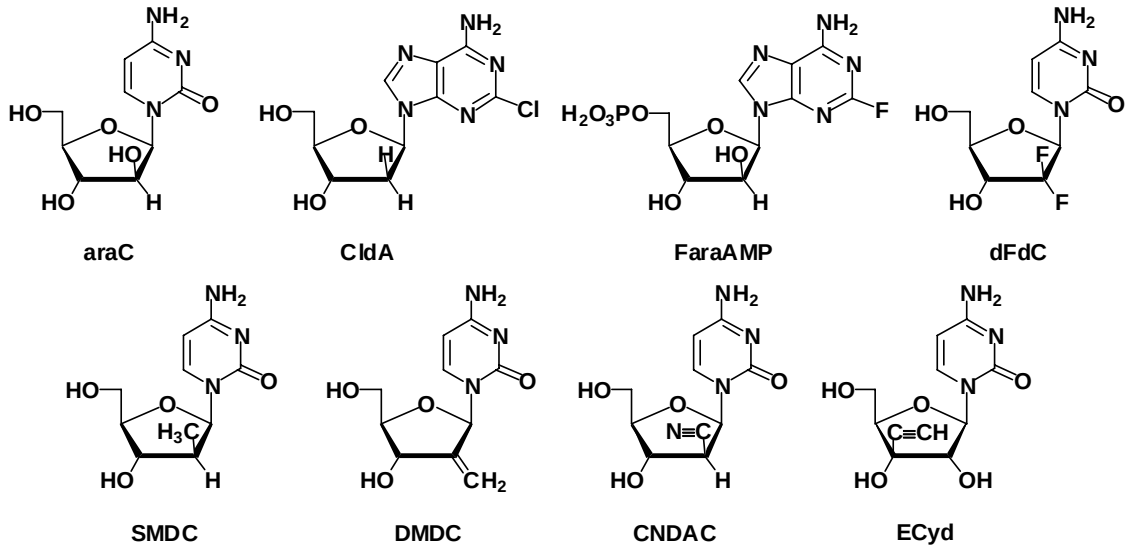


Figure 2.

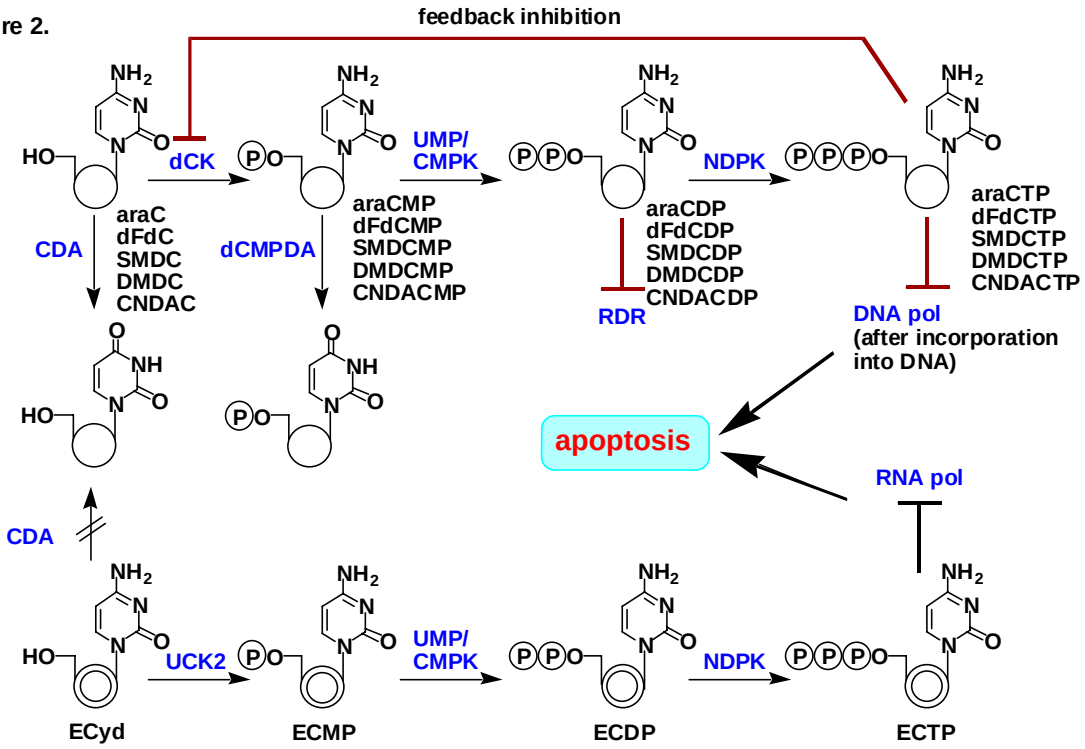
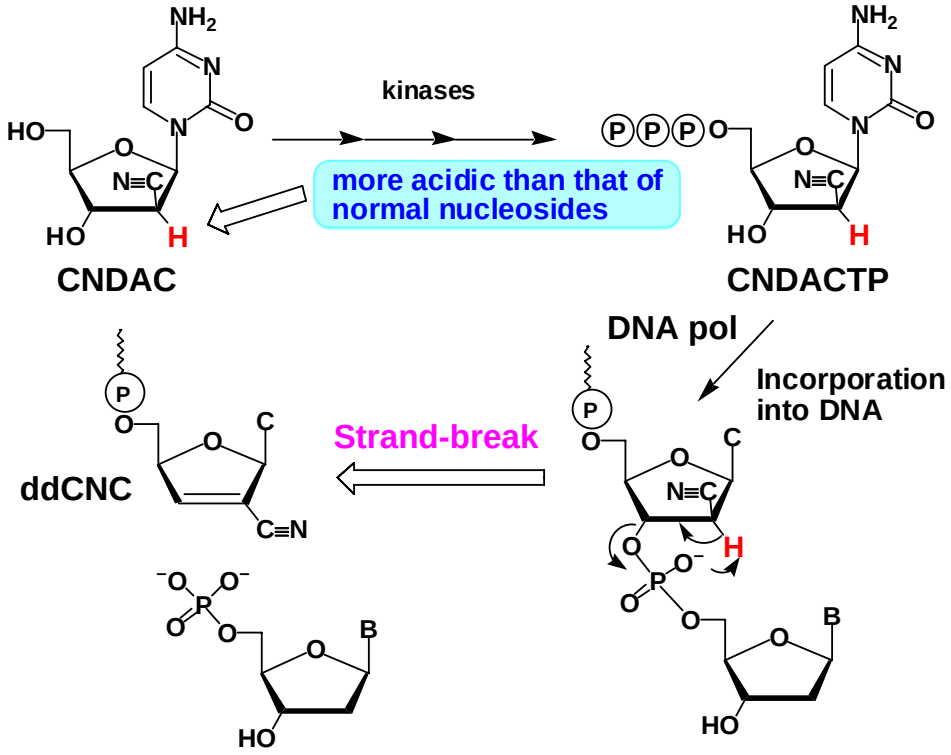


Figure 3.


kinases
more acidic than that of normal nucleosides
CNDAC
CNDACTP
DNA pol
Incorporation into DNA
Strand-break
ddCNC