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Jian Su · Yong-su Zhen · Chang-qing Qi · Ji-lan Hu

Antibiotic C3368-A, a fungus-derived nucleoside transport inhibitor, potentiates the activity of antitumor drugs

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Abstract Antibiotic C3368-A (CA) is produced by a fungus strain from a soil sample collected in Antarctica. CA markedly inhibited radiolabeled thymidine and uridine transport in mouse Ehrlich carcinoma cells, its 50% inhibitory concentration (IC_{50}) being 4.6 and 7.7 μM, respectively. In clonogenic assay, CA displayed a synergistic effect with methotrexate (MTX), mitomycin C (MMC), 5-fluorouracil (5FU), and Adriamycin (ADR) against human oral epidermoid carcinoma KB cells. CA also markedly enhanced the inhibitory effect of 5FU and ADR on the proliferation of human hepatoma BEL-7402 cells as determined by the *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (NAG) enzyme-reaction assay. 5FU or ADR cytotoxicity was not augmented by CA in human fetal lung 2BS cells. In vivo, CA significantly potentiated the inhibitory effect of MMC against colon carcinoma 26 in mice. No significant augmentation of toxicity by the combination was found in treated mice. The results suggest that CA, the newly found nucleoside-transport inhibitor, may be useful in potentiation of the effect of antitumor drugs.

Key Words CA · Nucleoside transport inhibitor · Synergism · Antitumor-drug activity

Introduction

There are two kinds of pathways of nucleotide biosynthesis (de novo pathways and salvage pathways) coexisting in mammalian cells, and the latter are always dominant in tumor cells [32]. Antimetabolites currently used in cancer chemotherapy are inhibitors of de novo pathways of nucleotide biosynthesis, but they are not capable of inhibiting the salvage pathways. When tumor cells are exposed to antimetabolites, the synthesis of nucleotides continues through salvage pathways and the antimetabolite activity can be offset by uptake of exogenous nucleosides. There are several kinds of nucleosides in the peripheral blood of animals, and their concentrations usually reach a level that is high enough to reverse the cytotoxicity of antimetabolites if tested in vitro [6, 23]. Dialyzed serum can increase the killing of HCT 116 carcinoma cells by methotrexate (MTX) [22]. These results indicate that exogenous nucleosides can prevent the nucleotide pools in tumor cells from sufficient decrease by antimetabolites. The reasons why antimetabolites do not show satisfactory therapeutic effects are attributed at least in part to the active nucleoside salvage in tumor cells [10]. Therefore, the question as to how the salvage pathways of nucleotide biosynthesis can be blocked is of concern in cancer chemotherapy [31].

Because transport is the initial step in the utilization of exogenous nucleosides, agents that inhibit nucleoside transport can be used to block the salvage pathways. Dipyridamole (DP), a potent nucleoside-transport inhibitor, has shown synergistic cytotoxicity with acivicin, an antimetabolite, in rat hepatoma 3924A cells. Moreover, exogenous nucleosides can protect tumor cells from acivicin cytotoxicity and DP can abolish the protection [33]. Similar synergism has been found in the combination of DP with different antimetabolites, including MTX, 5-fluorouracil (5FU), cytarabine (Ara-C), and *N*-phosphonacetyl-L-aspartate (PALA) [5, 6, 22, 24, 35]. Further studies have indicated that DP also potentiates the effects of Adriamycin (ADR) [15, 20], vincristine (VCR) [14], cisplatin [18, 19], etoposide (VP16), and vinblastine [15] and reverses multidrug resistance [28, 29]. Clinical studies on DP combined with several drugs in cancer chemotherapy are under way [1–4, 12, 13, 27].

J. Su · Y.-S. Zhen (✉) · C.-Q. Qi · J.-L. Hu
Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tiantan, Beijing 100050, China

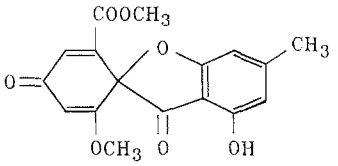

Fig. 1. Molecular structure of CA

The potential usefulness of nucleoside-transport inhibitors has attracted much attention. Besides DP, it is of interest to develop new substances that inhibit nucleoside transport and to investigate their applications. Studies have shown that microorganisms from Antarctica produce a variety of biologically active substances and may be new resources of microbial biopharmaceuticals [16]. Antibiotic C3368-A (CA), identified as bisdechlorogeodin (Fig. 1), is produced by *Chrysosporium verrucosum* Tubaki C3368 from a soil sample collected on King George Island, Antarctica [8]. The present study shows that CA is active as a blocker of nucleoside transport and potentiates the activity of antitumor drugs.

Materials and methods

Chemicals

CA was isolated from the fermentation liquor of the fungus strain, and its purity was over 96% as examined by high-performance liquid chromatography. [Methyl-^{3}H]-Thymidine and [^{3}H]-uridine were purchased from the Institute of Atomic Energy, Chinese Academy of Sciences (Beijing, China). NAG is a product of Sigma Chemical Co. (USA). The four antitumor drugs used included MTX (Shanghai Number 12 Pharmaceutical Works, Shanghai, China), 5FU (Haipu Pharmaceutical Works, Shanghai, China), mitomycin C (MMC; Kyowa Hakko Kogya Co., Japan), and ADR (Farmitalia Carlo Erba, Italy).

Nucleoside-transport assay

Ascites drawn from Ehrlich carcinoma-bearing mice was diluted with 0.85% NaCl and centrifuged at 1000 rpm for 5 min, after which the supernatant was removed. Cells resuspended in serum-free RPMI-1640 medium containing 0.05 *M* phosphate buffer (pH 7.3) were aliquoted into glass tubes with 7×10^6 cells/0.9 ml in each. After the addition of 0.1 ml of tested sample, the mixture was pre-incubated at 37°C in a water bath for 5 min; then, radiolabeled thymidine or uridine (1 μCi/tube) was added and the tube was kept at 37°C for 30 s. Immediately thereafter, ice-cold 0.85% NaCl was poured into the tube, the content of the tube was filtered onto a glass-fiber disc (2.5 cm in diameter), and the disc was washed twice with ice-cold 0.85%NaCl. The operation was performed one tube at a time; thus, the cells can be separated from the reaction medium almost instantaneously. After the addition of 0.1 ml of 0.1 *N* NaOH and subsequent drying, the radioactivity on discs in organic scintillation liquid was counted by a Beckman LS1201 liquid scintillation system as described previously [34]. Each sample concentration was tested in triplicate and the tubes without cells served as blanks. The blank was subtracted separately from all tested tubes.

Cell culture

Human oral epidermoid carcinoma KB cells, human hepatoma BEL-7402 cells, and human fetal lung 2BS cells were cultured in RPMI-1640 medium supplemented with 10% newbron calf serum, penicillin (100 U/ml). and streptomycin (100 μg/ml) at 37°C in an incubator containing 5%CO_2 and 95% relative humidity.

Clonogenic assay

KB cells in the exponential phase were seeded in 24-well plates at 100 cells/well. After 1 day of incubation, CA, MTX, MMC, or their combinations were added to the wells and maintained throughout the experiment. Triplicate wells were used for each concentration. After 7 days of incubation, colonies were counted under an inverted microscope.

NAG enzyme-reaction assay

For the NAG enzyme-reaction assay [21], logarithmically growing BEL-7402 or 2BS cells were seeded in 24-well plates at 1.5×10^4 cells/well. After 6 h of incubation, CA, 5FU, ADR, or their combinations were added to the medium. Triplicate wells were used for each concentration. After 72 h of culture, the supernatant of each well was removed completely and 0.2 ml of substrate buffer (0.05 *M* citrate buffer containing 1 m*M* NAG and 0.25% Triton X-100, pH 5) was added. The plates were then incubated at 37°C in 100% humidity for 5 h. Then, 3 ml of 50 m*M* glycine buffer [containing 5 m*M* ethylenediaminetetracetic acid (EDTA, pH 10.4)] was added to each well to terminate the reaction. Absorbance at 405 nm (A_{405}) was measured in a 721 spectrophotometer with a light distance of 1 cm.

Animal experiment

For the animal experiment [5], female BALB/c mice weighing 17–21 g were obtained from the Institute of Medical Laboratory Animals, Chinese Academy of Medical Sciences. A tumor fragment (2 mm in diameter) of colon carcinoma 26 was inoculated s.c. into the right armpit of the mouse. At 24 h postinoculation, treatment was started and given i.p. daily for 10 consecutive days. In the case of combination, CA was injected 30 min after MMC. Mice were killed 1 day after the last injection of drugs and the tumors were dissected and weighed. Specimens taken from the heart, lung, liver, spleen, kidney, and small intestine of mice were fixed in Bouin's solution. Histology sections were stained with hematoxylin and eosin and observed under a microscope. Femurs of all mice were removed and the bone marrow was washed out with 2 ml of 0.85% NaCl. After being diluted with 0.85% NaCl containing 1% acetic acid, the nucleated bone marrow cells were counted with a hemocytometer.

Evaluation of combination effects

The evaluation of two-drug (CA plus one of the antitumor drugs) combinations was done following a previously described method [9, 30], with modifications. The coefficient of drug interaction (CDI) was defined as the ratio between the percentage of colony survival, A_{405}, or tumor weight for a drug combination and the product of the percentage of those for the individual drugs. CDI values of < 1 (and $P < 0.05$, the combination group being compared with the group of antitumor drug alone by Student's *t*-test) were considered to show synergism; CDI > 1, antagonism; and CDI $= 1$, additivity.

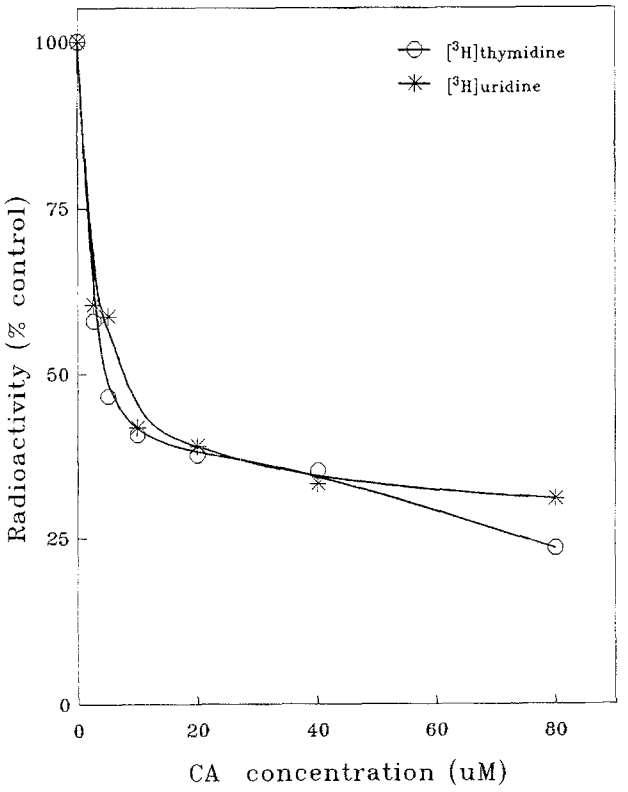

Fig. 2 Inhibition of nucleoside transport by CA in Ehrlich carcinoma cells. Ehrlich carcinoma cells from ascites were pre-incubated with various concentrations of CA for 5 min, then incubated with 1 μCi [^{3}H]-thymidine or [^{3}H]-uridine for 30 s. Points represent mean values for triplicate assays from 2 experiments. The IC_{50} values for inhibition of thymidine and uridine transport are 4.6 and 7.7 μ*M*, respectively

Results

Inhibition of nucleoside transport by CA

CA markedly inhibited radiolabeled thymidine and uridine transport into Ehrlich carcinoma cells. The IC_{50} values for thymidine and uridine transport were 4.6 and 7.7 μ*M*, respectively (Fig. 2).

Potentiation of MTX, 5FU, MMC and ADR cytotoxicity by CA in tumor cells

As determined by clonogenic assay, CA showed synergistic cytotoxicity with MTX, MMC, 5FU, and ADR in KB cells (Tables 1, 2). As shown by the NAG assay, CA markedly potentiated the growth-inhibitory effect of 5FU or ADR in hepatoma BEL-7402 cells (Figs. 3, 4). Significantly synergistic actions were demonstrated when the combination groups were compared with those of MTX, 5FU, MMC, or ADR alone, respectively.

Effect of CA on 5FU or ADR cytotoxicity in human fetal lung 2BS cells

As shown in Figs. 3 and 4, CA did not enhance 5FU or ADR cytotoxicity in human fetal lung 2BS cells. No significant difference was found between the groups of 5FU or ADR alone and the respective combination groups ($P > 0.05$).

Potentiation of MMC antitumor effect by CA in vivo

CA (50 mg/kg) alone showed no effect on the growth of colon carcinoma 26 in mice; however, the inhibitory effect of MMC was markedly enhanced by CA. The tumor-inhibition rate increased from 28.9%for MMC alone to 57.3% for CA plus MMC. In a comparison of MMC alone with MMC + CA, a synergistic effect was verified (CDI, 0.62; $P < 0.01$; Table 3).

Toxic effects of MMC and its combination with CA in mice

Bone marrow nucleated cells were counted at the end of treatment. The number of bone marrow cells was moderately reduced in MMC-treated mice, but no significant difference was found between groups treated with MMC alone versus MMC + CA ($P > 0.5$; Table 3). Evidently, CA did not exacerbate the hemopoietic suppression caused by MMC while the synergistic tumor-inhibitory effect emerged. By histopathologic examination, no toxicological change was found in the heart, lung, liver, spleen, kidney, or small intestine of any of the mice.

Discussion

In recent years, studies with DP have demonstrated the potential usefulness of nucleoside-transport inhibitors in cancer chemotherapy. However, DP binds extensively to serum proteins in vivo [3, 4, 12, 25], which may be one of the reasons why DP fails to potentiate antimetabolite action in some cases in animal experiments [24] and clinical cancer treatment. For better therapeutic efficacy, it is of interest to search for novel nucleoside-transport inhibitors and to explore their possible use in combination chemotherapy. Our studies reveal that CA is active in inhibiting nucleoside transport in tumor cells. This is the first report on a fungus-derived nucleoside-transport inhibitor showing synergism with antitumor drugs. The synergistic action of CA and two antimetabolites, MTX and 5FU, further confirms the idea proposed by Zhen et al. [33] that the combination of inhibitors acting on de novo nucleotide

Table 1 Potentiation of MTX and MMC cytotoxicity by CA in carcinoma KB cells[a]

Concentration (μM)			Number of colonies per well (mean ± SD)	Inhibition rate (%)	CDI	P
CA	MTX	MMC				
0	0	0	85.7 ± 4.5	–		
10	0	0	84.7 ± 2.1	1.2		
0	0.004	0	76.0 ± 2.0	11.3		
10	0.004	0	51.0 ± 4.0	40.5	0.68	< 0.001
0	0.008	0	43.0 ± 2.6	49.8		
10	0.008	0	24.3 ± 0.6	71.6	0.57	< 0.001
0	0	0.002	68.0 ± 3.0	20.7		
10	0	0.002	47.7 ± 4.2	44.3	0.71	< 0.005
0	0	0.004	25.7 ± 2.5	70.0		
10	0	0.004	10.0 ± 2.6	88.3	0.39	< 0.005

[a]Logarithmically growing KB cells were seeded in 24-well plates at 100 cells/well and the drugs were added 24 h later; triplicate wells were used for each concentration. Colonies were counted after 6 days of exposure to drugs

Table 2 Potentiation of 5FU and ADR cytotoxicity by CA in carcinoma KB cells[a]

Concentration (μM)			Number of colonies per well (mean ± SD)	Inhibition rate (%)	CDI	P
CA	5FU	ADR				
0	0	0	81.0 ± 6.2	–		
10	0	0	80.7 ± 0.6	0.4		
0	1	0	73.3 ± 7.8	9.5		
10	1	0	37.0 ± 3.6	54.3	0.51	< 0.001
0	2	0	57.0 ± 8.7	29.6		
10	2	0	27.7 ± 4.9	65.8	0.49	< 0.005
0	0	0.004	61.0 ± 6.6	24.7		
10	0	0.004	40.0 ± 4.6	50.6	0.66	< 0.01
0	0	0.008	21.0 ± 1.0	74.1		
10	0	0.008	5.3 ± 1.2	93.4	0.25	< 0.001

[a]Logarithmically growing KB cells were seeded in 24-well plates at 100 cells/well and the drugs were added 24 h later; triplicate wells were used for each concentration. Colonies were counted after 6 days of exposure to drugs

biosynthesis and those acting on salvage transport may provide synergistic antitumor effects.

Besides demonstrating synergism with antimetabolites, CA also augmented the cytotoxicity of two non-antimetabolites, MMC and ADR, in tumor cells. The possible mechanism for this synergism may be beyond the realm of nucleoside-transport inhibition. Previous studies on DP have revealed similar synergistic action. As reported, DP potentiated the cytotoxicity of several non-antimetabolites, including vinblastine, VCR, ADR, cisplatin, and VP16, and the synergism occurred simultaneously with an increased steady-state cellular content of drugs [14, 15, 18, 20]. However, there was no relationship between the extent of efflux inhibition and the magnitude of the DP-induced increase in cellular drug content, indicating that DP may have other effects as well [15]. Shalinsky et al. [28] found that the ability of DP in altering the steady-state concentration of drugs in cells could not predict accurately whether the interaction would be truly synergistic. There must be complex mechanisms involved in the synergistic effects of nucleoside-transport inhibitors and antitumor drugs. For elucidation of the mechanism of CA potentiating non-antimetabolite activity, the effects of CA on the uptake, retention, and steady-state concentration of drugs in tumor cells are being investigated.

While potentiating the antitumor activity of drugs, DP may enhance their toxicity as well. Previous studies found that DP enhanced MTX, 5FU, and PALA cytotoxicity in human bone marrow cells, PHA-stimulated human lymphocytes and human keratinocytes [4, 6, 17, 26]. DP reduced VCR efflux and increased cellular VCR accumulation in malignant lymphoid cells as well as in phytohemagglutinin (PHA)-stimulated and unstimulated lymphocytes from normal donors, but the efflux of VCR from normal lymphocytes was more rapid than that from malignant lymphoid cells [14]. As determined by clonogenic assay, DP increased the cytotoxicity of fluorodeoxyuridine

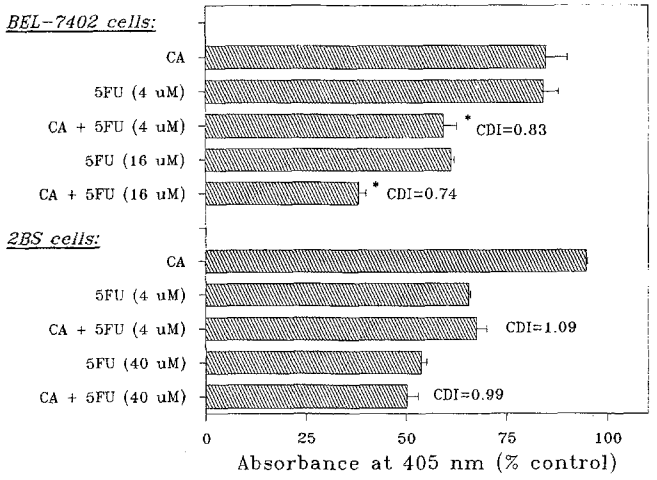

Fig. 3 Effect of CA on 5FU cytotoxicity in hepatoma BEL-7402 cells and fetal lung 2BS cells. Logarithmically growing BEL-7402 cells and 2BS cells were seeded in 24-well plates at 1.5×10^4 cells/well. Drugs were added 6 h later and the NAG assay was performed after 3 days. CA was added at 40 μM. Bars represent mean values $\pm$ SD for triplicate assays. $^*P < 0.025$ (significantly different from 5FU alone)

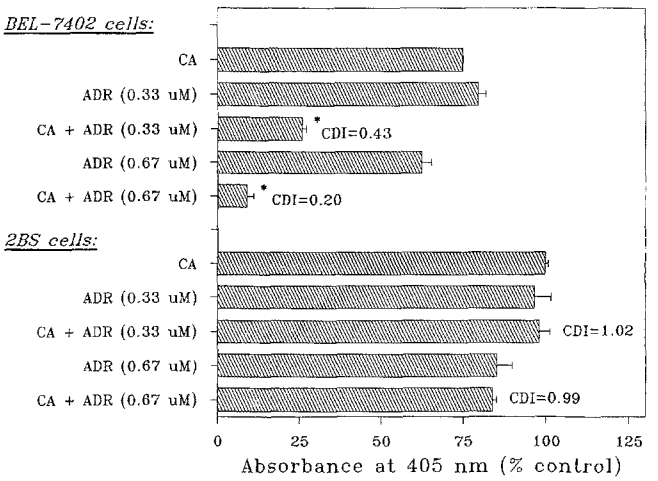

Fig. 4 Effect of CA on ADR cytotoxicity in hepatoma BEL-7402 cells and fetal lung 2BS cells. Logarithmically growing BEL-7402 cells and 2BS cells were seeded in 24-well plates at 1.5×10^4 cells/well. Drugs were added 6 h later and the NAG assay was performed after 3 days. CA was added at 40 μM. Bars represent mean values $\pm$ SD for triplicate assays $^*P < 0.001$ (significantly different from ADR alone)

Table 3 Effects of MMC and its combination with CA on the growth of colon carcinoma 26 and the numbers of bone marrow cells in mice[a]

Treatment	Dose (mg/kg)		Body weight change (g)	Tumor weight		Bone marrow cells	
	MMC	CA		g(mean $\pm$ SD)	Inhibition (%)	10^6/femur(mean $\pm$ SD)	% control
Control	0	0	− 0.41	1.68 $\pm$ 0,28	–	7.26 $\pm$ 2.91	–
CA	0	50	− 0.77	1.63 $\pm$ 0.18	3.0	7.04 $\pm$ 1.94	97.0
MMC	0.5	0	− 1.72	1.20 $\pm$ 0.35	28.9	4.87 $\pm$ 1.91	67.1
MMC + CA	0.5	50	− 0.48	0.72 $\pm$ 0.08	57.3[b]	5.57 $\pm$ 1.62	76.7[c]

[a]BALB/c mice were inoculated s.c. with colon carcinoma 26. Drugs were given 24 h later and i.p. daily for 10 consecutive days. There were 7 mice in each group, and no animal died during the experiment
[b]Synergistic action (CDI, 0.62; $P < 0.005$, significantly different from MMC alone)
[c]No significant difference from MMC alone ($P > 0.4$)

(FUdR) in human colon-cancner cell lines, but considerably fewer cytotoxic effects were observed for DP plus FuDR against human bone marrow colony-forming units [1].

Our studies found no CA-induced increase in the cytotoxicity of 5FU or ADR in a non malignant cell line, human fetal lung cells. It is suggested that the augmentation of antitumor drug cytotoxicity by nucleoside-transport inhibitors may to some degree be discriminative against cultured malignant and non-malignant cells. The mechanisms of selectivity need to be expounded yet.

CA also enhanced the inhibitory effect of MMC on colon carcinoma 26 in mice without markedly affecting the bone marrow toxicity of MMC in treated mice. In addition, no pathological changes in the heart, lung, liver, spleen, kidney, or small intestine were found in any of the treated mice. The present results were similar to those obtained for DP in combination chemotherapy with MTX or 5FU in mice [5]. Although the studies by Gingrich et al. [11] showed that the toxicity of cisplatin, carboplatin, and VP16 was increased by high-dose DP in combination therapy of nude mice bearing human bladder cancer xenografts, Cao et al. [5] documented that DP given at therapeutic doses might not increase 5FU or MTX toxicity in mice.

Our results show that CA, a newly found fungus-derived inhibitor of nucleoside transport, potentiates the antitumor effects of MTX, 5FU, MMC, and ADR in vitro and that of MMC in mice, whereas it does not enhance 5FU and ADR cytotoxicity to non-malignant cells or MMC toxicity in the hosts. It is suggested that CA as a nucleoside-transport inhibitor may be potentially useful in combination cancer chemotheraphy.

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