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The mechanism of action of quinocarmycin citrate (KW 2152) on mouse L1210 cells in vitro

Ryunosuke Kanamaru, Yukari Konishi, Chikashi Ishioka, Hideki Kakuta, Toshiaki Sato, Akira Ishikawa, Mitsuo Asamura, and Akira Wakui

Department of Clinical Cancer Chemotherapy, The Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1, Seiryo-machi, Sendai 980, Japan

Summary. The effects of the antitumor antobiotic, quinocarmycin citrate (KW 2152), on L1210 cells were studied in vitro. The cellular growth was completely inhibited at 10^{-6} *M* KW 2152, and after 2 days no viable cell was seen. The incorporation fo ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-leucine into the acid-insoluble fraction was not affected at 10^{-4} *M* for 1 h; however, when the cells were treated with 10^{-6} *M* for 24 h, the radioactivity appearing in the acid-insoluble fraction was reduced to 20%, 30%, and 48%, respectively, of the control.

The single strand scission of the DNA of L1210 cells was seen at 10^{-7} *M* for 24 h, as revealed by an alkaline, sucrose density gradient. However, no damage to plasmid pBR322 was observed even at 10^{-6} *M* KW 2152 for 24 h, as revealed by 0.8% agarose gel electrophoresis, indicating that some soluble factors of the cells might contribute to the damage to the DNA of L1210 cells. The processing of pre-rRNA of the cells was not inhibited at 10^{-6} *M* of the drug for 24 h of incubation.

Introduction

Quinocarmycin citrate (KW 2152) is an antibiotic with a chemical formula of 2a, 3, 4, 5, 6, 6a, 7, 11b-octahydro-11-methoxy-12-methyl-3,6-imino-1H-2-oxa-11C-azanaphth(1,2,3,-cd)azulene-5-carboxylic acid monocitrate (shown in Fig. 1), which was extracted from the culture broth of *Streptomyces melanovinaceus* [4, 6].

Since quinocarmycin is unstable in aqueous solution, for the evaluation of its anticarcinogenic effect on experimental animals bearing tumors or on cancer patients, a citrate salt of quinocarmycin was synthesized. This compound is more stable, with more than 95% of its activity retained after 72 h in phosphate buffer (pH 7.2) at 37° C [2]. It has been reported to be active against *Bacillus subtilis, Staphylooccus aureus,* and *Klebsiella pneumoniae,* but not against gram-negative bacteria [6].

KW 2152 also shows activity in vitro against various experimental tumors in rodents, such as L1210, B16 melanoma, and P388 and has been found to prolong life in tumor-bearing rats [2]. In addition, KW 2152 has been reported to be active against leukemia P388, L1210, and B16 melanoma refractory to MMC, ADR, or DTIC and has markedly prolonged the life span of rats bearing these tumor cells [2].

In human tumor cells xenografted s.c. in nude mice, KW 2152 has significantly inhibited the growth of MX-1 mammary carcinoma, and all the tumors were cured by the i.v. administration of 4.4 mg/kg per day for 7 days [2].

In an in vitro experiment, Fujimoto et al. have reported that this compound significantly inhibited the RNA synthesis of P388 leukemic cells. These authors have also reported the 50% inhibitory concentration for RNA synthesis to be 10^{-5} *M* and that for DNA synthesis, 3×10^{-3} *M*. Thus, DNA synthesis is 30-fold less sensitive than RNA synthesis [2]. However, the concentration required for the inhibition of DNA synthesis in this experiment was too high, hence the mechanism of action was studied more precisely using L1210 cells.

Materials and methods

Drugs. Quinocarmycin citrate (KW 2152) was generously supplied by Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.

This molecule is an antibiotic obtained from *Streptomyces melanovinaceus*; the chemical structure is shown in Fig. 1.

Tumor cells. L1210 cells maintained by serial passage were introduced into RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 μg/ml 2-mercaptoethanol (2ME).

The effects of KW 2152 on the incorporation of ^{3}H-labeled precursors into acid-insoluble fractions of the cells. L1210 cells were cultured with [6-^{3}H]-thymidine (^{3}H-TdR, 19.3 Ci/mmol), [6-^{3}H]-uridine (^{3}H-UR, 4.2 Ci/mmol), or L[3,4,5-^{3}H]-leucine (^{3}H-Leu, 147.0 Ci/mmol) in the presence or absence of KW 2152. After the desired time of incubation, the cells were washed three times with phosphate-buffered saline (PBS) and suspended in 5 ml PBS. The cells thus treated were then trapped on a glass fiber filter paper (Whatman GF/C 25 mm) and washed with

Offprint requests to: R. Kanamaru

Abbreviations used: MMC, mitomycin C; ADR, adriamycin; DTIC, dacarbazine; FCS, fetal calf serum; ^{3}H-TdR, ^{3}H-thymidine; ^{3}H-UR, ^{3}H-uridine; ^{3}H-Leu, L-^{3}H-leucine; PBS, phosphate-buffered saline; TCA, trichloracetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; cccDNA, covalently closed circular DNA; ocDNA, open circular DNA; rRNA, ribosomal RNA

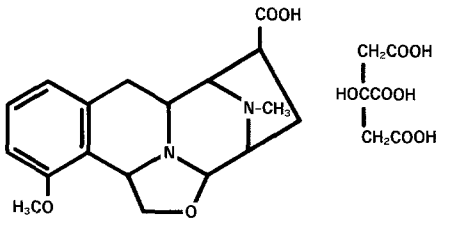

Fig. 1. Chemical structure of quinocarmycin (KW 2152)

15 ml PBS, 30 ml cold 5% TCA, and 15 ml 95% ethanol, successively. After drying, the radioactivity on glass fiber filter paper was counted in the scintillation fluid (2,5-diphenyl oxazol, 5 g; toluene 1l) with a liquid scintillation spectrometer.

Detection of the damage to single strand DNA. Various concentrations of KW 2152 were added to the cells labeled with 1 μCi/ml ^{3}H-TdR for 24 h, were layered on 5%–20% alkaline sucrose density gradient containing 0.3 *M* NaOH, 0.7 *M* NaCl, 0.01 *M* Na_2EDTA, and 0.01 *M* TRIS solution in an RPS 27.1 swing rotor. The centrifugation was carried out at 24,000 rpm at 15° C for 2.5 h. After centrifugation, the fractions were collected from the top of the gradient with the aid of a Hitachi density fractionator and the radioactivity was counted with a Beckman Model LS 250 scintillation spectrometer after the scintillator was added [1, 3, 5].

Detection of the damage to plasmid DNA. DNA from pBR322 was used to assess the direct action of KW 2152 on the DNA strand. As a reaction mixture, 0.3 μg pBR322 was dissolved in 50 m*M* TRIS-HCl buffer (pH 8.0) in the presence or absence of 1 m*M* dithiothreitol, with varied concentrations of KW 2152, for 24 h and was run on a 0.8% agarose gel in 25 m*M* TRIS acetate buffer (pH 8.0). After electrophoresis for 1.5 h, the bands were visualized with 0.5 μg/ml ethidium bromide.

Analysis of ribosomal RNA. Whole cellular RNA was extracted with the hot SDS phenol method, and the RNA was layered on 5%–30% sucrose density gradient and centrifuged at 21,000 rpm for 18 h in a RPS27-2 rotor in a Hitachi 65P ultracentrifuge. After centrifugation, the gradient was developed with the aid of the fractionator, as described above.

Results

Inhibitory effects of KW 2152 on the growth of L1210 cells

Figure 2 shows the effect of KW 2152 on the growth of L1210 cells. At concentrations lower than 10^{-7} *M*, the growth of the cells was not even influenced by continuous exposure to the drug; however, at 10^{-6} *M*, the growth was inhibited completely, and after 2 days no viable cell was seen.

Effect of KW 2152 on DNA, RNA, and protein synthesis

As indicated in Fig. 3(a), almost no significant inhibition was observed in the incorporation of ^{3}H-TdR into the acid-insoluble fraction of L1210 cells at less than 10^{-5} *M* KW 2152 up to 3 h of incubation, and the inhibitory effect

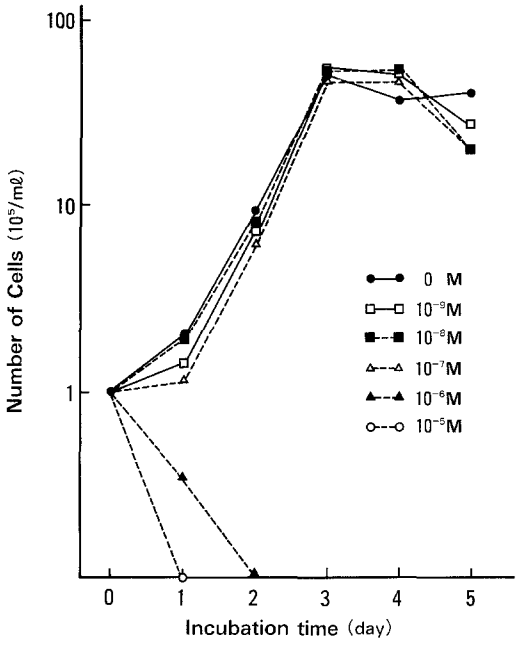

Fig. 2. Effect of KW 2152 on the growth of L1210 cells. 1×10^5 L1210 cells were incubated in PRMI 1640 medium supplemented with 10% FCS with or without KW 2152 at varied concentrations, and the number of the cells were counted after various periods of incubation

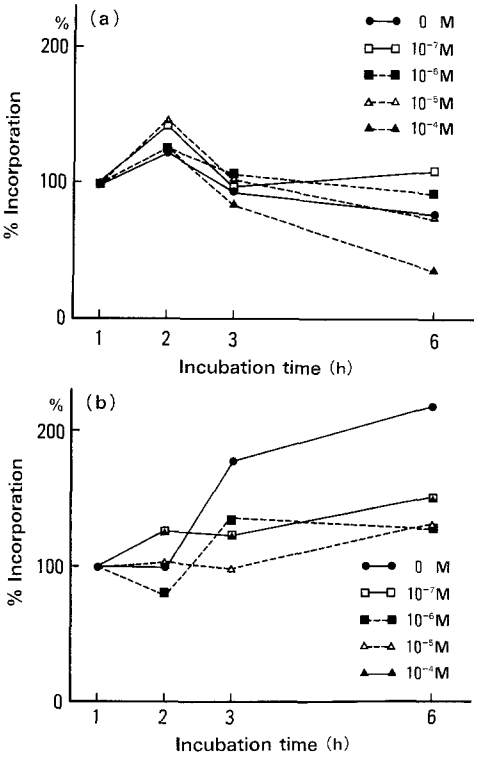

Fig. 3. Effect of KW 2152 on the incorporation of ^{3}H-TdR and ^{3}H-UR into the acid-insoluble fraction of L1210 cells. 5×10^5/2 ml L1210 cells were incubated with ^{3}H-TdR or ^{3}H-UR for 1 h in the presence of various concentrations of KW 2152 after the desired time of incubation. **(a)** incorporation of ^{3}H-TdR; **(b)** incorporation of ^{3}H-UR. ●——●, control; □——□, 10^{-7} *M*; ■——■, 10^{-6} *M*; △——△, 10^{-5} *M*; ▲——▲, 10^{-4} *M* KW 2152. The repeated experiment was carried out independently, and a similar result was obtained in the second experiment at each point

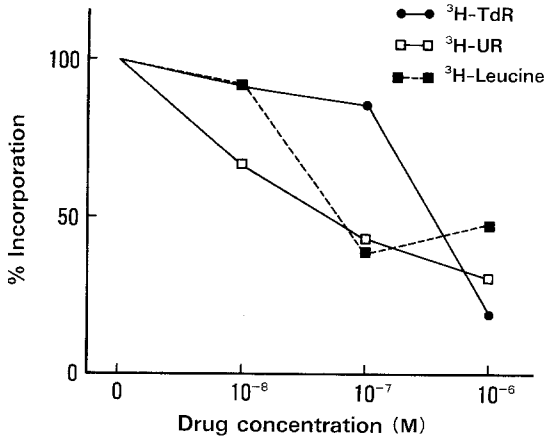

Fig. 4. Effect of KW 2152 on the incorporation of ^{3}H-TdR, ^{3}H-UR, and ^{3}H-Leucine into the acid-insoluble fraction of L1210 cells. 5×10^5/2 ml L1210 cells were incubated with 1 μCi/ml ^{3}H-TdR, ^{3}H-UR, or ^{3}H-Leucine in the presence of varied concentrations of KW 2152. Percentage of incorporation of: ●———●, ^{3}H-TdR; □———□, ^{3}H-UR; and ■———■, ^{3}H-Leu. The repeated experiment was carried out independently, and a similar result was obtained in the second experiment at each point

was observed at 10^{-4} *M* after 6 h. The incorporation of ^{3}H-UR was not influenced up to 6 h of incubation at 10^{-4} *M* KW 2152, as indicated in Fig. 3(b). However, when the cells were treated with KW 2152 for 24 h, the incorporation of ^{3}H-TdR, ^{3}H-UR, or ^{3}H-Leu into the acid-insoluble fraction was reduced to 20%, 30%, and 48%, respectively, of the control, even at concentrations as low as 10^{-6} *M*, as seen in Fig. 4.

Sedimentation behavior of the DNA of L1210 cells treated with KW 2152 on alkaline sucrose density gradient

Figure 5 shows the sedimentation behavior on alkaline sucrose density gradient centrifugation of the DNA of L1210 cells treated with KW 2152 for 24 h. In the control cells,

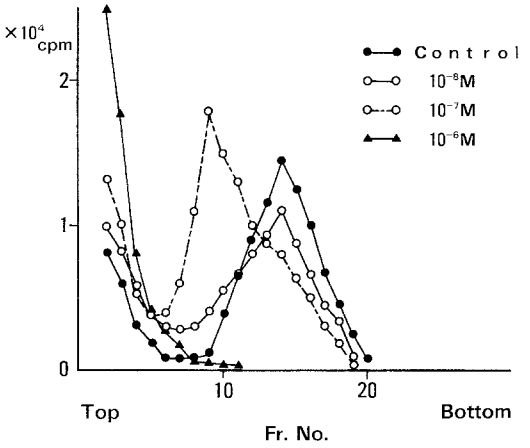

Fig. 5. The profile of alkaline sucrose density-gradient centrifugation of the DNA of L1210 cells treated with KW 2152 for 24 h. The cells labeled with 1 μCi/ml ^{3}H-TdR for 24 h were treated with varied concentrations of KW 2152 for 24 h. The cells thus treated were centrifuged on an alkali sucrose density gradient at 24,000 rpm for 2.5 h at 15° C. The fractions were collected from the top of the gradient. The experiment was repeated, and the result was similar in the second experiment

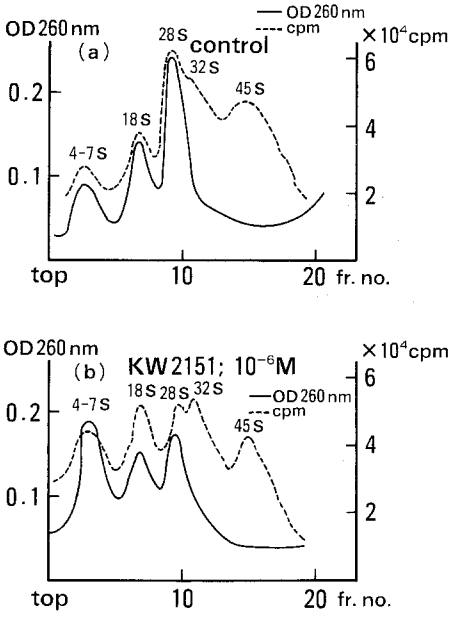

Fig. 6. The effects of KW 2152 on the processing of ribosomal RNA of L1210 cells. RNA was extracted from L1210 cells incubated with 1 μCi ^{3}H-UR for 2 h after treatment with 10^{-6} *M* KW 2152 for 24 h. For the separation of RNA, a 5%–30% sucrose density gradient was run as described in *Materials and methods.* (*a*) control; (*b*) cells treated with KW 2152; ———, optical density 260 nm; -----, cpm

the main peak is located at fraction 12; however, in the cells treated with 10^{-6} *M* KW 2152, the main peak shifted to the top of the gradient.

In the cells treated with 10^{-7} *M* KW 2152, the main peak is between the position of the main peak of the control cells and the top of the gradient. These results show that the single strand scission of DNA occurred during the KW 2152 treatment for 24 h.

The effect of KW 2152 on the processing of the ribosomal RNA of L1210 cells

Figure 6 shows the processing of the rRNA of L1210 cells labeled with ^{3}H-UR. The cells were cultured with or without 10^{-6} *M* KW 2152 for 24 h, then ^{3}H-UR was added for 2 h and the RNA was extracted from the cells. The RNA was centrifuged on the sucrose density gradient. On the sucrose density gradient pattern, the ribosomal RNA was slightly degraded in the cells treated with KW 2152 compared with the RNA of the control cells in Fig. 6(a); however, the processing was not disturbed, as shown by the dotted line in Fig. 6(b).

The effect of KW 2152 on plasmid pBR322

The effect of KW 2152 on plasmid DNA was investigated using pBR322. As shown in Fig. 7, there was no change in the electrophoretic DNA pattern at drug concentrations between 10^{-6} *M* and 10^{-8} *M* after 24 h of treatment. Under the experimental conditions, the presence of 1 m*M* DTT had no effect on the cleavage of DNA, and heating of the DNA at 70° C for 2 h after treatment with KW 2152 also resulted in no change in its electrophoretic mobility (data not shown).

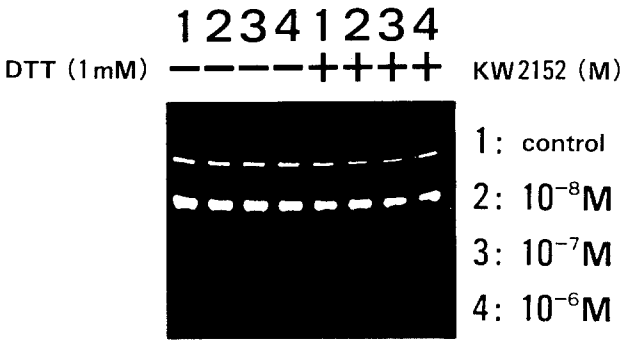

Fig. 7. The effects of KW 2152 on plasmid DNA. pBR322 was treated with various concentrations of KW 2152 in the presence or absence of 1 m*M* DTT for 24 h

Discussion

KW 2152 was synthesized as an anticancer drug showing remarkable life-prolonging activity in P388-, B16 melanoma-, and Lewis lung carcinoma-bearing mice. Moreover, this compound has shown a growth inhibitory effect on murine and human cancer cell lines xenografted in nude mice [2]. The IC_{50} of KW 2152 for L1210 cells has been reported to be 1.5×10^{-4} *M* and 7.2×10^{-7} *M* after 1 h and 72 h of exposure, respectively. The cells were seeded in the dishes 24 h after exposure [2]. These results clearly indicate that KW 2152 is more potent in cells exposed for longer periods.

Thus, the schedule dependency of KW 2152 suggested that it might act as an antimetabolite; however, the competition of various nucleotides with the growth inhibitory effect of KW 2152 on HeLa S_3 cells were not observed. Therefore, its action as an antimetabolite could not be proven.

Fujimoto et al. have reported that RNA synthesis is sensitive to KW 2152, and DNA synthesis was shown to be almost 30-fold less sensitive than RNA synthesis in P388 leukemia [2]. However, the exposure time was only 60 min and the dose they used was 10^{-4} *M*, which is too high a concentration. Although RNA synthesis was inhibited by 50% at 10^{-5} *M* KW 2152, DNA and protein syntheses were not inhibited after 60 min of incubation [2]. In the present study, however, at an exposure time of 24 h, the 1-h incorporation of ^{3}H-TdR, ^{3}H-UR, and ^{3}H-Leu into the acid-insoluble fraction of the cells was 20%, 31%, and 47%, respectively, of the control, at 10^{-6} *M* compared with 10^{-4} *M* in 1 h in the aformentioned experiment. The damage to the DNA strand was investigated by alkaline sucrose density-gradient centrifugation [1, 3, 5].

When the cells were exposed to the drug at 10^{-5} *M* for 1 h, no appreciable breakage of the DNA strand was observed (data not shown); however, single strand scission of the DNA was induced after 24 h incubation with 10^{-6}–10^{-7} *M* KW 2152, in a dose-dependent manner.

Tomita et al. have reported the effect of quinocarmycin on cccDNA and ocDNA of the PM2 phage; they observed a reduction in the amount of cccDNA and an increase in ocDNA [7].

In the present experiment, plasmid DNA pBR322 was used, and the effect of KW 2152 was studied in the presence or absence of DTT. However, no decrease in cccDNA or ocDNA was observed even after 24 h of treatment at 10^{-6}–10^{-8} *M*. The facts strongly suggest that some soluble cellular component is involved, such as proteins and/or RNA, which will convert KW 2152 to an active form capable of causing damage to cellular DNA. The metabolic pathway of KW 2152 is not yet known; however, a study is now in progress in this laboratory. The mode of damage to DNA caused by KW 2152 might be different from that of conventionally used anticancer drugs.

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