DIAZAQUINOMYCIN A, A NEW ANTIFOLATE ANTIBIOTIC, INHIBITS THYMIDYLATE SYNTHASE

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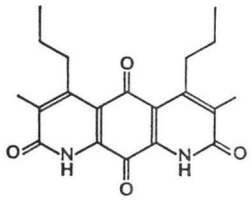
(Received for publication May 9, 1985)

The inhibitory effect of diazaquinomycin A (DQM) on the growth of *Enterococcus faecium* was completely reversed by thymidine. Relatively large amounts of folate, dihydrofolate or leucovorin also reversed the activity of DQM. DQM showed cytotoxicity against Vero and Raji cells and the cytotoxicity was partially reversed by leucovorin or thymidine. With enzyme experiments, it was found that DQM inhibits TMP synthases from *E. faecium* and Ehrlich ascites carcinoma competitively with 5,10-methylenetetrahydrofolate, one of the substrates for the enzyme reaction.

Diazaquinomycin A (DQM, Fig. 1) has been isolated and characterized as a new neutral quinoid antibiotic active against Gram-positive bacteria by ŌMURA *et al.*[1,2]. DQM was found as an antifolate antibiotic which activity was reversed by leucovorin (5-formyltetrahydrofolic acid) or thymidine (TdR) in the course of our screening work for new antifolates from microorganisms[3].

Fig. 1. Structure of diazaquinomycin A.



The present paper deals with the site of action of DQM in the folate metabolic pathway in bacteria and animal cells, compared with the known synthetic antifolates, trimethoprim (TP) and methotrexate (MT), and 5-fluorouracil (5-FU).

Materials and Methods

Bacterial Strain and Growth

Enterococcus faecium IFO 3181 maintained in this laboratory was used. The bacterium, which had been kept as a stab culture in Lactobacilli Inoculum Broth "Nissui"[3], was transferred into a test tube containing 10 ml of WOOD and GUNSALUS' medium[4] (yeast extract 1%, Tryptose 1%, K_2HPO_4 0.5%, glucose 0.3%) and incubated for 12 hours at 37°C to give a seed culture. The cells were washed four times with sterile water and the cell suspension was transferred at the rate of 5% into a test tube containing the medium mentioned below and then incubated statically at 37°C. The growth was monitored by measuring absorbance at 660 nm with a Coleman Junior II spectrometer.

In the experiments for growth inhibition and its reversal, Folic Acid Assay Medium "Nissui"[3] containing folate 1.0 ng/ml was used and various concentrations (1, 10, 100 ng/ml) of several potential reversing agents were further supplemented. The drugs (DQM, TP, 5-FU) were applied to the test tube at the final concentration of 0.1 μg/ml. Folate-related compounds, TP and 5-FU were sterilized by membrane filtration. DQM solution, 50 μl, in $CHCl_3$ - MeOH was applied to sterile paper disk (ϕ8 mm) and the dried disk was put into the medium.

In some further experiments, the synthetic medium containing only one folate-related compound, folate, dihydrofolate (DHF), leucovorin or TdR was used.

Animal Cell Culture

Vero cells were grown and maintained in EAGLE's minimum essential medium supplemented with 5% calf serum, benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) as monolayer culture. Logarithmic phase cells were harvested by treating with trypsin (0.05%) and EDTA (0.01%) in calcium- and magnesium-free phosphate buffered saline solution. After washed with the growth medium described above, cells were incubated in wells of 96-well flat bottom microplates (Corning cell wells). Each wells contained 0.1 ml of the fresh growth medium supplemented with drugs, folate-related compounds and 5×10^3 cells. DQM, MT and 5-FU were dissolved in DMSO and added to the medium at the final concentration of 1% of DMSO. Control contained DMSO at the same rate. Folate-related compounds were dissolved in the growth medium. The cells were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. After incubation for 1~4 days, the cells were washed with calcium- and magnesium-free phosphate buffered saline and stained with methylrosaniline solution[5] (methylrosaniline 1 g, formaline 10 ml, ethanol 100 ml, NaCl 1.7 g and distilled water 90 ml). After washing with water, cell growth was measured with a microplate photometer (Corona Electric Company, MTP-12) at 550 nm.

Culture of Raji cells were grown and maintained in RPMI 1640 medium with added 10% fetal calf serum, benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml). Logarithmic phase cells were washed with a fresh medium which consisted of folate-free RPMI 1640 medium, fetal calf serum and antibiotics discribed above and diluted to approximate 10^5 cells/ml prior to each experiment. The drugs and folate-related compounds were added to the medium. After incubation, the number of viable cells was counted with a haemocytometer by trypan-blue exclusion method.

Preparation of Thymidylate Synthase (TMP Synthase, EC 2.1.1.45.) from *E. faecium*

Preparation of the enzyme from wet cells (35 g) of *E. faecium* IFO 3181 obtained by the incubation (37°C, 4 hours) in 9 liters of WOOD and GUNSALUS' medium was carried out at 0~4°C as described by BLAKLEY and MCDOUGALL[6] with some modifications. The cells were suspended in 245 ml of 50 mM Tris-HCl buffer (pH 7.2, 50 mM KCl, 10 mM 2-mercaptoethanol and 1 mM EDTA), collected by centrifugation and then disrupted with quartz sands (95 g).

The buffer (100 ml) was added to the disrupted cells. Debris was removed by centrifugation at $12,000\times g$ for 1 hour. The supernatant fraction was dialyzed against an excess amount of the buffer, treated with 0.03 volume of 20% streptomycin sulfate, and centrifuged at $12,000\times g$ for 1 hour. An ammonium sulfate-saturated solution (1.5 volume, 0°C) was added gradually to the supernatant fraction. The flocculated precipitate was removed by centrifugation. A powder of solid ammonium sulfate was added gradually to the supernatant fraction to bring to 95% saturation with ammonium sulfate at 0°C. The precipitation obtained by centrifugation was dissolved in the buffer and dialyzed against the buffer. The dialyzed solution was applied to a Sephadex G-75 column (235 ml) after concentrated with dry Sephadex G-75 and developed with the above buffer. The enzymically active fractions from the column were pooled and used as an enzyme preparation of TMP synthase.

Preparation of TMP Synthase from Ehrlich Ascites Carcinoma

The enzyme preparation was obtained according to the method described by ROBERTS[7] with some modifications. All procedures were carried out at 0~4°C. Ehrlich ascites carcinoma cells were harvested 5 days after intraperitoneal transplantation to 4 mice. The cells were placed in 100 ml of EDTA solution (1 mg/ml, pH 7.5) in 5% glucose, collected by centrifugation at $8,000\times g$ for 5 minutes, and washed with the solution. The cells (*ca.* 9 g) were resuspended in 9 ml of cold 0.01 M Tris-buffer (pH 7.0) containing 0.25 M sucrose, disrupted with a Teflon-homogenizer, and centrifuged at $30,000\times g$ for 30 minutes. The supernatant fraction was used as enzyme preparation.

Assay of TMP Synthase

The activity of TMP synthase from *E. faecium* was assayed as discribed by DUNLAP[8] with some modifications. A stock solution of 2 mM (±)-L-methylenetetrahydrofolate was prepared by dissolving 6 mg of (±)-L-tetrahydrofolate (THF) in 5 ml of a solution containing 0.05 M $NaHCO_3$, 0.07 M formaldehyde and 0.25 M 2-mercaptoethanol, directly before being used for assay, and kept in an ice bath

under a gentle stream of nitrogen. Tris-HCl buffer (60 mM, pH 7.6, 2.25 ml) containing 24 mM $MgCl_2$ and enzyme solution (0.05 ml) were mixed and incubated for 5 minutes at 30°C. (±)-L-5,10-Methylene-THF solution (0.4 ml) was added to the mixture and incubated for 5 minutes at 30°C. The reaction was initiated by the addition of 0.1 ml of 1 mM dUMP to 1.0 ml of the above mixture. The reaction was monitored by the change in absorbance at 340 nm at 30°C.

The assay of TMP synthase from Ehrlich ascites carcinoma was performed as discribed by CALVERT *et al.*[9] with some modifications. An incubation mixture (0.5 ml) contained [5-^{3}H]dUMP 23.6 nmol, 0.5 μCi; DL-THF 50 nmol; formaldehyde 322 μmol; 2-mercaptoethanol 142 μmol; Tris-HCl buffer (pH 7.4) 5 μmol; and NaF 50 μmol. Reaction was started by adding 0.2 ml of the enzyme preparation, and stopped by adding 2 ml of iced water after 1-hour incubation at 37°C. Dowex 1 (Cl^-) resin (1.0 ml) was then added to the mixture and centrifuged. The radioactivity of 1.0 ml of the supernatant fraction was mixed with 5 ml of aqueous count scintillant (ACSII, Amersham) and counted in a liquid scintillation counter (Aloka, LSC-653).

Preparation of DHF Reductase (EC 1.5.1.3.) from *E. faecium* and Rat Liver

The enzyme from *E. faecium* was prepared according to the method of HILLCOAT and BLAKLEY[10] with some modifications. The wet cells (*ca.* 27 g) of *E. faecium* was disrupted with quartz sands as described above. Forty milliliters of 0.01 M phosphate buffer (pH 7.2) containing 1 mM EDTA was added to the disrupted cells and centrifuged at 8,000×*g* for 30 minutes. The supernatant fraction was applied to ammonium sulfate fractionation after treatment with streptomycin sulfate (final concentration, 1.0%). The 53~80% saturated ammonium sulfate precipitated fraction was dialyzed against the above buffer. The dialyzed material concentrated by dry Sephadex G-75 was applied to Sephadex G-75 column and eluted with the same buffer. The enzymically active fractions from the column were pooled and used as enzyme preparation.

Preparation of the enzyme from rat liver was carried out as described by BURCHALL and HITCHINGS[11].

Assay of DHF Reductase

The assay of DHF reductases from *E. faecium* and rat liver was performed as described by HILLCOAT and BLAKLEY[10] and BURCHALL and HITCHINGS[11], respectively.

Results

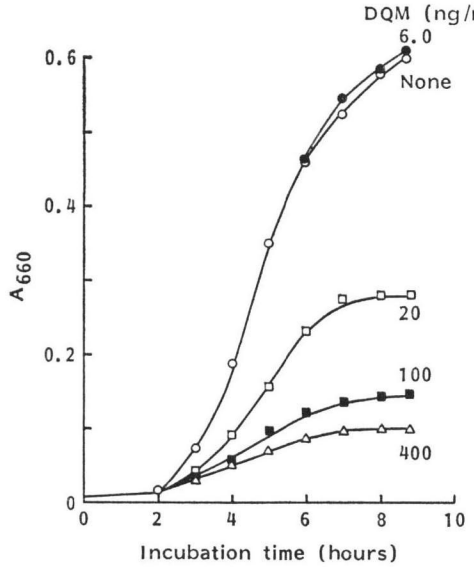
Effect of DQM on the Growth of *E. faecium*

The activity of DQM against a growing culture of *E. faecium* was examined. Because DQM is hardly soluble in water, a paper disk which was dipped in a DQM solution in $CHCl_3$ - MeOH (4: 1) and dried was put into the medium. The inhibitory zone of DQM by paper disk method against the bacterium was not proportional to the amount of DQM and was constant at the concentration over 0.5 μg/ml. On the contrary, the dose response of DQM to the bacterial growth was observed in the above method (Fig. 2). Because a significant inhibition was observed at 100 ng/ml, in reversal experiments 100 ng/ml of DQM was used.

Reversal by Folate-related Compounds of Growth Inhibition of DQM in *E. faecium*

Folate-related compounds were added to the medium containing DQM, TM or 5-FU at the concentration of 100 ng/ml, and the effect of the compounds on the growth of *E. faecium* was examined (Fig. 3). The antibacterial activity of DQM was completely reversed by the addition of DHF and leucovorin at the concentration of 100 ng/ml, but the reversal by DHF and leucovorin at the concentration of 2.0 ng/ml was hardly observed. On the other hand, the activity of TP was completely reversed by the addition of leucovorin at the concentration of 2.0 ng/ml. Although the reversal by

Fig. 2. Effect of DQM on the growth of *E. faecium*.



DHF at the same concentration was not observed, the activity of TP was completely reversed by the addition of the high concentration (100 ng/ml) of DHF. The addition of pteroate or folate showed the same effect as DHF on the antibacterial activity of DQM and TP (data not shown).

More detail reversal experiments were examined. The medium supplemented with only one folate-related compound was used. DQM showed antibacterial activity in the medium which contained 1 ng/ml of folate, DHF or leucovorin, but did not showed the activity in the medium supplemented with TdR at any concentration. Moreover, the activity of DQM was completely reversed by folate-related compounds when the medium contained 100 ng/ml of them (Fig. 4). On the other hand, the activity of TP was observed only when the medium contained 1 ng/ml of folate or DHF. It was not observed in the medium supplemented with leucovorin or TdR at any concentration. One-hundred nanograms per milliliter of folate or DHF showed reversal effect on its activity. 5-FU showed antibacterial activity in the medium supplemented with

Fig. 3. Reversal by DHF and leucovorin of the growth inhibition by DQM and TP in *E. faecium*.
○ None; ● drug (DQM or TM); □ drug + DHF (2 ng/ml); ■ drug + DHF (100 ng/ml); △ drug + leucovorin (2 ng/ml); ▲ drug + leucovorin (100 ng/ml).

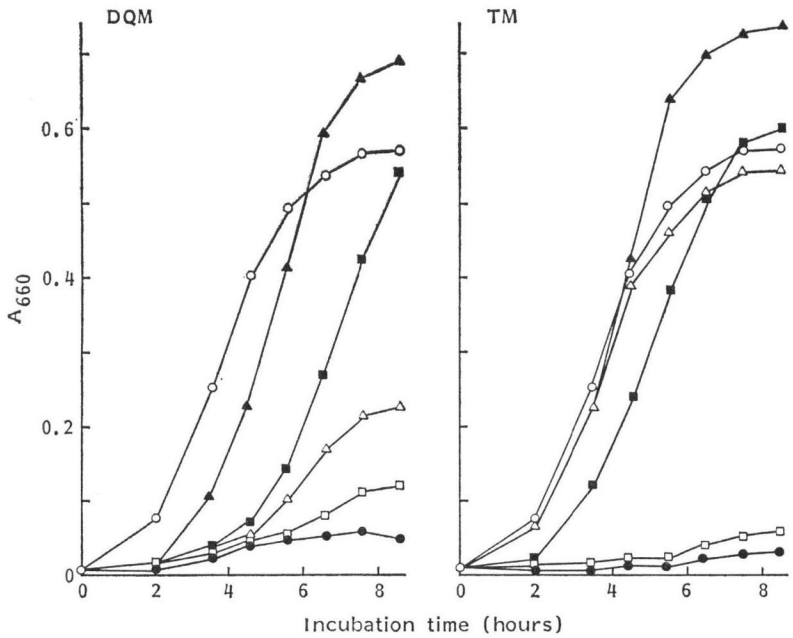
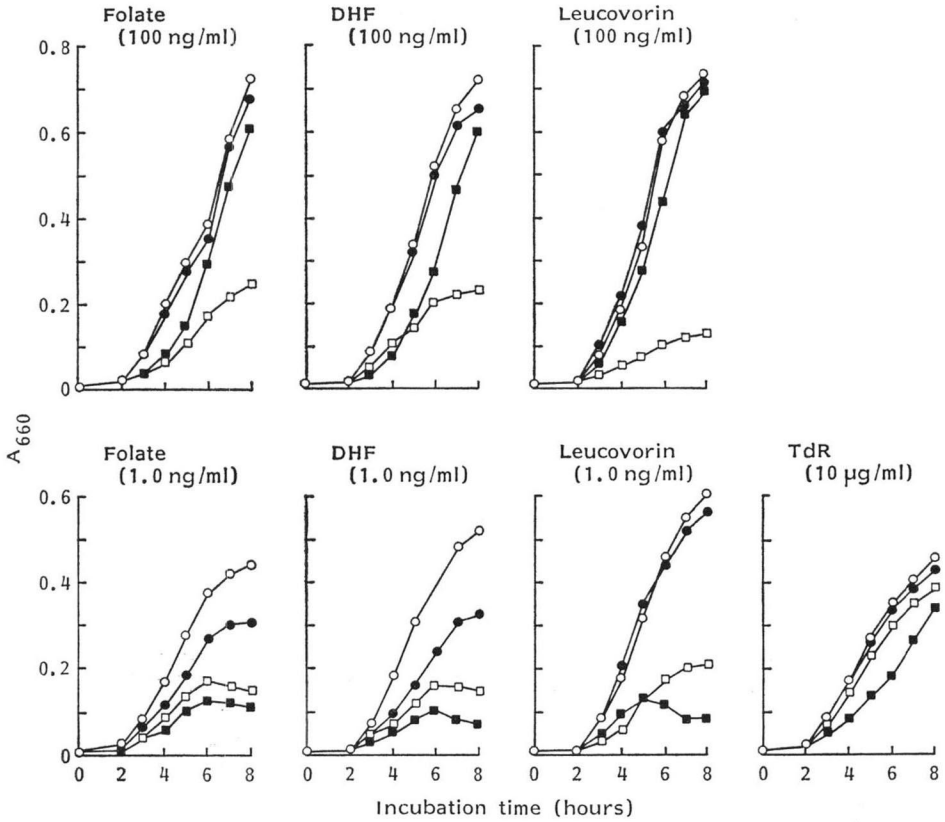


Fig. 4. Growth inhibition by DQM, TP and 5-FU in the presence of folate-related compounds.
○ No drugs; ■ DQM; ● TP; □ 5-FU.



folate, DHF or leucovorin at any concentration, but it did not show the activity when the medium contained TdR (Fig. 4).

Inhibition by DQM of the Growth of Vero and Raji Cells and the Reversal by Folate-related Compounds

DQM showed cytotoxicity against both Vero and Raji cells; the ID_{50} values were 0.86 and 0.23 μg/ml, respectively, which are similar order as those of 5-FU (0.7 and 0.9 μg/ml, respectively).

The cytotoxicity of DQM (1.0 μg/ml) against Vero cells was partially reversed by leucovorin alone (Fig. 5). The toxicity of MT (1.0 μg/ml) was reversed completely by leucovorin and partially by DHF. On the other hand, the toxicity of 5-FU (1.0 μg/ml) was not reversed by folate, DHF and leucovorin. TdR (1.0, 10 and 100 ng/ml) showed on reversal effect on the toxicity of the above three drugs.

With Raji cells, the cytotoxicity of DQM (0.125 μg/ml) was reversed by TdR plus inosine and leucovorin (Fig. 6), but folate and DHF had no reversal effect. When 0.25 μg/ml of DQM was used, TdR plus inosine exhibited only partial reversion. The cytotoxicity of MT (1.0 μg/ml) was completely reversed by leucovorin and TdR plus inosine, but that of 5-FU was not reversed by folate, DHF, leucovorin and TdR plus inosine at all.

Although some deviations of reversion depending on organism or drug were observed, the above

Fig. 5. Reversal by DHF and leucovorin of the cytotoxicity of DQM, MT and 5-FU in Vero cells.
○ None; ● drug (DQM, MT or 5-FU); □ drug + DHF (20 μg/ml); △ drug + leucovorin (20 μg/ml).

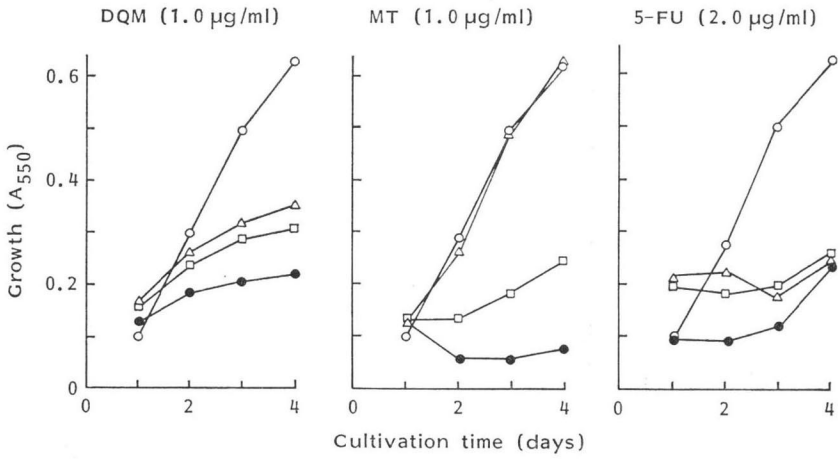
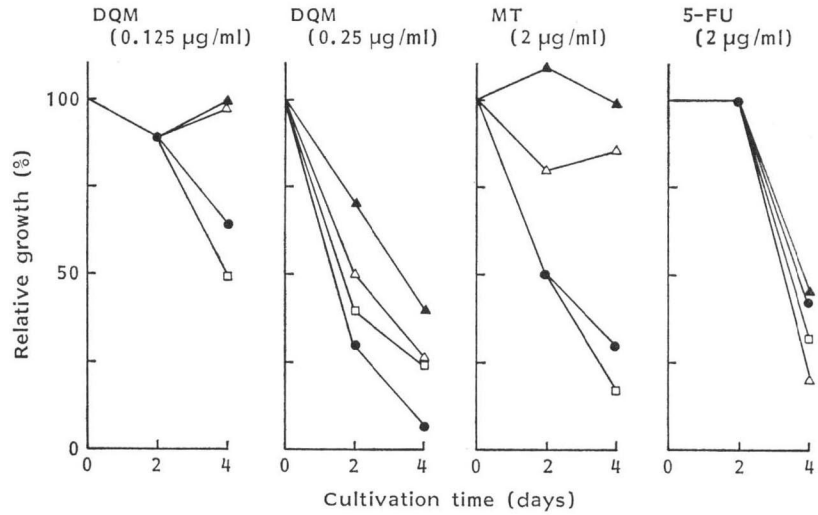


Fig. 6. Reversal by DHF, leucovorin and TdR plus inosine of the cytotoxicity of DQM, MT and 5-FU in Raji cells.
● Drug (DQM, MT or 5-FU); □ drug + DHF (10 μg/ml); △ drug + leucovorin (10 μg/ml); ▲ drug + TdR plus inosine (10 μg/ml).



results suggest that the site of inhibition of DQM lies on TMP synthase. The deviations may be due to the differences in cell permeability or inactivation of DQM in cells.

Inhibition of TMP Synthase by DQM

The inhibitory effect of DQM on TMP synthases from *E. faecium* and Ehrlich ascites carcinoma was examined. Because DQM was extremely insoluble in water, a DQM solution in DMSO was added to the buffer, and the mixture was sufficiently sonicated and at once subjected to assay to obtain reproducible values for its inhibitory activity.

With *E. faecium* enzyme, *Km* value was 274 μM for 5,10-methylene-THF and *Ki* value of DQM

Fig. 7. Inhibitory action of DQM on thymidylate synthase from *E. faecium*.
Km=274 μM, *Ki*=36 μM.

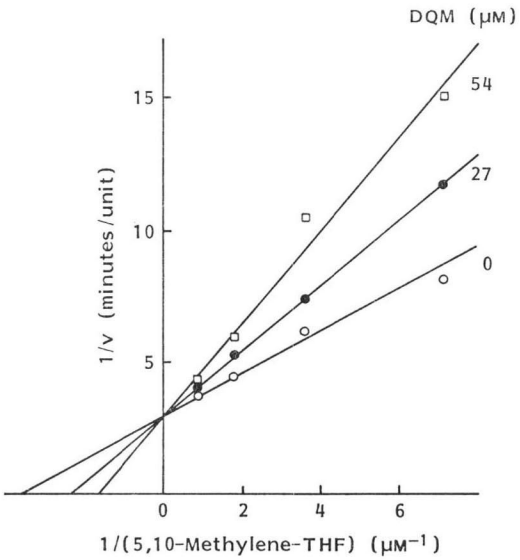


Fig. 8. Inhibitory action of DQM on thymidylate synthase from Ehrlich ascites carcinoma cells.
Km=45 μM, *Ki*=14 μM.

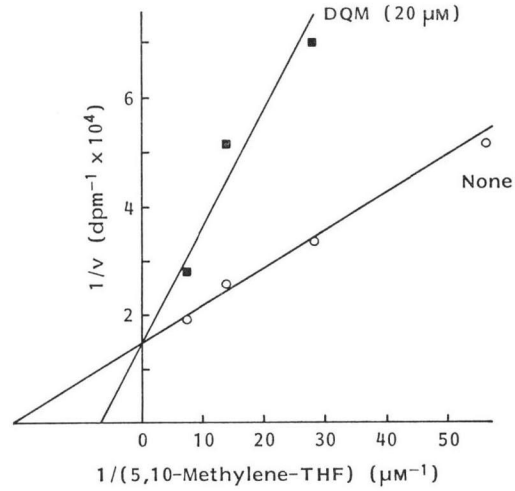
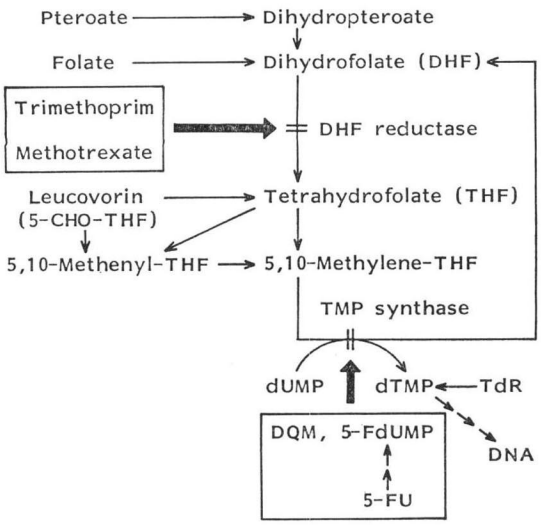


Fig. 9. Metabolic pathway of folate-related compounds and inhibitors.



was 36 μM (Fig. 7). With Ehrlich ascites carcinoma enzyme, *Km* was 45 μM and *Ki* was 14 μM (Fig. 8). The results of Lineweaver-Burk plot and Dixon plot showed that DQM inhibits the both enzymes competitively with the substrate 5,10-methylene-THF.

On the other hand, DQM showed no effect on the activity of DHF reductases from *E. faecium* and rat liver although 33 ng/ml of TM inhibited the enzyme from *E. faecium* by 59% and 33 ng/ml of MT inhibited rat liver enzyme by 78%.

Discussion and Conclusion

Fig. 9 shows the metabolic pathway of folate-related compounds and inhibitors. TP and MT which are clinically used as antibacterial or anticancer drugs, respectively, inhibit DHF reductase[12]. 5-FU which is one of the most important drugs among clinically useful anticancer ones inhibits TMP synthase after converted to 5-fluoro-dUMP in cells[13]. Thus, folate metabolic pathway is thought to be an important target for chemotherapy. At present, however, there are no clinically useful, natural products which inhibit specifically folate metabolism. So, DQM was investigated to be compared with the above synthetic antifolates in *E. faecium* and cell culture.

DQM inhibited the growth of *E. faecium* but it showed no activity in the medium supplemented with TdR. This pattern was similar to that of 5-FU and different from TP. However, a large amount of folate, DHF or leucovorin showed reversal effect on the activity of DQM. These compounds had no effect on the activity of 5-FU. On the other hand, the activity of TM was reversed by a large

amount of folate or DHF which is the substrate of DHF reductase. The above results suggest that DQM was differentiated from the type of antifolates[12] which inhibit DHF reductase as TM and MT, and that DQM inhibits TMP synthase competitively with 5,10-methylene-THF. In fact, DQM inhibited TMP synthase from *E. faecium*.

In animal cells, DQM showed also inhibitory effect. The cytotoxicity of DQM was partially reversed by leucovorin and TdR plus inosine. DQM also inhibited TMP synthase from Ehrlich ascites carcinoma competitively with 5,10-methylene-THF.

TMP synthase is thought to be an attractive target for cancer chemotherapy[13] because the enzyme is the only one *de novo* route to TMP and it is a pivotal enzyme in the biosynthesis of DNA. The activity of the enzyme is substantially elevated in proliferating cells. Further, there are indications in MT-resistant cells which over-produce DHF reductase that TMP synthase may become rate-limiting for growth in the presence of a large amount of MT[14]. Most of folic acid analogs act through inhibition of DHF reductase and generally, analogs of 5,10-methylene-THF as inhibitors of TMP synthase are not as potent as dUMP analogs. Recently, an effective inhibitor of TMP synthase has been sought[8,15~17] since such a compound be expected to have activity equivalent to or superior to MT. Folate analogs should be more effective than pyrimidines since they do not require metabolic activation and are poor substrates for degradation[16]. DQM had no inhibitory activity against DHF reductase but inhibited TMP synthase.

FRIEDKIN *et al.* found that a TMP synthase preparation from *Escherichia coli* contained an inhibitor against the enzyme. It was identified as 5-formyltetrahydropteroyloligoglutamate[18,19]. Another naturally occurring inhibitor of TMP synthase is a protein which arises during phage infection of *Bacillus subtilis*[20]. DQM is a low molecular weight antibiotic which has an unique symmetrical quinoid structure. In this mean, DQM is thought to be a first antifolate antibiotic and a novel type of inhibitor of TMP synthase. Unfortunately, the intraperitoneal administration of DQM exhibited no antitumor activity against L-1210 mouse leukemia and Ehrlich ascites carcinoma because of its low solubility. Preparation of derivatives with higher solubility is of interest in creating new antitumor drugs.

Acknowledgment

The authors wish to thank Dr. K. KOMIYAMA for providing Ehrlich ascites carcinoma cells.

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