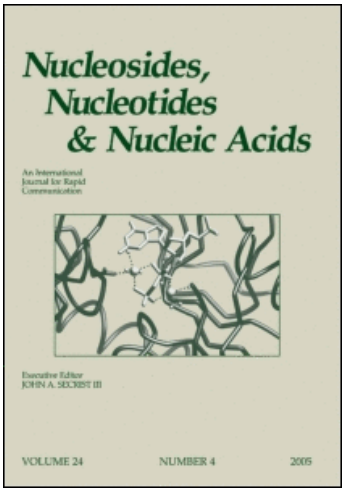

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A New Antitumor Nucleoside, 1-(3-*C*-Ethynyl-β-$_d$-*ribo*-pentofuranosyl)-cytosine (ECyd), Is a Potent Inhibitor of RNA Synthesis

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A NEW ANTITUMOR NUCLEOSIDE, 1-(3-*C*-ETHYNYL-β-D-*RIBO*-PENTOFURANOSYL)CYTOSINE (ECyd), IS A POTENT INHIBITOR OF RNA SYNTHESIS

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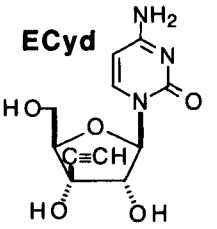
ABSTRACT: The antitumor activity, metabolism, and mechanism of action of a newly developed antitumor nucleoside, 1-(3-*C*-Ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) are described.

INTRODUCTION

The development of nucleoside antimetabolites is important for progress in anticancer chemotherapy. We have been developing 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC)[1] and 1-(2-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)cytosine (CNDAC)[2] as antitumor agents, especially for solid tumors. These compounds have been used in clinical studies. Many solid tumor cells grow slowly,



and drugs have few chances to encounter S-phase, in which DNA synthesis occurs. Therefore, it is not enough for nucleoside antimetabolites to only inhibit DNA synthesis in slow-growing solid tumor cells. It has been reported that these 2'-deoxycytidine analogues also inhibit RNA synthesis to some extent. Therefore, in our search for more potent inhibitors of tumor cell growth, we designed 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)-cytosine (ECyd) and -uracil (EUrd), which we expected would inhibit both DNA and RNA syntheses. We have already reported the synthesis of these nucleosides and the structure-activity relationships both the nucleobase and sugar moieties.[3] Of these, ECyd was found

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to be a potent inhibitor of tumor cell growth in vitro and in vivo. In this report, we describe its antitumor activity, metabolism, and mechanism of action.

Antitumor Activity

We evaluated ECyd against 36 human solid tumor cell lines, including eight stomach, four colon, ten lung, three breast, two pancreas, two bladder, two osteo, one leiomyo, one fibro, two melanoma, and one nasopharyngeal tumor cell lines *in vitro*. ECyd was effective against 22 cell lines with IC_{50} values in the nanomolar to subnanomolar range, and was ineffective against two cell lines.[3b,4] We next examined the antitumor effects of ECyd against human tumors (three stomach, three colon, two pancreas, one renal, one breast, and one bile duct cancers) as xenografts in nude mice, in comparison with the effect of 5-FU. ECyd had a potent antitumor effect on 9 of 11 tumor xenografts and a moderate effect on Hucc-T1 bile duct and BxPC-3 pancreas tumor xenografts, while 5-FU (administered intravenously 14 consecutive days at a dose of 15 mg/kg) had a potent antitumor effect only on H-81 stomach tumor and moderate antitumor effects on other human tumors. The doses of ECyd and 5-FU used in this study were each the respective maximum nontoxic doses. Since multiple-dose schedules were not tested, we may not have used these drugs under optimal conditions.[3b,4]

In gastric cancer, peritoneal dissemination is the most frequent cause of non-curative resection and recurrence after curative resection. At the present time, there is no effective treatment for this disease. We recently developed a new animal model for this disease by the intraperitoneal (ip) inoculation of highly metastatic human gastric cancer cell line MKN-45P in nude mice. When EUrd was administered ip at a dose of 10 mg/kg in this model, it showed excellent antitumor activity (T/C > 227%).

Metabolism

The metabolism of ECyd in mouse mammary tumor FM3A cells was studied using [cytosine-5-^{3}H]ECyd. ECyd is not deaminated by cytidine deaminase, and is a relatively good substrate of cellular uridine/cytidine kinase (UCK), which converts ECyd to its monophosphate (ECMP).[3c,5] ECMP is further converted into its diphosphate (ECDP) and triphosphate (ECTP) in tumor cells.[5-7] The amount of ECTP time- and dose-dependently increased in the cells. The intracellular stability of ECTP was compared with that of araCTP. After the incubation of ECyd (3.0 μM) or araC (44 μM), acid-soluble fractions were prepared from the drug-treated FM3A cells and analyzed using HPLC. AraCTP was eliminated from the cells with a half-life of less than 10 min, while ECTP had a half-life of about 79 h.[5] Intracellular ECTP was about 480 times more stable than araCTP. Therefore, ECyd is on a "closed" metabolic pathway and accumulates as a dead-end metabolite, ECTP.

Mechanism of Action

When FM3A cells were treated with ECyd (3.0 μM) or Actinomycin D (0.21 μM), nucleoli, in which ribosomal RNA is mainly synthesized, shrank before the cells began to

die. The time required for ECyd and Actinomycin D to reduce the percentage of cells with intact nucleoli to 50% was 3.5 and 2.4 h, respectively. In contrast, araC (44 μM) did not affect nucleoli.[5] At the same time, RNA synthesis, but not DNA synthesis, was inhibited to 30% by ECyd at the same concentration. Therefore, ECyd predominantly inhibits RNA synthesis. However, this inhibition of RNA synthesis was not accompanied by decreases in pools of rNTP precursors for RNA synthesis. Therefore, the inhibition of RNA synthesis must involve the inhibition of RNA polymerization by ECTP. In preliminary experiments, RNA polymerase was inhibited competitively by ECTP in isolated nuclei of FM3A cells. The *Ki* value of ECTP was 21 nM (apparent *Km* value of CTP was 8.0 μM). On the other hand, ECDP did not inhibit ribonucleoside diphosphate reductase from *E. coli* at up to 1 mM.[8] Thus, ECyd is a rather pure inhibitor of RNA polymerase after its conversion to ECTP, and this action is predominantly responsible for inhibiting tumor cell growth.

Induction of Apoptosis

Accompanying ECyd-induced cell death in FM3A[9] and MKN-45[4] cells with the wild-type *p53* gene, about 100-200 kbp-sized DNA and internucleosomal DNA fragmentation were observed by gel electrophoresis. However, in tumor cells with a mutated *p53* gene or which are deficient in the *p53* gene, such as MKN-28 (point mutation) and KATO III (deficient) cells, treatment with ECyd caused cell death by necrosis but not apoptosis.

Recently, it has been well documented that proteases play a key role in the signal pathways of apoptosis. Therefore, we investigated the effects of protease inhibitors on ECyd (3.0 μM)-induced cell death. Z-Asp-CH_2-DCB (a caspase inhibitor, 100 μM), TLCK and TPCK (serine protease inhibitors, 200 and 5.0 μM, respectively) effectively blocked cell death, whereas 1, 10-phenanthroline (a metalloprotease inhibitor, 100 μM), pepstatin A (an aspartic protease inhibitor, 100 μM), and antipain (a cystein protease inhibitor, 100 μM) failed to exert such inhibitory effects.[9] On gel electrophoretic analysis, Z-Asp-CH_2-DCB prevented DNA fragmentation. On the other hand, antipain failed to prevent DNA fragmentation. These data suggest that caspases play a key role in a sequence of cell-death events which lead to DNA fragmentation.

CONCLUSION

Low doses of 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) show potent antitumor activity against several human solid tumors xenografted into nude mice and rats. ECyd-induced cell death is accompanied by apoptosis when cells have the wild-type *p53* gene, while ECyd induces necrosis when cells have the mutated *p53* gene or are deficient in the *p53* gene. ECyd is phosphorylated by uridine/cytidine kinase (UCK) to its 5'-monophosphate, and is further converted into 5'-triphosphate (ECTP), which inhibits RNA polymerase quite potently. UCK-dependent chemotherapy is highly promising because UCK is much more active in various human tumor tissues than in non-neoplastic tissues.[10]

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