MILDIOMYCIN: A NUCLEOSIDE ANTIBIOTIC THAT INHIBITS PROTEIN SYNTHESIS

ELENA FEDUCHI, MONTSE COSÍN and LUIS CARRASCO

Departamento de Microbiología, Centro de Biología Molecular,
Canto Blanco, Madrid-28049, Spain

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Mildiomycin, a new nucleoside antibiotic, selectively inhibits protein synthesis in HeLa cells, and is less active in the inhibition of RNA or DNA synthesis. An increased inhibition of translation by mildiomycin is observed in cultured HeLa cells when they are permeabilized by encephalomyocarditis virus. This observation suggests that this antibiotic does not easily pass through the cell membrane, as occurs with other nucleoside and aminoglycoside antibiotics. The inhibition of translation is also observed in cell-free systems, such as endogenous protein synthesis in a rabbit reticulocyte lysate or the synthesis of polyphenylalanine directed by poly (U). Finally the mode of action of mildiomycin was investigated and the results suggest that the compound blocks the peptidyl-transferase center.

Mildiomycin is a nucleoside antibiotic isolated from the culture filtrate of *Streptoverticillium rimofaciens*[1,2]. Mildiomycin has a strong inhibitory effect against powdery mildew of barley and it also inhibits some *Mycobacterium* and *Rhodotorula* species[1]. The complete structure of mildiomycin has been elucidated[3] and is similar to other nucleoside antibiotics such as blasticidin S, gougerotin and anthelmycin[4]. These three antibiotics are inhibitors of protein synthesis, both in eukaryotic and prokaryotic organisms[4]. These compounds block the peptidyl-transferase center, located on the larger ribosomal subunit[4]. In this work we describe that mildiomycin is also an inhibitor of translation both in intact cells and in cell-free systems.

Materials and Methods

Cells

Human HeLa cells were grown and propagated in Dulbecco modified EAGLE's medium in the presence of 10% newborn calf serum. Yeast cells were grown in YEP medium containing in 1 liter; yeast extract 10 g, peptone 20 g, glucose 20 g. *Escherichia coli* cells were grown in MC medium[5] containing in 1 liter; $MgSO_4 \cdot 7H_2O$ 1 g, citric acid 2.1 g, $K_2HPO_4 \cdot 3H_2O$ 13.7 g, $NaNH_4PO_4 \cdot 4H_2O$ 3.3 g and supplemented with diaminopimelic acid 10 μg/ml, lysine 200 μg/ml, glucose 0.4%, thiamine 2 μg/ml.

Encephalomyocarditis Virus

Encephalomyocarditis virus was grown and titrated as previously described[6].

Rabbit Reticulocyte Lysate

Rabbits were made anaemic by daily injection of phenylhydrazine solution as described[7]. The recovery and lysis of reticulocytes has also been reported[7].

Rat and Yeast Ribosomes and Supernatant Fraction

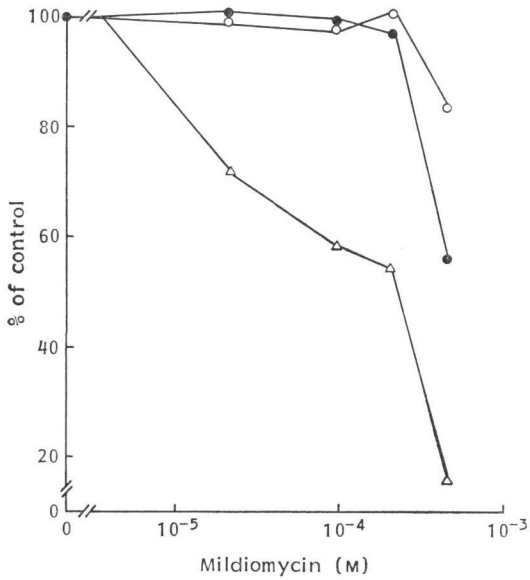
Washed ribosomes and supernatant were recovered from homogenized rat livers as described before[8]. Yeast ribosomes were prepared as previously described[8].

[^{3}H]Phe-tRNA and *N*-Ac[^{3}H]Phe-tRNA

These substrates were prepared from *Escherichia coli* tRNA (Sigma) that was labeled with [^{3}H]-

Fig. 1. Effect of mildiomycin on macromolecular synthesis by HeLa cells.

Cells were incubated 3 hours with mildiomycin and the level of DNA, RNA, and protein synthesis were measured by incubation with 1 μCi [^{3}H]thymidine (●), 1 μCi [^{3}H]uridine (○) or 0.2 μCi [^{35}S]-methionine (△) respectively. The reactions were stopped and processed as described[9].



phenylalanine (38.6 Ci/mmol) (The Radiochemical Centre, Amersham). *N*-Ac[^{3}H]Phe-tRNA was prepared by acetylation of [^{3}H]Phe-tRNA followed by purification on a Sephadex G-25 column[9].

Cell-free Assays

Protein synthesis in the rabbit reticulocyte lysate was essentially as previously reported[7]. The assays for polyphenylalanine synthesis and the enzymatic and nonenzymatic binding of substrates to the ribosomes has also been described[10].

Other Chemicals and Reagents

Poly (U), dithiotreitol and GTP were from Sigma. 2-Mercaptoethanol from Fluka. Millipore HAWP 2400 from Millipore Corp. Mildiomycin was a generous gift of Takeda Chem. Ind., Ltd. Blasticidin S was a generous gift of Dr. M. TANAKA (Institute of Applied Microbiology, Tokyo). Anisomycin from Pfizer. Gougerotin from Calbiochem. Anthelmycin from Lilly Laboratories. Trichodermin from Leo Pharmaceutical Products.

Results

Mildiomycin was first tested for the inhibition of macromolecular synthesis in cultured HeLa cells. For this purpose cells were incubated with different concentrations of this antibiotic and DNA, RNA and protein synthesis was estimated. Fig. 1 shows that translation in HeLa cells is inhibited by mildiomycin to a greater extent as compared to DNA and RNA synthesis. The partial inhibition of DNA synthesis obtained at 5×10^{-4} M mildiomycin might be a secondary consequence of the inhibition of protein synthesis. The comparison of the effect on translation of gougerotin, anthelmycin and blasticidin S in several cells is shown in Table 1. Mildiomycin was active not only

Table 1. Effect of some nucleoside antibiotics on protein synthesis in different cells.

Additions	Yeast		*Escherichia coli*		HeLa	
	cpm	% control	cpm	% control	cpm	% control
None	365,500	100	42,147	100	22,484	100
Mildiomycin 5×10^{-4} M	69,770	19	—	—	—	—
Mildiomycin 2×10^{-4} M	315,477	86	22,714	53	—	—
Mildiomycin 10^{-4} M	335,063	92	37,968	90	—	—
Gougerotin 10^{-4} M	236,076	64	10,238	24	18,828	84
Anthelmycin 10^{-4} M	397,546	109	10,493	25	23,194	103
Blasticidin S 10^{-4} M	17,651	5	2,689	6	4,474	20

Yeast and *E. coli* cells were incubated at 35°C for 30 minutes in YEP or MC medium respectively[5]. HeLa cells were incubated at 37°C for 30 minutes in DMEM medium containing 10% calf serum. Cells were labeled with 0.2 μCi of [^{35}S]methionine in the presence of the indicated concentration of antibiotics. At the end of the incubation period 1 ml of 5% TCA was added and processed as described[9].

Fig. 2. Effect of mildiomycin (panel A) and hygromycin B (panel B) on protein synthesis in control HeLa cells (●) or permeabilized by EMC virus (△).

The indicated concentrations of antibiotics and EMC virus were added simultaneously to culture HeLa cells and the level of protein synthesis was measured 1 hour later by 1 hour pulse with 0.2 μCi/ml [^{35}S]methionine. The cells were processed as described[6].

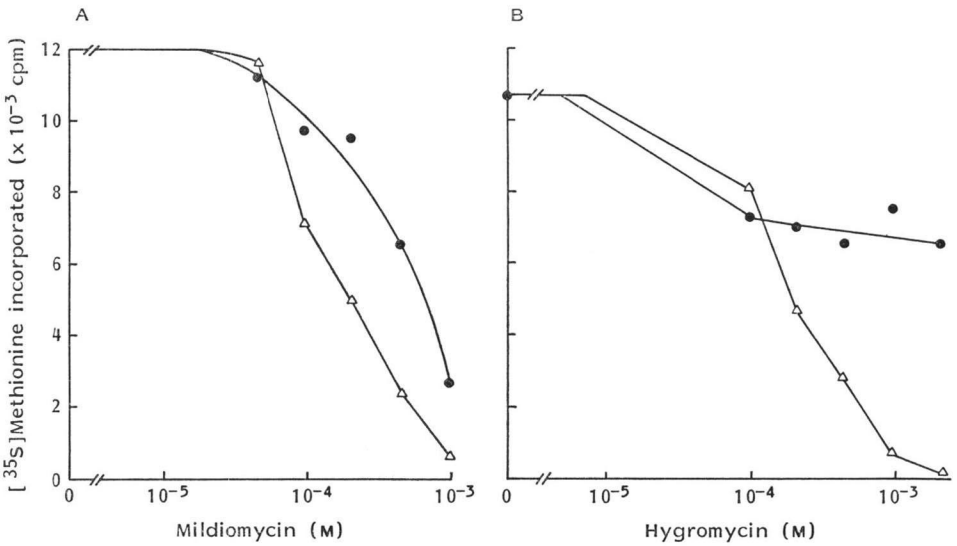


Fig. 3. Effect of mildiomycin on the synthesis of polyphenylalanine directed by poly (U) (●) and on endogenous protein synthesis in a S-30 rabbit reticulocyte lysate (○).

The synthesis of polyphenylalanine was carried out on 100 μl reaction mixtures as described[9]. The reaction mixtures were incubated at 30°C for 20 minutes and 2 ml of 5% TCA were added to stop the reaction. The mixtures were boiled for 15 minutes and then filtered on GF/C filters and counted in a liquid scintillation counter. Endogenous protein synthesis by S-30 rabbit reticulocyte cell free system was carried out as described[7]. Reaction mixtures containing 100 μl were incubated at 30°C for 20 minutes. To stop the reaction 0.5 ml NaOH, 1 ml H_2O, 200 μl H_2O_2 were added. After 20 minutes of incubation at 30°C, 2 ml of 25% TCA were added and the samples filtered and counted as described above.

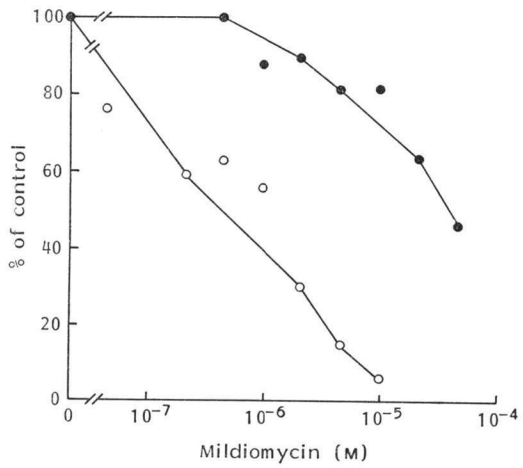


Table 2. Non-enzymatic binding of [^{3}H]Phe-tRNA and *N*-Ac[^{3}H]Phe-tRNA to ribosomes.

Additions	*N*-Ac[^{3}H]Phe-tRNA bound				[^{3}H]Phe-tRNA bound			
	Yeast		Rat		Yeast		Rat	
	cpm	% control	cpm	% control	cpm	% control	cpm	% control
None	1,500	100	4,908	100	3,483	100	12,767	100
Mildiomycin 10^{-4} M	1,822	121	4,866	98	5,439	156	14,072	110
Mildiomycin 5×10^{-4} M	1,849	123	3,211	65	3,700	106	13,630	107
Mildiomycin 10^{-5} M	1,953	130	5,022	101	3,333	96	12,856	102

100 μl reaction mixtures containing the indicated concentration of mildiomycin and yeast or rat ribosomes were incubated for 30 minutes at 30°C or 37°C respectively. The assay was carried out as already described[9].

Table 3. Enzymatic binding of [^{3}H]Phe-tRNA to rat ribosomes.

Addition	cpm	% control
−EF1−GTP	2,213	—
+EF1+GTP	13,483	100
Mildiomycin 5×10^{-4} M	10,268	76
Mildiomycin 2×10^{-4} M	11,974	89

100 μl reaction mixtures containing the indicated concentration of mildiomycin and rat ribosomes were incubated at 37°C. The assay was carried out as already described[9].

Table 4. Effect of mildiomycin on the puromycin reaction.

Addition	Peptidyl [^{3}H]-puromycin formed	
	cpm	% control
Polysomes	470	—
Complete	4,180	100
Anisomycin 10^{-4} M	436	10
Trichodermin 10^{-4} M	848	20
Mildiomycin 10^{-4} M	2,816	67

The puromycin reaction was carried out using yeast polysomes under high potassium concentration following the method already described[12].

in HeLa cells as shown in Fig. 1, but also in yeast and *E. coli* cells.

Nucleoside antibiotics, particularly gougerotin and anthelmycin are poor inhibitors of protein synthesis in intact cells (Table 1). The reason for this low activity lies in the hydrophilic nature of these molecules, and therefore transverse the cellular membrane very poorly. The hydrophilic nature of mildiomycin also suggests that this compound does not cross the cell membrane easily. To test this possibility, the experiment shown in Fig. 2 was carried out. We have previously described that animal viruses permeabilize cells to antibiotics and compounds that do not pass through the cell membrane[10]. Thus, the action of hydrophilic antibiotics is enhanced by the presence of these viruses. Fig. 2A shows that indeed when human HeLa cells are incubated with encephalomyocarditis virus a higher inhibition of protein synthesis by mildiomycin is observed. As a control, a similar experiment was performed with hygromycin B, an aminoglycoside antibiotic known to be highly impermeable (Fig. 2B).

To known whether the inhibition of translation by mildiomycin observed in intact cells was due to a direct effect in the protein synthesizing machinery or to an indirect effect, we analyzed the *in vitro* synthesis of proteins. Fig. 3 indicates that the synthesis of proteins in a S-30 rabbit reticulocyte lysate is blocked by mildiomycin under relatively low concentrations. Thus, 50% inhibition is achieved by a concentration of mildiomycin around 5×10^{-7} M. The synthesis of polyphenylalanine directed by poly (U) is also sensitive to mildiomycin but to a lesser extent as compared to the S-30 system. This result is consistent with previous observations on these two systems.

Finally the mechanism of action of this antibiotic was studied. First we tested the non-enzymatic binding of Phe-tRNA and *N*-Ac-Phe-tRNA to the ribosome directed by poly (U) (Table 2). Also the enzymatic binding of Phe-tRNA to the ribosome was analyzed (Table 3). No effect on these reactions

was observed by the presence of mildiomycin. These results were in agreement with previous findings indicating that mildiomycin related nucleoside antibiotics had no effect on these reactions. To test if mildiomycin blocked peptide-bond formation, we analyzed its action on the peptidyl-puromycin reaction using yeast ribosomes. Table 4 shows that indeed some inhibition of this step is achieved by mildiomycin, although its potency is lower than anisomycin or trichodermin.

Discussion

The discovery of nucleoside antibiotics has greatly increased in the last ten years[11]. The discovery of new antibiotics is normally followed by the analysis of their mode of action. Knowledge of the mechanism of action of a given compound is important since this provides a new tool to study certain cellular functions. This is particularly evident for a number of inhibitors of protein synthesis[4].

The new nucleoside antibiotic mildiomycin was found to be highly inhibitory against *Mycobacterium phlei* and *Rhodotorula rubra*, but only slightly active against several other microorganisms[1]. This lack of activity might be due at least in part to the poor entry of this compound into cells because the inhibitory effect of this compound increased when the cells are permeabilized by animal viruses (Fig. 2)[10]. Our present results suggest that the mode of action of this antibiotic is on protein synthesis, since RNA synthesis was not affected and the slight inhibition observed on DNA synthesis can be explained by the effect observed on translation. With mildiomycin present, the binding of substrates to ribosomes is unaffected, whereas an inhibition of peptide-bond formation is apparent. This suggests that the target of mildiomycin action is the peptidyl-transferase center.

Acknowledgments

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