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Mechanism of tumor cell killing by HO-221, a novel antitumor compound

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Summary. The mechanism of tumor cell killing by HO-221, a novel benzoylphenylurea derivative that shows broad-spectrum antitumor activities, was studied. HO-221 strongly inhibited the activity of mammalian DNA polymerase α but not that of DNA polymerases β or γ. The inhibition was equivalent to that induced by aphidicolin and ara-CTP, which were selective inhibitors of the enzyme. Furthermore, the inhibition by HO-221 of DNA polymerase α was found to be non-competitive with respect to dCTP as a substrate, unlike that induced by aphidicolin and ara-CTP. The inhibition was reduced the addition of an excess of DNA polymerase α but not by excess amounts of activated DNA as a template primer. These results suggest that HO-221 inhibits the activity of DNA polymerase α by direct interaction with the enzyme in contrast to the impairment of template activity through intercalation into DNA induced by anthracycline compounds. On the other hand, HO-221 showed almost no effect on RNA polymerase activity, the reverse transcriptase activity of avian myeloblastosis virus or protein synthesis in a cell-free system. The flow-cytometry analysis revealed that HO-221 accumulated HL-60 cells in G1–S phases at a low concentration but increased the number of cells in the G1 phase at a higher concentration, stopping cell-cycle progression. The results suggest a correlation between cell-cycle progression and inhibition by HO-221 of DNA polymerase α, which plays a role in DNA replication during the S phase in living cells.

Introduction

HO-221, *N*-[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl) urea, is a novel antitumor compound that was chosen from many related compounds for the present study because of its excellent antitumor activities and its lack of particular toxicities in animals. We have previously reported that this compound shows significant activity against various murine tumors and human xenografts and is especially effective against solid tumors [3]. HO-221 also shows no cross-resistance to ten antitumor agents that are clinically used for the treatment of human cancer. In the present study, we attempted to clarify the mechanism of tumor cell killing by HO-221 underlying its antitumor effect.

Abbreviations: Ara-C, cytosine arabinoside; *ara-CTP*, 1-β-D-arabinofuranosylcytosine-5′-triphosphate; *ACNU*, nimustine; *BCNU*, carmustine; *HN2*, nitrogen mustard, *ACM*, actinomycin D; *Cy-ic*, cyclohexyl isocyanate; *ADM*, Adriamycin; *DM*, daunomycin; *CDDP*, cisplatin; *ddTTP*, 2′,3′-deoxythymidine 5′-triphosphate

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Materials and methods

Chemicals. *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl) urea (HO-221; molecular weight, 492.67 Da) was synthethized. For in vitro study, it was dissolved in 12.5% dimethyl β-cyclodextrin solution. 1-β-D-arabinofuranosyl cytosine 5′-triphosphate (ara-CTP), actinomycin D (ACM), nitrogen mustard (HN2), 2′,3′-deoxythymidine 5′-triphosphate (ddTTP), rifampicin and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo.); aphidicolin, from Wako Chemical Co. (Osaka, Japan); cyclohexyl isocyanate (Cy-ic), from Aldrich Chemical Co. (Milwaukee, Wis.); nimustine (ACNU), from Sankyo Co. (Tokyo); carmustine (BCNU), from Bristol-Myers Pharmaceutical Co. (Syracuse, N.Y.); daunomycin (DM), from Meiji Seika Kaisya Co. (Tokyo); Adriamycin (ADM), from Kyowa Hakko Kogyo Co. (Tokyo); cisplatin (CDDP), from Nippon Kayaku Co. (Tokyo); α-amanitin, from Boehringer Ingelheim GmbH (FRG); emetine, from Fluka Chemie AG (Switzerland); [^{3}H]-dTTP (3.75 Ci/mmol), [5,6-^{3}H]-UTP ([^{3}H]-UTP, 5 Ci/mmol), [6-^{3}H]-thymidine ([^{3}H]-TdR, 19.3 Ci/mmol), [6-^{3}H]-uridine, ([^{3}H]-UR, 4.2 Ci/mmol) and L-[3,4,5-^{3}H]-leucine ([^{3}H]-Leu, 147 Ci/mmol), from New England Nuclear Co. (Boston, Mass.); and activated calf-thymus DNA from Cooper Biochemical Co. (Malvern, Pa.).

Enzymes. Calf-thymus DNA polymerase α and wheat-germ RNA polymerase II were purchased from Pharmacia Co. (Sweden). DNA polymerases α, β and γ were partially purified from KB cells synchronized for growth by hydroxyurea treatment according to the methods of Yoshida et al. [8]. The reverse transcriptase of avian myeloblastosis virus (AMV) was purchased from Bethesda Research Laboratories (Gaithersburg, Md.).

Tumor cells. Mouse leukemia L1210 cells were grown in RPMI 1640 medium with 10% fetal calf serum. Mouse leukemia P388 was main-

tained by serial i.p. passage in DBA2 mice and the tumor cells were obtained on days 8 after tumor inoculation according to NCI protocols [1]. Human KB cells (human carcinoma of the nasopharynx) and HL-60 cells (human promyelocytic leukemia) were grown in minimum essential medium (MEM) and RPMI 1640 with 10% fetal calf serum, respectively.

Inhibition of macromolecular synthesis. The synthesis of DNA, RNA and protein in these cell systems was measured by th incorporation of [^{3}H]-TdR, [^{3}H]-UR and [^{3}H]-Leu into the acid-insoluble fractions. HO-221 or Ara-C was added to L1210 or KB cell suspensions (1×10^6 cells/ml) to give the desired concentration. After 1 or 24 h incubation, tritiated nucleosides were added to a final concentration of 0.001 mCi/ml and the incubation was then continued for 1 h at 37° C. The cells were washed three times with phosphate-buffered saline (PBS) and suspended in the same buffer. The cells were then trapped on glass-fiber filter paper (Whatman GF/C) and successively washed with PBS, cold 5% TCA and 95% ethanol. After drying, the radioactivity on the filter paper was counted in the scintillation fluids.

Assay of DNA polymerases. The standard reaction mixture (0.25 ml) for calf-thymus DNA polymerase α contained 60 mM TRIS-HCl (pH 7.5); 6 mM $MgCl_2$; 5 mM 2-mercaptoethanol; 4 mM adenosine 5′-triphosphate (ATP); 0.04 mM each of dATP, dCTP and dGTP; 0.025 mM [^{3}H]-dTTP; 0.05 mg activated calf-thymus DNA; and 0.3 units DNA polymerase α. When DNA polymerases from KB cells were used, the reaction mixture (0.25 ml) for the α-enzyme contained 50 mM TRIS-HCl (pH 7.5); 2.5 mM $MgCl_2$; 2 mM 2-mercaptoethanol; 4 mM ATP; 0.1 mM each of dATP, dCTP and dGTP; 0.02 mM [^{3}H]-dTTP; 0.125 mg activated calf-thymus DNA; 0.05 mg bovine serum albumin (BSA); and the α-enzyme fraction. The reaction mixture for the β-enzyme contained 50 mM TRIS-HCl (pH8.5); 10 mM $MgCl_2$; 50 mM KCl; 10 mM *N*-ethylmaleimide; 0.1 mM each of dATP, dCTP and dGTP; 0.02 mM [^{3}H]-dTTP; 0.125 mg activated calf-thymus DNA; 0.05 mg BSA; and the β-enzyme fraction. The reaction mixture for the γ-enzyme comprised 50 mM TRIS-HCl (pH 8), 0.5 mM $MnCl_2$, 50 mM KCl, 50 mM potassium phosphate, 2 mM 2-mercaptoethanol, 0.05 mM [^{3}H]-dTTP, 0.0125 mg poly-(rA).(rT)12, 0.05 mg BSA and the γ-enzyme fraction.

After incubation at 37° C for 30 or 60 min, acid-insoluble radioactivity was measured. For assay as to whether HO-221 interacts with DNA polymerase α in the inhibition of DNA synthesis, the enzyme activity was measured by the addition of activated calf-thymus DNA (0.05–0.4 mg/ml) to reaction mixture containing 5×10^{-5} M HO-221, 2×10^{-5} M DM, 1×10^{-4} M aphidicolin and 2×10^{-4} M ara-CTP, respectively. For determination as to whether HO-221 interacts with DNA as a template primer, the same experiment was done by adding DNA polymerase (0.2–1 unit) to 1×10^{-4} M of HO-221, 6×10^{-5} M DM, 1×10^{-4} M aphidicolin and 1×10^{-3} M ara-CTP, respectively. Furthermore, for evaluation of the mode of inhibition by HO-221 of DNA polymerase α, data were plotted according to Lineweaver and Burk.

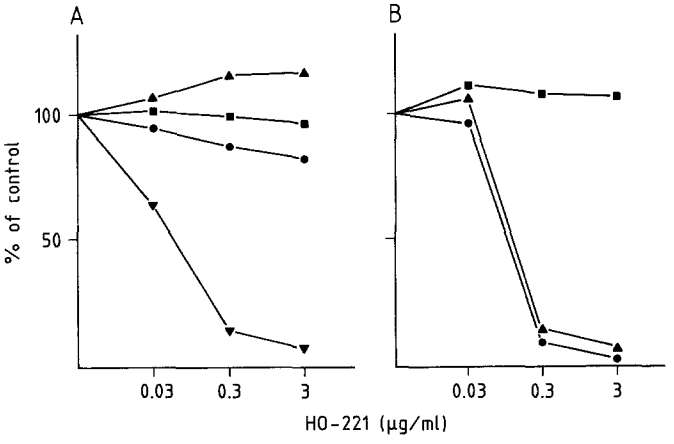

Fig. 1 A, B. Effect of HO-221 on the DNA, RNA and protein synthesis of **A** L1210 cells after 1 h and **B** KB cells after 24 h. HO-221 dissolved in dimethyl-β-cyclodextrin solution was added to the cell suspension (10^6 cells/ml). The results are expressed as the percentage of incorporation of tritium related to the control value. The incorporation of [^{3}H]-UR (▲) and [^{3}H]-Leu (■) by L1210 cells that had been incubated with Ara-C (▼) was almost the same as that of cells that had been treated with HO-221. ●, [^{3}H]-TdR

Assay of RNA polymerase. The standard reaction mixture (0.1 ml) for wheat-germ RNA polymerase II contained 50 mM TRIS-HCl (pH 7.5); 0.15 M NaCL; 10 mM $MgCl_2$; 0.5 mM ethylenediaminetetraacetic acid (EDTA); 0.1 mM DTT; 0.2 mM each of ATP, CTP and GTP; 0.02 mM [^{3}H]-UTP; 0.01 mg calf-thymus DNA; and 0.4 unit RNA polymerase. After incubation at 25° C for 30 min, acid-insoluble radioactivity was measured.

Assay of protein synthesis in a cell-free system. This assay was done mainly by using a kit (reticulocyte-lysate translation system) from New England Nuclear Co. The reaction mixture (0.025 ml) contained 0.01 ml rabbit reticulocyte lysate, 84.4 mM potassium acetate, 0.78 mM magnesium acetate, [^{3}H]-Leu (0.02 mCi) and 0.0001 mg rabbit globin mRNA. After incubation at 37° C for 30 min, acid-insoluble radioactivity was measured. The assay was performed according to the method of Pelham and Jackson [6].

Assay of reverse transcriptase. The standard reaction mixture (0.05 ml) contained 100 mM TRIS-HCl (pH 8.3); 0.05 M KCl; 10 mM $MgCl_2$; 10 mM DTT; 0.05 mM each of dATP, dCTP and dGTP; 0.025 mM dTTP; 0.02 mM [^{3}H]-dTTP; 0.001 mg rabbit globin mRNA; oligo-dT12-18 (0.02 mg/ml); and 0.5 unit AMV reverse transcriptase. After incubation at 37° C for 20 min, acid-insoluble radioactivity was measured [4, 7].

Cell-cycle analysis. HL-60 cells at 10^6/ml were treated with HO-221 at 37° C for 24 and 48 h, and the number of cells was counted using a Coulter counter. The cells (5×10^6) were washed with PBS, then fixed with cold 70% ethanol. The cells were digested with 1 mg/ml RNase in PBS at 37° C for 30 min and then suspended in the same volume of propidium iodide staining solution in PBS (1 mg/ml) [2]. The stained cells were kept at 4° C before cell-cycle analysis using a flow cytometer (Cytofluorograf system 50H, Ortho Co.).

Results

Effect of HO-221 on nucleic acid and protein synthesis

As shown in Fig. 1, the incorporation of [^{3}H]-TdR by L1210 cells and that of [^{3}H]-TdR and [^{3}H]-UR by KB cells (acid-insoluble fractions) was slightly inhibited by HO-221. No inhibition of the incorporation of [^{3}H]-Leu was observed at a drug concentration of 0.003 mg/ml. On the other hand, ara-C markedly inhibited the incorporation of [^{3}H]-TdR by L1210 cells. The selectivity of the inhibition by HO-221 of DNA and RNA synthesis was not clarified in this experiment.

Effect of HO-221 on DNA polymerases

All antitumor agents used inhibited DNA polymerase α activity in a dose-dependent manner (Fig. 2). The concentrations required to inhibit cell growth by 50% (IC_{50}) for individual agents were 1.1×10^{-4} M for HO-221, 3.3×10^{-5} M for aphidicolin and 2×10^{-4} M for ara-CTP; these were selective inhibitors of DNA polymerase α activity. Comparison of these values revealed that the inhibitory activity of HO-221 was equivalent to that of aphidi-

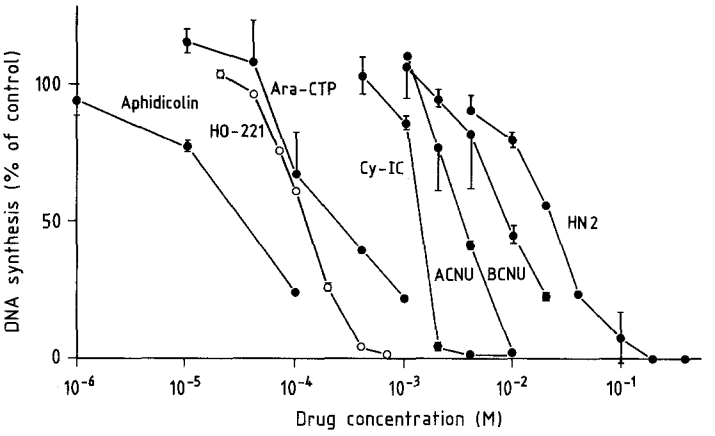

Fig. 2. Effect of HO-221 on DNA polymerase α activity. The activity of the enzyme from calf thymus with activated DNA was measured in the presence of various antitumor agents. The results are expressed as the percentage of incorporation of [^{3}H]-dTTP relative to the control value, which was about 60,000 cpm

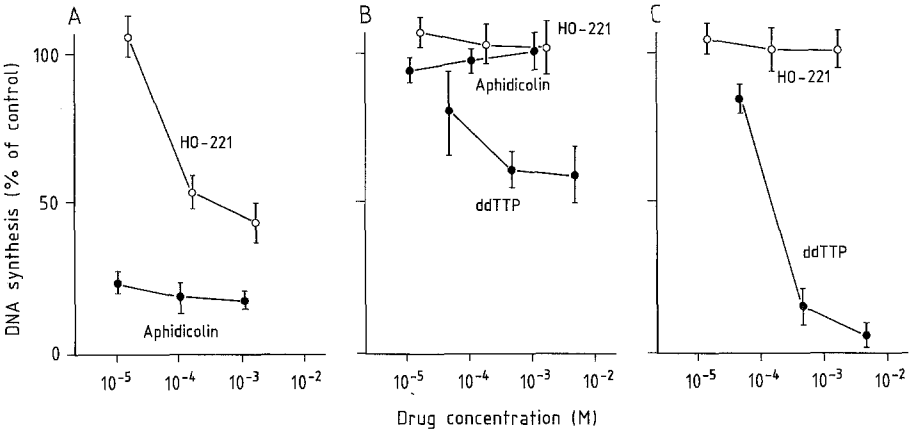

Fig. 3 A–C. Effect of HO-221 on DNA polymerase **A** α, **B** β and **C** γ from KB cells. Aphidicolin and ddTTP were the selective inhibitors for the α- and for the β- and γ-enzymes, respectively. The results are expressed as the percentage of incorporation of [^{3}H]-dTTP relative to the control value

colin and ara-CTP. On the other hand, inhibition by alkylating agents (ACNU, BCNU, Cy-ic, HN2) was weak compared with that induced by the selective inhibitors. As shown in Fig. 3, HO-221 also inhibited the activity of DNA polymerase α from KB cells but not that of DNA polymerases β or γ.

Mode of inhibition of DNA polymerase α activity by HO-221

The inhibition of DNA polymerase α by DM was reduced by the addition of an excess of activated DNA as a template primer, but inhibition by HO-221 remained unchanged, as did that by aphidicolin or ara-CTP. On the other hand, the inhibition by HO-221 was reduced by the addition of an excess of DNA polymerase α, in contrast to that by the other three agents (Fig. 4). As shown in Fig. 5, the mode of inhibition by HO-221 of the enzyme according to double reciprocal plot analysis was non-competitive with respect to dCTP, a deoxynucleoside triphosphate. On the other hand, aphidicolin and ara-CTP showed a competitive inhibition pattern.

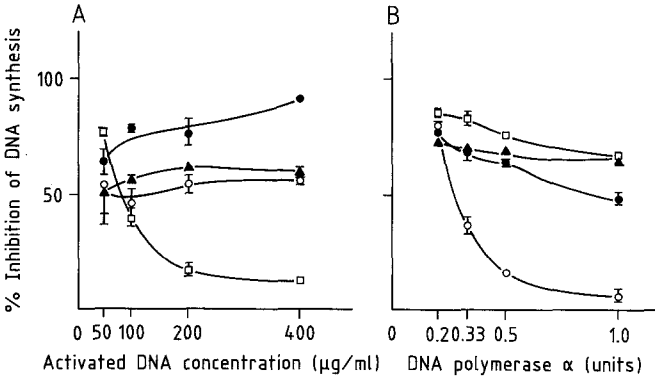

Fig. 4 A, B. Effect of **A** activated DNA and **B** DNA polymerase α on the inhibition of DNA polymerase α activity by HO-221 (○). The activity was measured by the addition of various amounts of activated DNA or enzyme in the presence of fixed concentrations of antitumor agents. ●, Aphidicolin; ▲, ara-CTP; □, daunomycin

Effect of HO-221 on RNA polymerase and protein synthesis

Table 1 shows that HO-221 did not affect the activity of either RNA polymerase II or protein synthesis in a cell-free system, in contrast to the inhibitory effect of α-amanitin and actinomycin D on RNA polymerase and that of emetine and cycloheximide on protein synthesis.

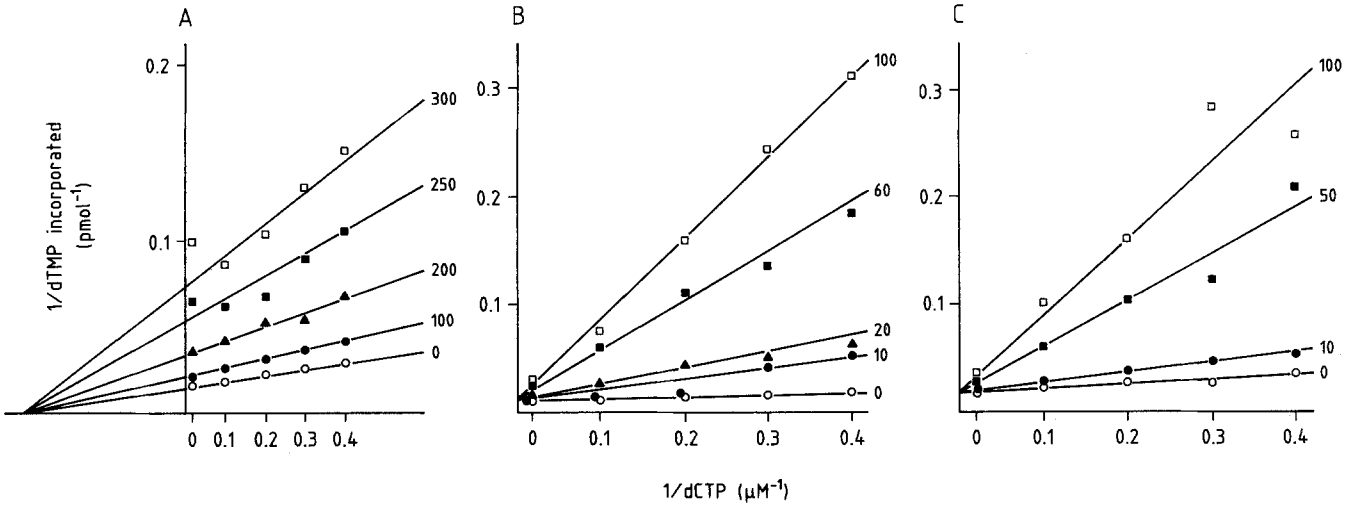

Fig. 5 A – C. Double reciprocal plot of the inhibition by **A** HO-221, **B** ara-CTP and **C** aphidicolin of DNA polymerase, expressed in μM. Each of the three drugs was added to the reaction mixture as indicated in the figure, and the concentration of dCTP was varied in the presence of fixed concentrations of the agents. Data are plotted according to Lineweaver and Burk

Table 1. Effect of HO-221 on RNA polymerase II-dependent RNA synthesis and protein synthesis

Drug	Inhibition of RNA polymerase II activity
	IC_{50} (M)
HO-221	2.6×10^{-3}
Ara-CTP	5.4×10^{-4}
Adriamycin	9.0×10^{-6}
Actinomycin D	3.4×10^{-7}
α-Amanitin	1.4×10^{-8}

Drug	Inhibition of protein synthesis
	IC_{50} (M)
HO-221	$>1.0 \times 10^{-3}$
Emetine	1.0×10^{-6}
Cycloheximide	1.0×10^{-6}
Adriamycin	3.0×10^{-5}
Ara-CTP	4.0×10^{-4}
Cisplatin	$>1.0 \times 10^{-4}$

The activity of wheat-germ RNA polymerase II was measured by incorporation of [^{3}H]-UTP into the acid-insoluble fraction after incubation at 25° C for 30 min (control; 50,000 cpm). After protein synthesis, the reaction mixture containing rabbit reticulocyte lysate and rabbit globin mRNA was incubated at 37° C for 30 min, and then the incorporation of [^{3}H]-Leu into the acid-insoluble fraction was measured (control; 30,000 cpm)

Table 2. Effect of HO-221 on the reverse transcriptase activity of AMV

Drug	Concentration % of		
	(M)	control	IC_{50} (M)
HO-221	1.6×10^{-4}	95	
	8×10^{-4}	96	$>1.6 \times 10^{-3}$
	1.6×10^{-3}	86	
Adriamycin	1×10^{-5}	117	
	1×10^{-4}	64	1.9×10^{-4}
	1×10^{-3}	15	
Actinomycin D	1×10^{-5}	121	$>1 \times 10^{-4}$
	1×10^{-4}	63	
Rifampicin	1×10^{-5}	100	$6..5 \times 10^{-5}$
	1×10^{-4}	39	
Ara-CTP	1×10^{-4}	72	
	1×10^{-3}	45	3.5×10^{-4}
	1×10^{-2}	2	

Radioactivity incorporated in control samples (drug-free) amounted to 38,000 cpm

Effect of HO-221 on AMV reverse transcriptase

Inhibition of AMV reverse transcriptase was observed for rifampicin, Adriamycin, actinomycin D and ara-CTP. In contrast, HO-221 showed no effect on the activity of this enzyme at 1.6×10^{-3} M (Table 2).

Effect of HO-221 on cell-cycle progression

Figure 6 shows that HO-221 accumulated HL-60 cells in the G1- S phases at a low concentration and showed S-phase accumulation at the median concentration used. At a higher concentration, HO-221 increased the number of cells in the G1 phase, stopping cell-cycle progression.

Discussion

HO-221 is a novel benzoylphenylurea derivative that shows significant activity against various experimental murine tumors and human xenografts. There is no cross-resistance between HO-221 and other known antitumor agents [3]. We suggest that in addition to its lack of partic-

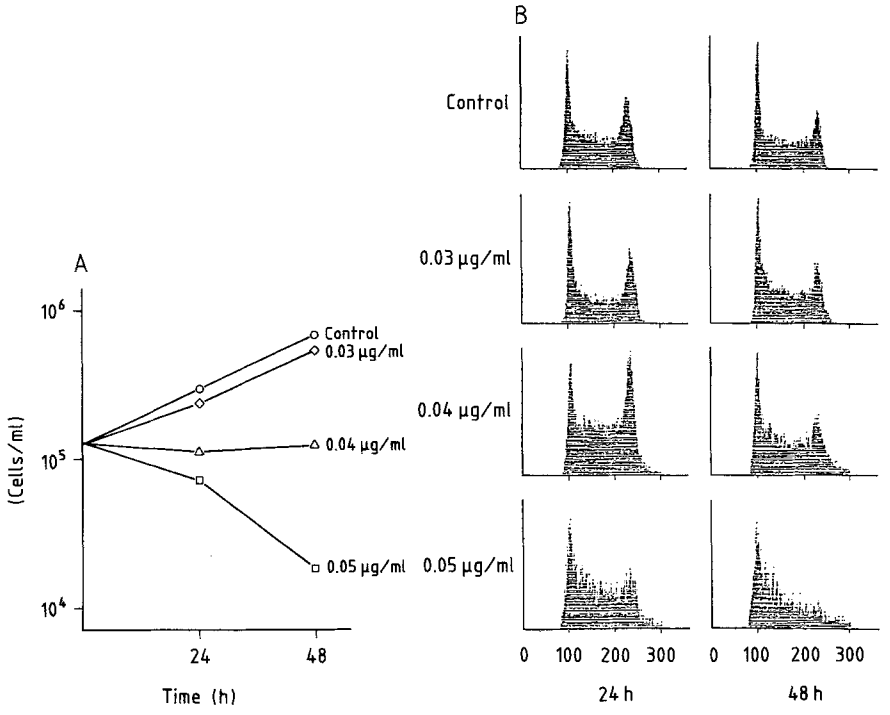

Fig. 6 A, B. Effect of HO-221 on the **A** cell growth and **B** cell-cycle progression of HL60 cells. The results show the changes in the number of viable cells and in the cell-cycle-phase distribution after 24 and 48 h treatment with HO-221

ular toxicities in animals, this compound is promising as a therapeutic drug for human cancer chemotherapy. In this report, we examined the mechanism of tumor cell killing underlying the antitumor effect of HO-221.

The effect of HO-221 on DNA and RNA synthesis in L1210 and KB cells was inhibitory as compared with that on protein synthesis. However, the selectivity of this inhibition relative to that of ara-C was not clarified. Similar results were also obtained in these cell lines using varying durations of exposure to HO-221 (data not shown). The inhibitory effect of HO-221 and six antitumor agents on DNA polymerase α was examined in vitro. HO-221 was found to inhibit the enzyme activity strongly. Comparison of IC_{50} values for the individual agents revealed that the inhibitory activity of HO-221 was equivalent to that of aphidicolin and ara-CTP, which are selective inhibitors of DNA polymerase α activity. Alkylating agents such as ACNU, BCNU and HN2, targets of which were DNA as both template and cellular proteins, weakly inhibited the activity of the enzyme (Fig. 2). Furthermore, HO-221 also inhibited the activity of DNA polymerase α partially purified from KB cells (human carcinoma of the nasopharynx). However, inhibition by HO-221 of two other types of enzyme from KB cells, DNA polymerase β and γ, was not observed in this assay. Therefore, HO-221 seems to exert selective inhibition on DNA polymerase α.

Ara-C has been used clinically for the treatment of acute leukemia and lymphoma. The antitumor activity this agent is attributed to the metabolite 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP), which strongly inhibits DNA polymerases α and β by competing only with dCTP [8]. Aphidicolin is also a selective inhibitor of DNA polymerase α but not of DNA polymerases β and γ [5]; its inhibition is competitive with respect to the four deoxynucleoside substrates [9]. On the other hand, the inhibition by HO-221 of DNA polymerase α was found to be non-competitive with respect to dCTP, unlike that of aphidicolin or ara-CTP (Fig. 5). HO-221 also showed the same mode of inhibition with respect to the other three deoxynucleoside substrates (dATP, dGTP and dTTP; data not shown). These results suggest that HO-221 is a potent selective inhibitor of the activity of DNA polymerase α whose mechanism of action is different from that of ara-C, an effective anti-leukemic agent.

Moreover, the inhibitory activity of HO-221 on DNA polymerase α in contrast to that of ara-CTP, aphidicolin and DM was reduced by the addition of an excess of the DNA polymerase. However, inhibition by HO-221 was not reduced by the addition of an excess of activated DNA as a template primer (Fig. 4). These results suggest that HO-221 inhibits the activity of DNA polymerase α by direct interaction with the enzyme as opposed to the impairment of template activity through intercalation into DNA induced by DM. On the other hand, HO-221 showed almost no effect on RNA polymerase activity, AMV reverse transcriptase activity or protein synthesis in a cell-free system. Furthermore, in our investigations of the effect of HO-221 on the cell-cycle progression of HL-60 cells, the compound accumulated cells in the G1 and S phases (Fig. 6). The results suggest a correlation between cell-cycle progression and the inhibition by HO-221 of DNA polymerase α, which plays a role in DNA replication during the S phase in living cells.

These data, coupled with the significant activity of HO-221 against experimental tumors and its lack of cross-resistance to known antitumor agents, may suggest that HO-221 is a unique therapeutic drug among antimetabolic antitumor agents for cancer chemotherapy.

References

1. Geran RI, Greenberg NH, MacDonald MM (1972) Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep 3(3): 1
2. Krishan A (1975) A rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodine staining. J Cell Biol 66: 188
3. Nakajima T, Masuda H, Okamoto T, Watanabe M, Yokoyama K, Yamada N, Tsukagoshi S, Taguchi T (1990) Antitumor activity on murine tumors of a novel antitumor benzoylphenylurea derivative, HO-221. Cancer Chemother Pharmacol (in press)
4. Nishio M, Kuroda A, Suzuki M, Ishimaru K, Nakamura S, Nomi R (1983) Retrostatin, a new specific enzyme inhibitor against avian myeloblastosis virus reverse transcriptase. J Antibiot 36: 761
5. Ikegami S, Taguchi T, Ohashi M, Oguro M, Nagano H, Mano Y (1978) Aphidicolin prevents mitotic cells division by interfering with the activity of DNA polymerase α. Nature 275: 458
6. Pelham HRB, Jackson RJ (1976) An efficient mRNA-dependent translation system from reticulocyte lysates. Eur J Biochem 67: 247
7. Wang LH, Duesberg P, Beemon K, Vogt PK (1975) Mapping RNase T1-resistant oligonucleotides of avian tumor virus RNAs. J Virol 16: 1051
8. Yoshida S, Yamada M, Masaki S (1977) Inhibition of DNA polymerase α and β of calf thymus by 1-β-arabinofuranosylcytosine-5′-triphosphate. Biochim Biophys Acta 477: 144
9. Oguro M, Suzuki-Hori C, Nabano H, Mano Y, Ikegami S (1979) The mode of inhibitory action by aphidicolin on eukaryotic DNA polymerase α. Eur J Biochem 97: 603