# ANTIVIRAL ACTIVITY OF MYCOPHENOLIC ACID

## STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. IV

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Mycophenolic acid shows significant antiviral activity in the agar diffusion plaque inhibition, plaque reduction and tube culture methods. Both DNA and RNA viruses are involved in the antiviral spectrum and the chemotherapeutic index of mycophenolic acid is high against viruses *in vitro*.

Many synthetic compounds have been found to show antiviral activity since the finding of cytopathic effect caused by virus infection. However, a few antiviral antibiotics were obtained using the tissue culture techniques for a screening. Gliotoxin[1], tenuazonic acid[2], trichothecin[3], verrucarrin A and brefeldin A[4] were reported to show antiviral activity *in vitro* among fungal metabolites.

In our screening for antiviral antibiotics using the agar diffusion method for assay and fungi as the screening organisms, it was found that mycophenolic acid is a potential antiviral agent[5]. In this paper the antiviral activity of mycophenolic acid is described.

### Isolation and Identification

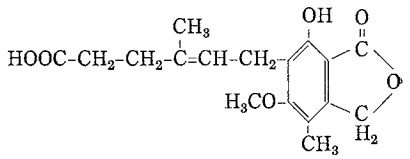
In primary screening, an acetone extract of the mycelium of a *Penicillium* sp., isolated from soil, showed a remarkable antiviral activity against herpes simplex virus strain HF (HSV) and vaccinia virus strain DIE (VAV) infected on primary chick embryo fibroblast cell monolayer (CEF). Later, it was found that the active principle was present not only in the mycelium but also in the culture filtrate. Both the culture filtrate and the mycelial extract inhibited the growth of *Candida albicans* strain Ch and *Bacillus subtilis* IAM 1026. The hypothesis that the antimicrobial activity was due to the substance responsible for the antiviral activity was suppored by the fact that the two activities showed the same Rf values when the extract and the culture filtrate were subjected to thin-layer chromatography. An agar diffusion assay using *C. albicans* as the test organism, therefore, was developed to guide fermentation, fractionation and isolation.

The fungus was aerobically grown for 96 hours at 27°C in a medium consisting of (g/liter) glucose 50, peptone 5, yeast extract 2, ammonium chloride 1, potassium phosphate monobasic 0.6, magnesium sulfate heptahydrate 0.4 and calcium carbonate 10. The fermentation broth was filtered through Celite and the culture filtrate was extracted with ethylacetate after adjusting to pH 3 with hydrochloric acid. The ethyl-

acetate layer was dried over anhydrous sodium sulfate and then concentrated in reduced pressure to a small volume. The resulting oily residue was chromatographed on silica gel. The active principle was eluted with benzene-methanol (95 : 5) and readily crystallized as large prisms when kept standing at 5°C overnight.

The antiviral antibiotic recrystallized from ethanol melted at 141~143°C. The infrared absorption spectrum suggested the presence of hydroxyl, ester or lactone, carboxylic acid, olefinic double bond and phenyl groupings. The presence of aromatic ring was supported by the strong peaks at m/e 77, 91 and 320, the parent peak (base peak), in the mass spectrum. The molecular formula, $C_{17}H_{20}O_6$, was assigned for the antibiotic on the basis of elementary analyses and the molecular weight, 320, determined by mass spectroscopy (Found C 63.59 %, H 6.23 %; Calcd. for $C_{17}H_{20}O_6$, C 63.74 %, H 6.29 %). All this evidence indicated that the antibiotic obtained is mycophenolic acid. Mycophenolic acid is an antibiotic effective against some bacteria and fungi (Fig. 1).

Fig. 1. Mycophenolic acid (6)



## Antiviral Activity in Agar-diffusion Method

Table 1 shows the dose-response relationship of antiviral activity of mycophenolic acid in the agar-diffusion method. Primary chick embryo fibroblast cell monolayers (CEF) were infected with the virus and after washing with EARLE's buffer, overlaid with the maintenance medium containing 1 % agar which was composed of EARLE's buffer supplemented with 5 % calf serum, 0.5 % lactalbumin hydrolyzate, 0.1 % yeast extract and 0.0007 % neutral red. Paper discs 8 mm in diameter which were impregnated with the antibiotic solution were placed on the solidified surface and the cultures were incubated for 2 days at 37°C in a humidified atmosphere containing 10 % $CO_2$. The appeared plaque-free protected zones were measured and the antiviral activity was expressed as diameter of the zones.

Table 1. Antiviral activity of mycophenolic acid by agar-diffusion plaque-inhibition method

| Mycophenolic acid (mg/ml) | Antiviral activity | | | |
|---|---|---|---|---|
| | NDV | | HSV | |
| | CTZ | PIZ | CTZ | PIZ |
| | mm | mm | mm | mm |
| 32.600 | — | 40 | — | 40 |
| 8.150 | — | 40 | — | 40 |
| 2.038 | — | 34 | — | 34 |
| 0.520 | — | 34 | — | 32 |
| 0.130 | — | 30 | — | 32 |
| 0.065 | — | 30 | — | 32 |

Newcastle disease virus strain Miyadera (NDV) and herpes simplex virus strain HF (HSV) were used. Antiviral activity was expressed as diameter of plaque-free protected zone (PIZ) on primary chick embryo fibroblast monolayer together with diameter of cytotoxic zone (CTZ). The paper-disc used was the product of Toyo Kagaku Co., Ltd. of 8 mm diameter and 1 mm thickness which is able to absorb 0.05 ml of solvent per disc.

Plaque formation by Newcastle disease virus strain Miyadera and HSV were suppressed in the same manner so that both DNA and RNA viruses are included in its antiviral spectrum. The index is >500 in this assay system because no cytotoxic zone appeared in the indicated assay range. The index is extremely high in comparision with those of other antiviral antibiotics such as tenuazonic acid and trichothecin.

### Antiviral Activity by the Tube Culture Method

Table 2 demonstrates the antiviral activity of the antibiotic in tube culture method using HeLa cells as a host. Monolayers of HeLa cells grown in tubes containing EARLE's buffer supplemented with 5 % calf serum, 0.5 % lactalbumin hydrolyzate and 0.1 % yeast extract were infected with the viruses and the monolayers were cultured in fresh medium containing given amounts of the antibiotic. Cytopathic effect caused by the virus multiplication was examined once a day for 5 days through direct microscopic observation. Cytopathic effect (CPE) by HSV, VAV, and measles virus strain Sugiyama was completely suppressed in the presence of 31.5~500 mcg/ml of mycophenolic acid, whereas CPE by NDV and adeno virus type 5 could not be suppressed even if the antibiotic was present at the highest well-tolerated dose. It is obscure why the antibiotic was ineffective against NDV in the tube culture, although it was effective in the agar-diffusion method. The index is >16 because CPE was inhibited at a concentration of 1.55 mcg/ml. Thus the antiviral spectrum includes both DNA and RNA viruses in the tube culture method as well as in the agar-diffusion method.

Table 2. Antiviral activity of mycophenolic acid by tube-culture dilution method

| Mycophenolic acid (mcg/ml) | CP effects of viruses | | | | |
|---|---|---|---|---|---|
| | Vaccinia | NDV | Adeno | Herpes | Measles |
| 0 | +++* | +++ | +++ | +++ | +++ |
| 31 | − | +++ | + | − | − |
| 62 | − | +++ | + | − | − |
| 125 | − | +++ | + | − | − |
| 250 | − | +++ | + | − | − |
| 500 | − | +++ | + | − | − |

* CP Effects stands for cytopathogenic effects of viruses.

The HeLa cell tubes containing mycophenolic acid dissolved in the maintenance media and appropriate virus dilution were incubated. The tubes were inspected for the possible cytopathogenic effect of viruses at 5 days.

Mycophenolic acid at 1,000 mcg/ml was found to be toxic, therefore, concentrations 500 mcg/ml or lower were used for inhibition study.

### Antiviral Activity in the Plaque Reduction Method

A plaque reduction assay was carried out to detemine the effective concentration using CEF-HSV system. The method used was the same as in the agar-diffusion assay except that 40 PFU per petri dish of 9 cm in diameter were infected. As shown in Table 3, numbers of plaques were reduced according to the increased concentration of mycophenolic acid. Complete plaque reduction was observed at a concentration of 31 mcg/ml. In this assay system the index is also high.

Mycophenolic acid exerts significant antiviral activity against a number of viruses in tissue cultures. It had long been considered that any antiviral agents with high indexes in either *in vivo* or *in vitro* might be specific in its antiviral spectrum. It is interesting that

Table 3. Antiviral activity of mycophenolic acid by the plaque-reduction method

| Mycophenolic acid (mcg/ml) | Antiviral activity (Numbers of plaques/Petri dish) | | | Plaque reduction (%) |
|---|---|---|---|---|
| | Exp 1 | Exp 2 | Exp 3 | |
| 0 | 41 | 35 | 37 | 0 |
| 7.5 | 8 | 13 | 10 | 72.6 |
| 31.0 | 0 | 0 | 0 | 100.0 |
| 125.0 | 0 | 0 | 0 | 100.0 |
| 500.0 | Toxic | Toxic | Toxic | |

The virus used was herpes simplex virus strain HF and the monolayer was primary chick embryo fibroblast grown in petri dishes 9 cm in diameter.

mycophenolic acid has a wide antiviral spectrum and high index *in vitro*.

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**Addendum**

After this manuscript had been completed, WILLIAMS *et al.* reported "Mycophenolic Acid" : Antiviral and Antitumor Properties"[7]. They reported that mycophenolic acid is effective against some experimental tumors and oncogenic viruses *in vivo*. Antiviral activity *in vitro* was also reported which is in good accordance with the data presented here.

**References**

1) RIGHTSEL, W. A.; H. G. SCHNEIDER, B. J. SLOAN, P. R. GRAF, F. A. MILLER, Q. R. BARTZ & J. EHRLICH : Antiviral activity of gliotoxin and gliotoxin acetate. Nature 204 : 1333~1334, 1964
2) MILLER, F. A.; W. A. RIGHTSEL, J. EHRLICH, J. C. FRENCH, Q. R. BARTZ & G. J. DIXON : Antiviral activity of tenuazonic acid. Nature 200 : 1338~1339, 1963
3) ARIMA, K.; A. TAKATSUKI, S. SUZUKI, K. ANDO & G. TAMURA : Antiviral activity of trichothecin. J. Antibiotics 21 : 158~159, 1968
4) TAMURA, G.; K. ANDO, S. SUZUKI, A. TAKATSUKI & K. ARIMA : Antiviral activity of brefeldin A and verrucarin A. J. Antibiotics 21 : 160~161, 1968
5) HERRMANN, E. C., Jr.; J. GABLIKS, C. ENGLE & P. L. PERLMAN : Agar diffusion method for detection and bioassay of antiviral antibiotics. Proc. Soc. Expt. Biol. & Med. 103 : 625~628, 1960
6) BIRKINSHAW, J. H.; H. RAISTRICK & D. J. ROSS : Studies in the biochemistry of micro-organisms. The molecular constitution of mycophenolic acid, a metabolic product of *Penicillium brevicompactum* DIERCKX. 3. Further observations on the structural formula for mycophenolic acid. Biochem. J. 50 : 630~634, 1952
7) WILLIAMS, R. H.; D. H. LIVELY, D. C. DELONG, J. C. CLINE, M. J. SWEENEY, G. A. POORE & S. H. LARSEN : Mycophenolic acid. Antiviral and antitumor properties. J. Antibiotics 21 : 463~464, 1968