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Antitumor effect of alpha isomer of anordrin *in vitro* and cell cycle arrest at G_1 phase

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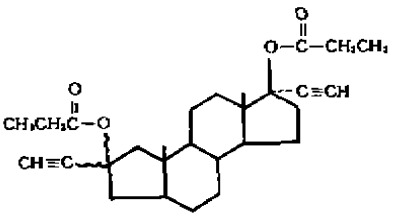
ABSTRACT Alpha isomer of anordrin (2α, 17α-diethyl-A-norandrostane-2β, 17β-diol dipropionate, α-Ano) inhibited mouse hepatoma (Hep A) and P388 cell growth *in vitro*. α-Ano (20 μg·ml^{-1}) inhibited the incorporation of [^{3}H]uridine and [^{3}H]thymidine into RNA and DNA within 3 h, but the inhibition of *L*-[^{3}H]lysine incoporating into protein was not obvious. α-Ano had no effect on the DNA-dependent RNA synthsis with purified nuclei of Hep A cells. It is suggested that the inhibition of RNA and DNA syntheses is the major cause of the cytostatic effect. α-Ano blocked the P388 cells at G_1/G_0 phase, and the delay in G_1/G_0 to S phase transition plays an important role in the inhibition of P388 cell growth.

KEY WORDS norandrostanes; anordrin; experimental liver neoplasms; leukemia P388; thymidine; uridine; cell cycle

Anordrin has been used as a contraceptive[1]. It was proved that the alpha isomer of anordrin could produce obvious antitumor action on some experimental tumors *in vitro* and *in vivo*, while the beta isomer had no effect [2]. The drug might be clinically useful for anticancer treatment because of its therapeutic action and low toxicity. In the present work, we investigated the mechanism of the anticancer action of alpha isomer of anordrin.



α-anordrin

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MATERIALS AND METHODS

Materials Anordrin was produced by Shanghai No 19 Pharmaceutical Factory. It is a mixture of alpha and beta isomers, and the alpha isomer (α-Ano) was isolated and purified by low pressure column chromatography of aluminum oxide in our institute[3]. α-Ano was initially dissolved in absolute ethanol (the final concentration of ethanol was below 0.1%) and stored at 4 C, used within 2 wk of preparation. [^{3}H]TdR (850 TBq·mol^{-1}), [^{3}H]UR (780 PBq·mol^{-1}), and [^{3}H]ATP (960 PBq·mol^{-1}) were purchased from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. *L*-[^{3}H]Lys (3070 PBq·mol^{-1}) was Amersham product, and propidium iodide (PI) was Sigma product. RNase A was purchased from Shanghai Institute of Biochemistry, Chinese Academy of Sciences.

Cell culture Mouse hepatoma (Hep A) and P388 cells were routinely cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 IU·ml^{-1}), and streptomycin (100 mg·L^{-1}). Cells were cultured at 37 C in 5% CO_2.

Cell growth determination Cells were seeded at 4 × 10^4/well in 96-well plates, and treated with α-Ano at the appropriate concentrations. They were counted with a hemocytometer at different time intervals in culture. Trypan blue exclusion was used as an indicator of viability.

Radioactive precussors incoporation assay Hep A cells were obtained from mice bearing ascites hepatoma, and washed with PBS twice. Suspension of 1 × 10^6 Hep A cells in 1 ml RPMI 1640 (10% FBS) was incubated at 37 C in a 5% CO_2 atmosphere, 30 min prior to harvest, [^{3}H]TdR (74 kBq·ml^{-1}), [^{3}H]UR (37 kBq·ml^{-1}) or *L*-[^{3}H]Lys (74 kBq·ml^{-1}) was added. TCA 10% was used to terminate the reaction. The mixture was filtered and washed with 5% TCA 3

times, the filters were dried, and radioactivity was measured in 5 ml of scintillator using a RackBeta Spectral Model 1219 liquid scintillation counter. Each time the average triplicate determinations was plotted.

DNA-dependent RNA synthesis in isolated nuclei As described in the literature[4], RNA synthesis was assayed *in vitro* with a medium of Tris-HCl (50 mmol·L^{-1}, pH 7.9), $Mg(Ac)_2$(5 mmol·L^{-1}), KCl (1 mmol·L^{-1}), glycerol (20%), 2-mercaptoethanol (12 mmol·L^{-1}), each of GPT, UTP, CTP 1 mmol·L^{-1}, ATP (0.01 mmol·L^{-1}), [^{3}H]ATP (14.8 MBq·ml^{-1}). The reaction was initiated by the addition of nuclei containing DNA (2 mg·ml^{-1}). As described in the literature[5], the nuclei were isolated from murine ascites hepatoma (Hep A) as the source of DNA template and RNA polymerase. The reaction mixture was incubated at 25 C for 30 min. RNA synthesis was terminated by the addition of 2 ml 5% TCA. Other procedures were the same as in the incorporation assay.

Flow cytometry P388 cells were seeded in RPMI 1640 supplemented with 10% FBS. After the anordrin exposure, cells were separated from the medium by centrifugation and washed with PBS. Cells were fixed with 10 ml 70% ethanol at 4 C for at least 12 h. Cells were treated with RNase A (40 mg·L^{-1}) for 30 min at 37 C, and then stained with PI (50 mg·L^{-1}) for 30 min in the dark. The PI stained nuclei were analyzed using a FACStar PLUS (Becton Dickinson) equipped with the Cellfit DNA analysis software package. The distribution of cells in G_1/G_0, S, and G_2/M phases of the cell cycle was determined by the sum of broadened rectangles method.

Statistics Data were expressed as $\bar{x}\pm s$ and evaluated by t test.

RESULTS

Growth of Hep A and P388 cells Continuous treatment of Hep A and P388 cells with α-Ano reduced the cell growth depending upon the drug concentrations. With α-Ano 1 mg·L^{-1}, a growth lag was seen in Hep A cells, and (10 mg·L^{-1}) caused a total cessation of growth of Hep A cells. P388 cells exhibited a similar sensitivity to α-Ano (Fig 1).

Macromolecular synthesis With various concentration of α-Ano, Hep A cells were incubated with [^{3}H]TdR, [^{5}H]UR, or L-[^{3}H]Lys, and the incorporation was assayed as radioactivity in the acid-precipitable fraction. With α-Ano 20 mg·L^{-1}, an inhibition of DNA and RNA syntheses was observed ($P<0.05$), but the inhibition of protein synthesis was not significant. At various time intervals (0.5, 1, 3 h) after α-Ano (20 mg·L^{-1}) treatment, the inhibitions of DNA, RNA, and protein were shown in Tab 1. The inhibition of RNA and DNA syntheses appeared almost immediately after drug exposure. At 3 h after α-Ano treatment, RNA and DNA were inhibited by 84% and 46% respectively, while the protein synthesis did not show any obvious change. The inhibition of RNA sythesis was more marked than that of DNA synthesis ($P<0.05$).

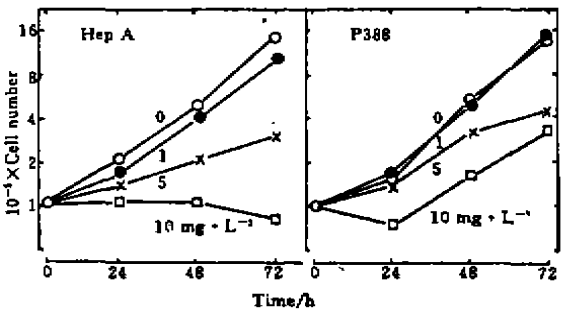

Fig 1. Effects of α-anordrin on growth of mouse hepatoma (Hep A) and leukemia P388 cells.

RNA polymerase activity The effect of α-Ano on the DNA-dependent RNA synthesis was studied using isolated nuclei from Hep A cells (Fig 2). As a control, dactinomycin at 5 mg·L^{-1} inhibited the RNA synthesis. α-Ano (20—80 mg·L^{-1}), inhibited the RNA synthesis of Hep A cells, did not exert a pronounced influence on the RNA polymerase activity.

P388 cell cycle distributions After α-Ano (10 mg·L^{-1}), the accumulation of cells in G_1/G_0 was remarkable ($P<0.05$), increasing from a control value of 31% to 47% at 48 h,

Tab 1. Effects of α-anordrin on incorporation of ^3H precursors into DNA, RNA, and protein of mouse hepatoma cells. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control. n=number of experiments.

α-Anordrin	n	$10^{-3}\times$Incorporation radioactivity/dpm		
		[^{3}H]TdR	[^{3}H]UR	*L*-[^{3}H]Lys
Concentration				
0 mg·L^{-1}	4	13.9±1.2	28±7	2.62±0.28
4	4	13.3±1.3^a	24±3^a	2.8±0.4^a
20	4	9.4±1.1^c	13.4±2.4^b	2.78±2.9
60	4	3.4±0.6^c	4.3±1.9^c	1.73±0.13^b
120	4	2.43±0.24^c	1.7±0.6^c	1.52±0.28^c
Exposure time				
0 h	3	16±1.4	26±3	2.50±0.27
0.5	3	8.1±1.1^c	8.2±2.4^c	2.2±0.3^a
1.0	3	11.1±1.8^c	8±4^c	2.2±0.3^a
3.0	3	9±5^b	4.0±0.6^c	2.0±0.4^a

while the S phase cells decreased from 62% to 45%, but the fraction of cells in the G_2/M phase remained nearly constant (Fig 3). In another experiment, exposure for 12 h with α-Ano caused an increase in G_1/G_0 cells and a decrease in S fraction ($P<0.05$). When α-Ano was removed, the fraction of G_1/G_0 was decreased, while that of S cells increased (Fig 3). These results suggested that after removal of α-Ano the G_1/G_0 cells could progress to S phase. Thus, the continuous presence of α-Ano was required for the G_1/G_0 phase block, and the G_1/G_0 arrest was reversible.

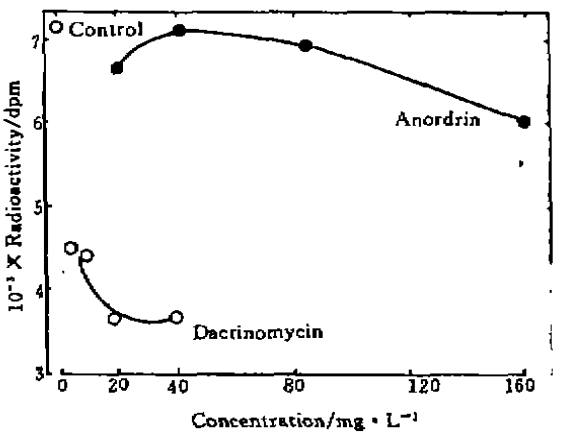

Fig 2. Effects of α-anordrin and dactinomycin on [^{3}H]ATP incorporating into RNA in isolated nuclei of mouse hepatoma cells. n=2 experiments.

DISCUSSION

The experiment of *in vitro* DNA-dependent RNA synthesis suggested that RNA polymerase was not the target of the drug. The mechanism of the inhibition of RNA synthesis of Hep A cells remains to be further elucidated. In our another experiment using calf thymus DNA by means of circular dichroism spectra (CD) method, it was proved that α-Ano could not affect the DNA template. It has been reported[7] that cytotoxic drugs affecting DNA template could cause the inhibition of DNA-dependent RNA synthesis. The present work showed that DNA template was not the target of α-Ano.

α-Ano could induce the alteration in cell cycle kinetics. It has been demonstrated that the delay in progression of cells through G_1/G_0 phase played an important role in the cessation of cell division. The reversibility of G_1/G_0 arrest can explain the fact that α-Ano is not a potent cytotoxic agent, but a cancer cell growth inhibitor. It is known that drugs

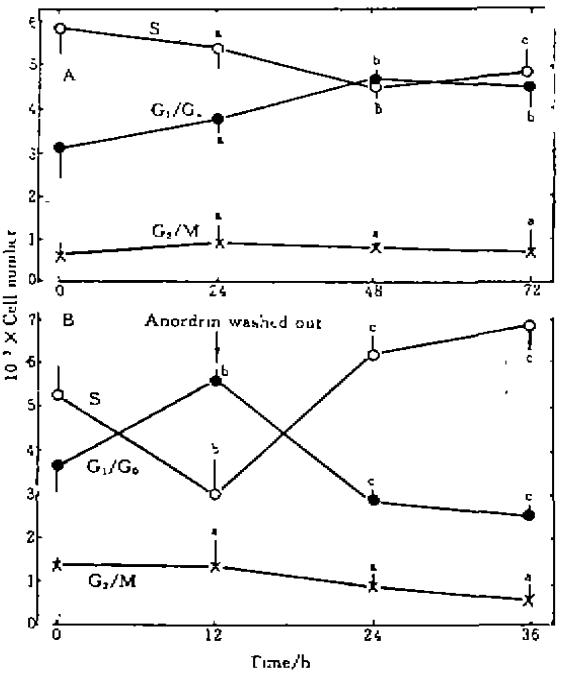

Fig 3. A. Number of cells in G_1/G_0, S and G_2/M phases during continuous exposure to α-anordrin 10 mg·L^{-1}. Control $n=5$; 24, 48, 72 h, all *vs* 0 h. $n=3$, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$. B. After 12 h of treatment with α-anordrin 10 mg·L^{-1}, the cells were placed in normal medium. $n=3$, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$; 12 h *vs* 0 h. The 24 h and 36 h *vs* 12 h. (n=number of experiments).

affecting calmodulin, protein kinase C, estrogen receptor, and other intracellular proteins can cause the arrest in G_1 phase. We have proved that α-Ano is a calmodulin antagonist in red blood cells. Calmodulin might be one of the targets of the drug's antiproliferative effect. Contraceptive studies showed α-Ano has a estrogen receptor binding affinity similar to tamoxifen[8]. It might be valuable to study the drug's anticancer effect in estrogen receptor or progestin receptor positive human cancer cell lines.

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α-双炔失碳酯的体外抗肿瘤作用及阻断细胞 G_1 期

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摘要　α-双炔失碳酯（α-Ano）对体外培养的小鼠肝癌细胞和 P388 细胞的生长均有抑制作用。20 μg·ml^{-1} 药物对小鼠肝癌细胞的 DNA 和 RNA 的合成均有明显抑制作用。利用抽提的小鼠肝癌细胞核进行 RNA 转录的无细胞系统实验，证明 α-Ano 对 RNA 聚合酶无影响。细胞动力学的研究资料表明，α-Ano 能使 P388 细胞阻断在 G_1/G_0 期，使它们不能进入到 S 期，从而抑制了 P388细胞的增殖。

关键词　失碳雄烷；双炔失碳酯；实验性肝肿瘤；白血病 P388；胸苷；尿苷；细胞周期