Biological Activity and Mechanistic Studies of Andrimid

MAYA P. SINGH*, MARY JO MROCZENSKI-WILDEY†, DEBORAH A. STEINBERG††, RAYMOND J. ANDERSEN[a], WILLIAM M. MAIESE and MICHAEL GREENSTEIN

Natural Products Research Section,
Wyeth-Ayerst Research,
American Home Products Corporation,
Pearl River, New York, 10965 U.S.A.
[a]Department of Chemistry, University of British Columbia,
Vancouver, B. C., Canada

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Andrimid (Fig. 1), a peptide-like antibiotic, has been isolated from the culture broths of three distinct species of bacteria[1~3]. It has been reported to exhibit moderate activity against *Bacillus* sp.[3] and very good activity against *Xanthomonas campestris* which causes bacterial blight in rice plants[4]. We tested the andrimid isolated from the fermentation broth of a marine *Pseudomonas fluorescens*[2] for *in vitro* activity against an expanded panel of bacteria and for cytotoxicity against a series of human tumor cell lines. We also investigated the specific target of antibacterial action, and the preliminary findings are reported here.

The *in vitro* minimum inhibitory concentrations (MICs) were determined by the broth microdilution method as described earlier[5]. Andrimid exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 1). Poor activity against wild-type Gram-negative bacteria compared to an *E. coli* strain with increased membrane permeability (*imp*) suggested andrimid had difficulty penetrating the outer membrane. Several-fold higher MBC values compared to MICs indicated a bacteriostatic action for andrimid. In addition, the antibacterial activity of andrimid was highly inoculum-dependent (MICs against *E. coli* (*imp*) at 10^7 cfu/ml and 10^5 cfu/ml inoculum densities were found to be 1 μg/ml and ≤ 0.015 μg/ml, respectively).

Effects on macromolecular processes were studied by the method described earlier[5]. Incorporation of isotopic precursors into acid-precipitable material of an exponential-phase culture of *E. coli* (*imp*) suggested preferential inhibition of RNA synthesis (IC_{50} was estimated to be 0.06 μg/ml for 5 minutes of andrimid treatment) (Table 2). After 15 minutes of drug treatment, DNA and protein synthesis were also inhibited in a dose-dependent manner (Fig. 2). Although DNA and protein synthesis remained inhibited until 60 minutes,

Fig. 1. Chemical structure of andrimid.

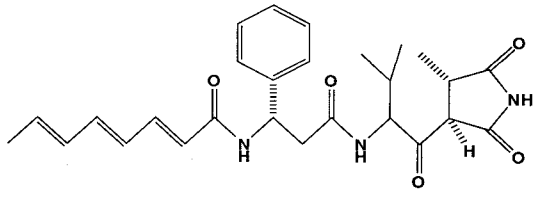


Table 1. Antimicrobial activity of andrimid.

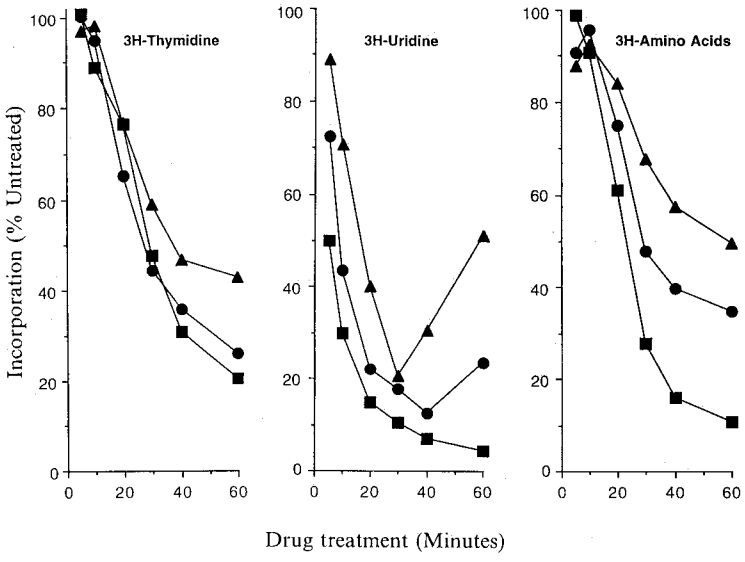
Organism	Andrimid		Penicillin G
	MIC	MBC	MIC
Staphylococcus aureus MSSA	1	>32	≤0.03
Staphylococcus aureus MRSA	1	4	32
Enterococcus faecalis vanco[S]	0.50	2	0.50
Enterococcus faecium vanco[R]	1	32	32
Bacillus subtilis	0.06	>32	≤0.03
Escherichia coli (*imp*)	≤0.015	0.25	2
Escherichia coli	4	16	32
Klebsiella pneumoniae	0.06	0.12	32
Proteus mirabilis	>32	>32	4
Pseudomonas aeruginosa	>32	>32	>64
Candida albicans	>32	>32	>64

Broth microdilution method; medium, YMB for Ca54, TSB for *Enterococcus*, and MHB for all other organisms; inoculum, 10^5 cfu/ml; incubation, 37°C for 20 h. MIC and MBC values are in μg/ml. MIC (MBC) for *E. coli* (*imp*) at 10^7 and 10^5 cfu/ ml inoculum densities determined in minimal medium were 1 (>32) and ≤0.015 (0.25) μg/ml, respectively.

Current address: †R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ 08869.
††IntraBiotics Pharmaceuticals, Inc., 816 Kifer Road, Sunnyvale, CA 94086.

Fig. 2. Effects of andrimid on macromolecular synthesis in *E. coli* (*imp*).

▲ 0.015 μg/ml, ● 0.03 μg/ml, ■ 0.06 μg/ml.



Exponential-phase cells were treated with the drug for 5-60 minutes and then were pulse labeled for 5 minutes with ^{3}H-thymidine, ^{3}H-uridine, ^{3}H-amino acids for measuring DNA, RNA, and protein syntheses, respectively.

RNA synthesis began to recover after 30 minutes of treatment with subinhibitory concentrations ($\leq$0.03 μg/ml) of andrimid. At this concentration, DNA and protein synthesis showed signs of recovery after 75 minutes (data not shown). None of the macromolecular synthesis showed recovery from inhibition when exposed to andrimid at $\geq$0.06 μg/ml. It appears that subinhibitory concentrations of andrimid do not lead to an irreversible damage to the cells.

Andrimid's effect on *E. coli* RNA polymerase was tested in an *in vitro* cell-free assay by a modified method described in the Worthington Enzyme Manual[6]. Andrimid, ciprofloxacin (a DNA gyrase inhibitor) and chloramphenicol (a protein synthesis inhibitor) did not inhibit the incorporation of ^{3}H-ATP into RNA at 1 ~ 10 μg/ml concentrations whereas rifampin (1 μg/ml) completely inhibited the same by inhibiting the RNA polymerase present in the reaction mixture.

Since andrimid showed no effect on RNA polymerase it was suspected of having some effect on the pyrimidine metabolic pathway. The pathways of *de novo* biosynthesis of the four essential pyrimidine nucleoside triphosphates by enteric bacteria is well established[7]. The biosynthesis of pyrimidine nucleotides, UTP and CTP, is accomplished by the sequential action of at least nine different enzymes. UMP kinase is an important enzyme which converts UMP into UDP. UDP is then converted into UTP which is incorporated into RNA. We tested the effect of andrimid and showdomycin (a known UMP-kinase inhibitor[8,9]) on freshly prepared crude UMP-kinase from *E. coli* (*imp*). Crude enzyme was prepared and assayed by a slightly modified method of BECK *et al.*[10] Briefly, an overnight culture of *E. coli* (*imp*) grown in minimal medium (50 ml) at 37°C, 200 rpm was washed with 0.9% NaCl by centrifugation (3000 rpm). Cells were resuspended in 5 ml of 0.1 M Tris-chloride (pH 7.8) containing 10 mM $MgCl_2$ and 2 mM mercaptoethanol, sonically extracted three times for 30 sec at 0°C, and centrifuged (20,000 rpm). The supernatant was dialyzed for 2 hours against 0.1 M Tris-chloride (pH 7.8) containing 10 mM $MgCl_2$ and 2 mM mercaptoethanol (0°C) and used immediately for assay. Assay reaction mixture (40 μl) contained: 26 μl of 0.1 M Tris-chloride (pH 7.8) containing 10 mM $MgCl_2$, 2 mM mercaptoethanol, 3 mM adenosine triphosphate (ATP) and 1 mM ^{3}H-uridine (500 μCi/ml); 4 μl of water or drug solution; and 10 μl of the enzyme preparation. The reaction mixture was incubated at 37°C and at appropriate times (30, 45, and 60 minutes) 5 μl of reaction mixture was spotted on thin-layer plates coated with poly(ethyleneimine)-cellulose (PEI, Emerck cat. #5722-6) and dried with hot air to stop the reaction. ^{3}H-Uridine and ^{3}H-uridine triphosphate (^{3}H-UTP) were used as controls. Plates were developed successively in 0.1, 0.3, 0.7, and 1.5 M LiCl solutions for 1, 5, 15, and 25 minutes, respectively. Plates were dried with warm air and exposed to preflashed X-ray film (Kodak, cat. #165 1454) for 5 days. Assay results were read from the developed X-ray films. Showdomycin (100 μg/ml) inhibited the formation of UDP and UTP during the first 30 minutes of reaction whereas andrimid (100 μg/ml) had no effect on the enzyme. Lower concentrations (1 and 10 μg/ml) of andrimid also did not have any effect. After 1 hour, a band corresponding to CTP developed in andrimid-treated, Pen G-treated, and untreated reaction mixtures but not in the showdomycin-treated mixture. These

Table 2. Effects on uptake of precursors and their incorporation into macromolecules of *E. coli* (*imp*).

Compound	Conc μg/ml	^{3}H-Tdr		^{3}H-Udr		^{3}H-AA	
		Uptake	Incorp	Uptake	Incorp	Uptake	Incorp
Andrimid	0.03	80	100	63	72	119	91
	0.06	87	100	57	50	99	98
	0.12	87	100	48	40	86	97
CPLX	0.25	68	8	99	81	91	102
RIF	0.25	150	107	31	5	55	37
CHL	8	96	90	97	93	34	13
POLY	8	3	1	7	1	22	3

Values are expressed as % of untreated control after 5 min preincubation with drugs and 5 min pulse labeling with isotopic precursors. Uptake = total amount of precursor inside cells (pool) after an instant saline wash. Incorp = precursor incorporated into TCA-insoluble macromolecules. ANDR, andrimid; CPLX, ciprofloxacin-HCl; RIF, rifampin; CHL, chloramphenicol; and POLY, polymyxinB-SO_4.

data suggest that andrimid is mechanistically different from showdomycin.

Uptake of isotopic precursors (^{3}H-Tdr, ^{3}H-Udr and ^{3}H-AA) into an exponential-phase culture of *E. coli* (*imp*) was determined by measuring the radioactivity in cells after a 5 minutes' pulse labeling and an instant wash with saline. The count represented the amount of precursor present in the cellular pool and those incorporated into macromolecules. In general, uptake of ^{3}H-Udr was decreased considerably whereas uptake of ^{3}H-Tdr and ^{3}H-AA were relatively unaffected by inhibitory concentrations of andrimid (Table 2). Comparison of uridine uptake and incorporation (into TCA precipitate) data revealed that the levels of ^{3}H-Udr taken up by the cells were similar to the levels incorporated into TCA-insoluble material of the cells. This data suggested that andrimid may have some influence on ^{3}H-Udr transport into cells. Since accurate determination of uridine transport in enteric bacteria is complicated by the presence of periplasmic nucleoside deaminases and phosphorylases[11], exact effect of andrimid could not be determined. Although no conclusive correlation between uridine uptake and incorporation could be established (Table 2), the similarity in the uptake and incorporation data may be explained by the "by-pass" mechanism present in bacteria. By this mechanism, exogenous uridine is directly incorporated into RNA without disturbing the cellular nucleotide pool, and therefore, reduced uptake of radiolabeled uridine is reflected by the reduced radioactivity in RNA[12].

Showdomycin, a nucleoside antibiotic, has been shown to be a competitive inhibitor of nucleoside transport in sensitive bacteria and has been shown to inhibit UMP-kinase[8,9]. Although andrimid is structurally different from showdomycin, andrimid contains a succinimide moiety instead of maleimide moiety present in showdomycin. We tested the antagonistic effects of adenine, cytosine, guanine, thymine, uracil, adenosine, cytidine, guanidine, thymidine, uridine, and selected cations ($1\sim50\,\mu$M concentration) on the antimicrobial activity of andrimid ($0.02\sim2\,\mu$M) against *E. coli* (*imp*) after $1\sim18$ hours incubation. None of the above compounds affected the growth inhibitory properties of andrimid, *i.e.* it does not appear to be a competitive inhibitor of nucleosides or their bases.

Effect of andrimid on intracellular potassium was studied by the method previously described[13]. Exponential-phase cells suspended in growth medium released 34% of their intracellular potassium when treated with andrimid (0.1 μg/ml) for 15 minutes. In contrast, exponential-phase cells resuspended in sucrose-phosphate buffer (0.1 M) did not release any intracellular potassium. Polymyxin B released $45\sim60$% of the the intracellular potassium under both conditions. Andrimid appeared to interfere with the membrane integrity of metabolically active cells which may be essential for the active transport of andrimid into the cell. Membrane damaging effect was either hampered by the osmotic protection provided by sucrose buffer, or andrimid was not able to reach the active site (membrane) due to the absence of active transport systems. Metabolic transformation of andrimd to an unknown active form also could not be ruled out.

Antiproliferative effects of andrimid on drug-sensitive and drug-resistant human tumor cell lines (ovarian: HTB161, A2780S and A2780DDP; and colon: MIP, SW620 and Caco2) were tested by sulforhodamine B dye assay[14]. Andrimid exhibited no antiproliferative activity at $1\sim10\,\mu$g/ml. Moderate activity ($20\sim35$% inhibition) was detected against ovarian tumor cell lines at 25 μg/ml.

Mechanistically andrimid appeared to have a preferential effect on uridine uptake and RNA synthesis in bacteria within the first $5\sim10$ minutes of treatment. However, it did not have inhibitory effects on RNA-polymerase and UMP-kinase. Although observations of delayed but nonspecific inhibition of other macro-

molecular processes and potassium leakage from exponential-phase cells are consistent with membrane-damaging effects of andrimid, further investigation is needed to elucidate the precise mechanism of antibacterial action. Andrimid is an interesting antibiotic, but its moderate Gram-positive activity and poor activity against normal Gram-negative bacteria would limit its clinical utility. Structural modifications to improve the spectrum of antibacterial activity and outer membrane penetration through wild type Gram-negative bacteria would be required.

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