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Isolation of Isoguanosine from *Croton tiglium* and Its Antitumor Activity

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This paper describes the isolation of isoguanosine from *Croton tiglium* L. and its cytotoxic effect against several tumor cell lines in culture and newly reports that isoguanosine has an antitumor activity against implanted S-180 ascitic tumor mice. Isoguanosine is effective at the dose of 24 mg/kg/day×5, with T/C value of 168%. Isoguanosine inhibits the growth of S-180 and Ehrlich solid tumor in mice at the optimal doses of 96 mg/kg/day×12 and 48 mg/kg/day×12, with 1-T/C values of 65% and 60%, respectively.

Key words: Isoguanosine, Anti-tumor activity

INTRODUCTION

Isoguanosine (Crotonoside or 2-Hydroxy adenosine) is one of naturally occuring nucleoside analogs of guanosine. It was first isolated from *Croton tiglium* L. in 1932 (Cherbuliez *et al.*, 1932), and then from butterfly wings of *Prioneris thestylis* (Vasu and David, 1985). The reports (Buell *et al.*, 1927) that it occurs in pig blood could not be confirmed and Furman et al isolated isoguanosine from an animal, the marine nudibranch mollusk *Diaulula Sandiegensis* (Fuhrman *et al.*, 1981).

This compound has been reported to possess various biological activities such as incorporating into mammalian nucleic acid (Lowry and Brown, 1952), stimulating the accumulation of cyclic AMP in the brain (Huang *et al.*, 1973), and inhibiting IMP (Vasu and David, 1985) pyrophosphorylase and glutamic acid dehydrogenase. Recently, the aqueous extract of *Croton tiglium* L. was shown to have a strong antitumor activity by one of the authors'laboratories searching for new antitumor drugs from traditional plants (Kim *et al.*, 1993). The active component of the aqueous extract was isolated by bioassay-guided fractionation and the chemical structure of which was presumed to be the isoguanosine (Kim *et al.*, 1994). According to literature survey, the antitumor activity of isoguanosine has not been confirmed. Skipper et al tested isoguanosine against leukemia L1210 in mice, but reported that it had a negligible antitumor activity (Skipper *et al.*, 19 59). In this studies, we have isolated isoguanosine from *Croton tiglium* L. and report that isoguanosine has a considerable antitumor activity against various cell lines both *in vitro* and *in vivo* tests. Especially isoguanosine was shown to be very effective against solid tumor and ascitic tumor.

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METERIALS AND METHODS

Meterial and Reagents

The seeds of *Croton tiglium* L. were purchased from Kyung Dong herb market in Seoul.

Experimental Animal and Tumor Cell-lines

DDY mice used for this study were purchased from Clea Japan Co. and produced by inbreeding more than 25 generations at Institute of Experimental Tumor. Male and female (21±1 g) DDY mice were used and devided into groups of 6 mice for each *in vivo* assay. Tumor cell lines for *in vitro* and *in vivo* studies were purchased from ATCC (American Tissue Culture Collection); Sp2/O (murine myeloma), P338 (murine leukemia), HL-60 (human leukemia), L-1210 (murine leukemia), and Ehrlich.

Isolation of Isoguanosine from *Croton tiglium* L.

Croton tiglium L. was washed with ethylether by continuous extraction to eliminate seed oils. The resulting

powder (5 g) was stirred with MeOH-H_2O (2 : 8) at 50℃ for 5 hrs and filtered, and then the residue was extracted again. The combined filterates were concentrated under reduced pressure. The aqueous suspension was centrifuged and the supernatant was reevaporated to yield the crude extract as an yellow powder A (0.3 g). Thirty grams of fibrous DEAE cellulose was stirred with 500 ml of 0.5 N HCl and left to hydrate at room temperature for 60 min. The supernatant was decanted, and the cellulose was filtered and washed with water on a funnel until the pH of the washings became 4.0. The cellulose was then stirred with 750 ml of 0.5 N NaOH for 30 min at room temperature. Decanting and water washing steps were repeated until the pH of the supernatant was to be 7.0. The DEAE cellulose was then allowed to equilibrate in 750 ml of 0.7 M boric acid for 16 hrs and then washed with water to neutrality. The cellulose was suspended in 0.15 M boric acid and washed until the desired pH of the buffer was attained (pH 4.0), and then it was packed into column (2.5 cm×30 cm). Yellow powder A dissolved in 0.15M boric acid 100ml was loaded on the column and gradiently eluted with boric acid. Resulting isoguanosine fractions in boric acid was evaporated to 2ml under reduced pressure, and then was applied on to the Lichroprep.RP-18 column and eluted with 70% MeOH. Lyophilization of the isoguanosine fractions gave light yellow powder 50 mg. mp 237-238 ℃ (decomp.)

Growth Inhibitory Effect of Isoguanosine

Tumor cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, streptomycin 0.1 mg/ml and penicillin 100 units/ml at 37℃. After incubation for 48 hrs at 37℃ in 5% carbon dioxide, the number of remaining cells was counted with a cell counter by MTT method (Mosmann, 1983) and cytotoxic activities of isoguanosine against each tumor cell-lines were evaluated in terms of the ED_{50}.

Assay of the Effect of Isoguanosine against S-180 and Ehrlich, in Mice

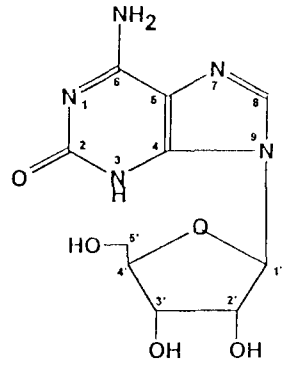
For ascitic tumors, female (21±1 g)DDY mice were inoculated intraperitineally with 1×10^7 cells of S-180 and devided into groups of 6 mice. Isoguanosine was administered intraperitioneally once a day for 5 days starting from 24 hrs after tumor implantation. Then mean survival time (MST) of each group was examined. Antitumor activity was evaluated in terms of the percentage of increased mean survival time over the control group,

MST (mean survival time) ratio (%)=
(MST of Treated group/MST of control)×100

For solid tumors, male (21±1 g) DDY mice were inoculated hypodermically on the left flank with 1×10^7 cells of S-180 and Ehrlich, respectively. Isoguanosine was administered hypodermically once a day for 12 days starting from 24 hrs after tumor implantation, and tumor weight of each group was examined. Anti-tumor activity was evaluated in terms of the percentage of reduced tumor weight (%Growth inhibition) over the control group. For the measuring tumor weight, width and length of solid tumor in experimental animal was measured externally by using caliper and weight was calculated according to the following fomular (Kyoichi *et al.*, 1988).

Tumor weight (mg)=1/2×length (mm)×width (mm)

$$\text{Growth inhibition (\%)} = \left(1-\frac{\text{mean tumor weight of treated group}}{\text{mean tumor weight of control group}}\right)\times100$$



Isoguanosine

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Isoguanosine from *Croton tiglium* L.

It was reported (Kim *et al.*, 1993) that *Croton tiglium* L. seeds include 30-50% oils, 10% proteins and small amount of albumin. Especially oils have been found to have phorbol ester which is well known as a strong tumor promotor (Slaga *et al.*, 1978). To eliminate phorbol ester, *Croton tiglium* L. seed was ground to powder and its oils were extracted by continuous extraction using soxhlet. The residual powder was extracted with hot water and fractionation of water soluble residue was performed by DEAE-cellulose-borate column chromatography designed by Pike and Rottman (Pike., 1974).

As listed in Table I, careful examination of the ^{1}H-NMR spectrum of isolated isoguanosine showed a adenosine and guanosine-like pattern. In particular, the anomeric proton of one doublet at δ 5.6 and other protons from δ 3.4 to δ 4.6 suggested the presence of ribose sugar and one singlet proton at δ 7.7 showed C-8 proton of isoguanosine. ^{13}C chemical shift data

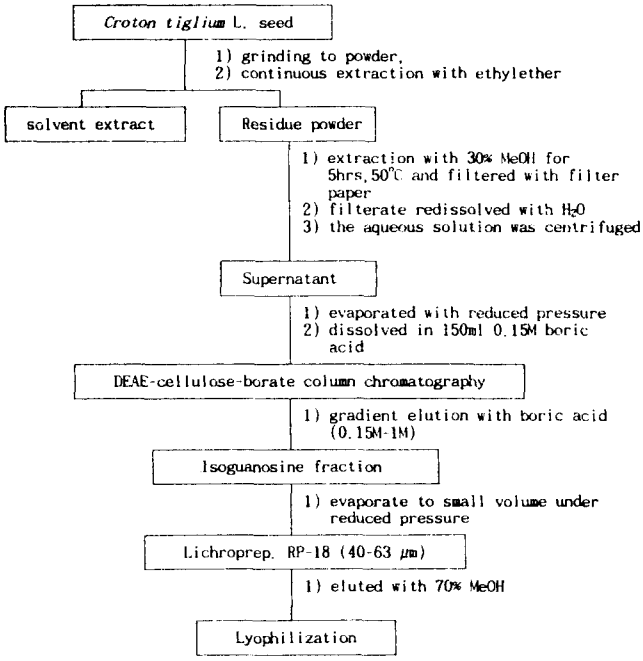


Fig. 1. Procedure for the Isolation of Isoguanosine from *Croton tiglium* L.

between 100-160 ppm showed the presence of signals typical for a purine base. Finally the comparison of NMR data with reported one(Divaker *et al.*, 1991) made us to confirm the isolated compound to be the isoguanosine.

Cytotoxic Activity of Isoguanosine against some Tumor Cell-lines

As mentioned above, isoguanosine has many biological activity. Its biological activity is similar to that of adenosine and other analogs and it is even more potent and much longer-acting than adenosine (Fredrick *et al.*, 1981). But antitumor activity of isoguanosine has not been reported. For our initial study of the proposed bioassay, we examined the cytotoxicity of isoguanosine against several tumor cell-lines in culture. The general method for the examination of the cytotoxicity effect of isoguanosine against some cultured tumor cell-lines is described in "Materials and Methods". The

Table I. NMR Data of Isoguanosine

Position	Chemical shift δ (ppm) from Me_4Si	
	^{1}H-NMR (500 MHz in D_2O)	^{13}C-NMR (125 MHz in DMSO-d_6)
C-1′	5.6 (doublet, 1H, J.6.4)	87.7
C-2′	4.6 (multiplet, 1H)	72.98
C-3′	4.11 (dd, 1H, J.2.7 and 4.9)	70.7
C-4′	3.9 (m, 1H)	85.94
C-5′ α	3.56 (dd, 1H, J.2.8 and 12.3)	61.6
β	3.66 (dd, 1H, J.2.8 amd 12.3)	
C-2		156
C-4		153.6
C-5		107.7
C-6		160.6
C-8	7.98 (singlet, 1H)	138

Table II. *In Vitro* Cytotoxicity of Isoguanosine with Several Tumor Cell-lines

Cell line	No.of cells/well	ED_{50} (g/48 hr)
Murine		
Leukemia P338	10,000	2.1×10^{-5} M
L5178Y	10,000	3.7×10^{-4} M
Myeloma Sp2/O	5,000	1.5×10^{-4} M
Human		
Leukemia HL60	20,000	7.0×10^{-5} M
Lymphoma Raji	10,000	4.0×10^{-4} M

effectiveness of isoguanosine was evaluated in terms of ED_{50} values. As are shown in Table II, ED_{50} values of isoguanosine was in the range of 30~40 μM against P338, HL-60 and Sp2/O cell-lines. It was found that isoguanosine had a considerable cytotoxicity against various tumor cell lines in culture.

Antitumor Activity of Isoguanosine against S-180 and Ehrlich in Mice

Isoguanosine was active against ascitic tumors and solid tumors in mice at doses of from 12 mg/kg to 96 mg/kg. However at dose of 400 mg/kg or more, severe toxicity appeared. Against implanted S-180 ascitic tumors in mice, antitumor activity of isogunosine was found to be most effective at dose of 24 mg/kg/days×5, with %T/C values of 168%, and 0.4 g loss

Table III. Anti-tumor Activity of Isoguanosine against Intraperitionally Inoculated S-180 Ascitic mice

Cell-line	Dose mg/kg/day×5	Weight change (g/mouse) on day 8(T-C)	Mean survival days[a] (T/C)	% T/C
S-180	12	−0.2	17.95/13.6	132
	24	−0.4	22.84/13.6	168
	48	−1.8	21.78/13.6	160
	96	−1.6	20.40/13.6	150

[a] mean survival days were calculated by the method in the NCI protocol.

Table IV. Anti-tumor Activity of Isoguanosine against hypodermically inoculated S-180 and Ehrlich solid tumor in mice

Cell-line	Dose mg/kg/day×12	Weight change (g/mouse) on day(T-C)	Growth inhibition % 1−T/C	Activity[b]
S-180	12	+0.3	20	+
	24	+0.7	35	+
	48	+0.6	60	++
	96	+0.5	65	++
Ehrlich	12	−0.2	−[a]	
	24	+0.1	30	+
	48	0.4	60	++
	96	+0.3	60	++

[a] Not detectable
[b] Citeria: +++ ≥70, ++ ≥50, + ≥20

in average weight changes per mouse by day 8. Also at 48 mg/kg dose of isoguanosine, the result turned out to be 160 %T/C with 1.8 g/mouse weight loss. Especially against the maligant solid tumors,the antitumor activity of isoguanosine was proved to be very high. The results against implanted S-180 and Ehrlich in tumor mice were quite remarkable (Table IV). When isoguanosine was administered to the mice with implanted S-180 solid tumor in the doses of 48 mg/kg /day×12 and 96 mg/kg/day×12, the %(1−T/C) values were 60% and 65%, respectively. And the average weight changes per mouse by day 12 were 0.6 g/mouse gain for 48 mg/kg/days×12 dosage schedule.

The assay indicated that the efficiency of isoguanosine more apparent *in vivo* studies than in vitro. When the antitumor activity of isoguanosine was compared with that of Ara-C (Karon *et al.*, 1969), the antitumor activity of isoguanosine was shown to have a similar activity with that of Ara-C *in vivo*.

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REFERENCES CITED

Buell, M. V. and Perkins, M. E., Cryst. guanine nucleoside. *J. Biol. Chem.*, 72, 745-748 (1927).

Cherbuliez, E. and Bernhard, K., Croton seed(1)crotonoside. *Helv. Chim. Acta.*, 15, 464, 978-982 (1932).

Divaker, K. J., Colin, B. R., Yogesh, S. S. and Karl, A. D., Conversion of guanosine into isoguanosine and derivatives. *J. Chem. Soc. Perkin Trans* I, 771-774 (1991).

Fuhrman, A. F., Geraldin, J. F., Ronald, J. N.and Harry, S. M., Isoguanosine; isolation from an animal. *Science*, 212, 557-558 (1981).

Hagen, C., Analysis in the variation in lymphocyte response to PHA (phytohemagglutinin) in normal subjects. *Biochem. Biophys. Acta.*, 293, 105-110 (1973).

Huang, M., Shimizu, H. and Daly, J. W., Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. *J. Med. Chem.*, 15, 462-468 (1972).

Karon, M. and Shirakawa. S., Rocus of action of 1-β-D-arabinofuranosyl cytosine in the cell cycles. *Cancer Res.*, 29, 687-696 (1969).

Kim, C. W., Moon, J. C. and Kim, J. B., Cytotoxic effects of extract (CP-2) from the mixture of Coptis and *Croton tiglium* L. of the various tumor cell-lines. *The Korean Central Journal of Medicine*, 58(3), 177-184 (1993).

Kim, J, H., Lee, S. J., Han, Y. B. and Kim, J. B., Identification of active compounds from *Croton tiglium* and *Coptis japonica* aqeous mixture (CP_2) and studies of it's cytotoxicity, *Kor. J. Pharmaeogn.*, 38(1), 31-37 (1994).

Kyoichi, S., Toshitaka, M. and Sueo, M. U., Antitumor activity and hematotoxicity of a new, substituted dihydrobenzo., FK 973, in mice, Cancer Res., 48, 1168-1172 (1988).

Lowry, B. A. and Brown, G. B., The utilization of purine nucleosides for nucleic acid synthesis in the rat. *J. Biol. Chem.*, **197**, 591 (1952).

Mosmann, T., Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. *J. Immumol. Methods*, 65, 55-63 (1983).

Pike, L. M. and Rottman, F., The determination of 2'-O-methyl nucleosides in RNA. *Anal. Biochem.*, 61, 367-378 (1974).

Skipper, H. E., Montgomery, J. A., Thomson, J. R. and Schabel, F. M., Structure-activity relations and cross-resistance observed on evaluation of a series of purine analogs against exptl. neoplasms. *Cancer Res.*, 19, 425 (1959).

Vasu, N. and David, A. Y., A New Synthesis of Isoguanosine. *J. Org. Chem.*, 50, 406-408 (1985).