Biological and Mechanistic Activities of Phenazine Antibiotics Produced by Culture LL-14I352

MAYA P. SINGH*, ANA T. MENENDEZ[a,†], PETER J. PETERSEN[b], WEI-DONG DING, WILLIAM M. MAIESE and MICHAEL GREENSTEIN

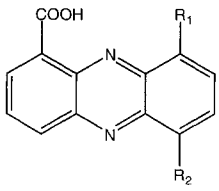
Natural Products, [b]Infectious Diseases and [a]Anticancer Research Sections, Wyeth-Ayerst Research, Pearl River, New York, 10965 U.S.A.

(Received for publication June 30, 1997)

In the course of screening microorganisms isolated from the marine environment, fermentation samples of culture LL-14I352 were found to exhibit activity in the Biochemical Induction Assay (BIA), which detects agents that directly or indirectly initiate DNA damage[1]. This culture was originally isolated from an orange tunicate collected from the Pacific Ocean in proximity to Fiji and was subsequently identified as a new halophilic marine bacterium[2]. The BIA-active compounds isolated from fermentations of this culture were found to be the new phenazine antibiotics LL-14I352α and β, which also exhibited activity in both our antibacterial and antitumor primary screens. Coincidently, a compound identical to LL-14I352α, pelagiomicin-A, was also discovered from a marine bacterium isolated from a different marine environment by IMAMURA *et al.*[3]. We identified an additional component, LL-14I352β, which was found to be less active than the α component. Further details of *in vitro* and *in vivo* antibacterial, cytotoxic, and antitumor activities and the mechanism of antibacterial action are reported here.

The *in vitro* antibacterial activities against 15 Gram-positive and 5 Gram-negative isolates were determined by the broth microdilution method[4]. LL-14I352α exhibited good Gram-positive activity (MIC, 0.25~2 μg/ml), but moderate to poor Gram-negative activity (MIC, 16~>128 μg/ml). This data is consistent with the activities reported for pelagiomicin A[3]. LL-14I352β, which lacks the amino acid residue (see Fig. 1), was approximately 40-fold less active (Gram-positive MIC, 8~>64 μg/ml) than the α component. The amino acid residue of the LL-14I352α may be facilitating its

Fig. 1. Structure of LL-14I352α and related compounds. LL-14I352α is identical to pelagiomicin A.



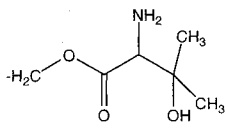
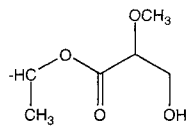
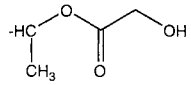
Compound	R_1	R_2
LL-14I352α	OCH_3	
LL-14I352β	OCH_3	$-CH_2OH$
DOB-41	H	
DC-86-M	H	

Table 1. Inhibitory effects of LL-14I352α and known antimicrobial agents on incorporation and uptake of radiolabeled precursors in *E. coli imp.*

Compound	Conc. (μg/ml)	Inhibition (% of untreated control)					
		^{3}H-Tdr		^{3}H-Udr		^{3}H-AA	
		Incorp	Uptake	Incorp	Uptake	Incorp	Uptake
LL-14I352α (2×MIC)	1	94	37	53	−3	16	19
(MIC)	0.50	87	32	40	−6	4	19
(1/2×MIC)	0.25	82	29	18	5	−2	33
Ciprofloxacin	0.25	87	43	7	−3	15	−5
Rifampin	0.25	10	−59	97	54	63	9
Chloramphenicol	8	10	−12	8	−9	84	55
Polymyxin B	8	99	97	98	93	97	78

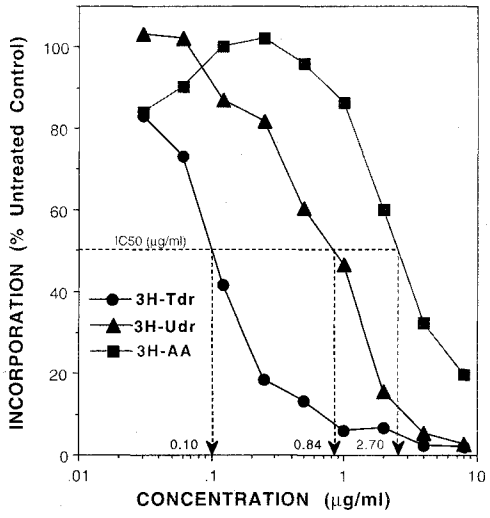
Data presented represents 5 minutes pretreatment and 5 minutes pulse labeling. Incorp=radiolabeled precursor incorporated into TCA-precipitable material. Uptake=total radiolabeled precursor remaining in the cells after an instant saline wash.

† Present address: Bristol-Myers Squibb Company, P. O. Box 4500, Princeton, NJ 08543

transport through the cytoplasmic membrane. The phenazine-type antibiotics (DOB-41 and DC-86-M) isolated from a *Streptomyces* sp.[5] and a *Pseudomonas* sp.[6] have also been reported to exhibit superior Gram-positive activity. LL-14I352α exhibited good activity (MIC/MBC, 0.5/2 μg/ml) against *E. coli* carrying an *imp* outer membrane mutation, but poor activity against wild type *E. coli* suggesting a permeability problem with this compound. MICs increased two-fold with 100-fold increase in inoculum density, and the effect was bactericidal (MBC was only 1~2 fold higher than MIC). LL-14I252α did not appear to bind to DNA or serum as the addition of exogenous DNA (200 μg/ml of herring sperm or calf thymus) or horse serum (4% v/v) into the medium did not affect activity against *E. coli imp*. The MIC of adriamycin (a known DNA-binding agent) increased 8-fold in presence of DNA, and the presence of horse serum considerably reduced the antibacterial activity of pyroindomycin, a known serum-bound antibiotic (data not shown).

Inhibition of DNA, RNA, and protein synthesis were determined by measuring the incorporation of the radiolabeled precursors ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA, respectively, into TCA-precipitable material prepared from logarithmic-phase cultures of *E. coli imp*[4]. Within 5 minutes of treatment with 1/2× MIC of LL-14I352α, DNA synthesis was inhibited by 82%, whereas RNA and protein syntheses were only marginally affected (Table 1). During the same period, the control drugs ciprofloxacin, rifampin, and chloramphenicol predominantly inhibited DNA, RNA and protein synthesis, respectively. Polymyxin B inhibited incorporation into all three macromolecules. The concentrations of LL-14I352α required for 50% inhibition (IC_{50}) of DNA, RNA, and protein syntheses within 10 minutes (5 minutes preincubation with drug and 5 minutes pulse labeling) were 0.10, 0.84, and 2.70 μg/ml, respectively (Fig. 2). After 15 minutes, the IC_{50}s for protein synthesis decreased to 1.40 μg/ml, whereas the IC_{50}s for DNA and RNA syntheses remained almost unchanged at 0.12 μg/ml and 0.80 μg/ml, respectively (data not shown). Although inhibition of the various macromolecular processes was concentration dependent, DNA synthesis was preferentially inhibited at all concentrations. The effect of drugs on the cellular uptake of radiolabeled precursors was determined by measuring radioactivity retained in saline-washed cells under the same experimental conditions. For each drug tested, uptake of the three radiolabeled precursors was unaffected relative to the specific inhibition of incorporation into TCA-precipitable material (Table 1). BIA activity of LL-14I352α at a concentration as low as 0.08 μg/spot further confirmed its DNA-damaging activity in bacteria.

Fig. 2. Effects of LL-14I352α on macromolecular synthesis in *E. coli imp*.



Exponential-phase cells were treated with various concentrations of the drug for 5 minutes and were then pulse-labeled for 5 minutes with ^{3}H-Tdr, ^{3}H-Udr, or ^{3}H-AA for measuring DNA, RNA, or protein synthesis, respectively. The arrows indicate the IC_{50}s (concentrations at which incorporation was inhibited by 50%) for each macromolecular process.

Table 2. *In vitro* cytotoxic activity (IC_{50}, μg/ml) of LL-14I352 antibiotics against selected human tumor cell lines.

Cell name	Tumor type/Properties	LL-14I352α	LL-14I352β	Adriamycin
A2780S	Ovarian/solid	0.18	0.74	0.004
A2780DDP	Ovarian/solid/DNA damaging agent resistant	0.60	6.30	0.05
SW620	Colon/solid/MDR drug sensitive	0.53	1.80	0.09
MIP	Colon/solid/MDR drug resistant	0.61	0.84	1.00
CCRF-CEM	T-cell leukemia/suspension/MDR drug sensitive	0.70	6.90	0.05
NEC	Normal endothelial cell/solid/MDR drug sensitive	2.70	7.30	0.008

Method: KUBOTA *et al.* Colorimetric chemosensitivity testing using sulforhodamine B. J. Surgical Oncology 52: 83~88, 1993.

Bleomycin, a known DNA-damaging agent, gave a positive BIA response at a minimum concentration of 0.003 μg per spot.

Since LL-14I352α and β were found to be BIA active and to inhibit preferentially DNA synthesis, they were tested for cytotoxicity against five human tumor cell lines with defined properties and against a normal bovine cell line (Table 2). Other phenazines isolated from diverse sources have been found to exhibit antitumor activity[7,8]. The mean IC_{50} values for LL-14I352α and β were 0.88 μg/ml and 3.98 μg/ml, respectively, suggesting that the α component is 4.5 times more cytotoxic than the β. The presence of the amino acid residue in the α component may be facilitating its entry into the cells (Fig. 1). The ratio of the IC_{50} value for A2780DDP, a cell line with enhanced DNA repair capability, to that of its parent A2780S[9] for adriamycin, LL-14I352β, and LL-14I352α were 12.5, 8.5, and 3.3, respectively. These data suggest that the α component is less responsive to the enhanced DNA repair capability of A2780DDP. Antibiotic LL-14I352α and β were equipotent against a MIP multidrug-resistant colon carcinoma cell line and SW620, a comparable drug-sensitive line, suggesting that these compounds are unaffected by the *p*-glycoprotein-mediated drug efflux pump[10~12]. LL-14I352α appeared to be somewhat more active against the tumor cell lines than against the normal endothelial line.

LL-14I352α and LL-14I352β were inactive in an *in vivo* P388 murine leukemia model at concentrations up to 8 mg/kg. Pelagiomicin A also was inactive against P388 in *in vitro* testing[3]. Testing has not been conducted, however, in solid tumor models. Acute toxicity was observed at 16 mg/kg. LL-14I352α and β also failed to protect mice against a lethal *S. aureus* infection at doses up to 8 mg/kg. The LD_{50} for LL-14I352α was estimated to be 16 mg/kg, but the β component exhibited no toxicity at doses up to 32 mg/kg. Lack of *in vivo* activity and a poor toxicological profile would most likely limit the therapeutic usefulness of LL14I352α.

References

1) GREENSTEIN, M.; M. J. WILDEY & W. M. MAIESE: The biochemical induction assay and its application in the detection of the calicheamicins. *In* Enediyne Antibiotics as Antitumor Agents. *Ed.*, D. B. BORDERS & T. W. DOYLE, pp. 17~27, Marcel Dekker Inc, NY, 1993
2) BERNAN, V. & D. A. MONTENEGRO: Personal communication
3) IMAMURA, N.; M. NISHIJIMA, T. TAKADERA, K. ADACHI, M. SAKAI & H. SANO: New anticancer antibiotic pelagiomicin, produced by a new marine bacterium *Pelagiobacter variabilis*. J. Antibiotics 50: 8~12, 1997
4) SINGH, M. P.; P. J. PETERSEN, N. V. JACOBUS, W. M. MAIESE, M. GREENSTEIN & D. A. STEINBERG: Mechanistic studies and biological activity of bioxalomycin α_2, a novel antibiotic produced by *S. viridodiastaticus* subsp. "*litoralis*" LL-31F508. Antimicrob. Agents Chemother. 38: 1808~1812, 1994
5) TAKAHASHI, K.; I. TAKAHASHI, M. MORIMOTO & F. TOMITA: DC-86-M, a novel antitumor antibiotic II. Structure determination and biological activities. J. Antibiotics 39: 624~628, 1986
6) SHOJI, J.; R. SAKAZAKI, H. NAKAI, Y. TERUI, T. HATTORI, O. SHIRATORI, E. KONDO & T. KONISHI: Isolation of a new phenazine antibiotic, DOB-41, from *Pseudomonas* species. J. Antibiotics 41: 589~594, 1988
7) NAKAIKE, S.; T. YAMAGISHI, K. NANAUMI, S. OTOMO & S. TSUKAGOSHI: Cell-killing activity and kinetic analysis of a novel antitumor compound NC-190, a benzo[*a*]phenazine derivative. Jpn. J. Cancer Res. 83: 402~409, 1992
8) TARUI, M.; M. DOI, T. ISHIDA, M. INOUE, S. NAKAIKE & K. KITAMURA: DNA-binding characterization of a novel anti-tumor benzo[*a*]phenazine derivative NC-182: spectroscopic and viscometric studies. Biochem. J. 304: 271~279, 1994
9) PRATESI, G.; M. TORTERETO, C. CORTI, R. GIARDINI & F. ZUNINO: Successful local regional therapy with topotecan of intraperitoneally growing human ovarian carcinoma xenografts. British J. Cancer 71: 525~528, 1995
10) ALVAREZ, M.; K. PAULL, A. MONKS, C. HOSE, J. S. LEE, J. WEINSTEIN, M. GREVER, S. BATES & T. FOJO: Generation of a resistance profile by quantitation of mdr-1/p-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen. J. Clin. Invest. 95: 2205~2214, 1995
11) CHIN, K. V. & B. LIU: Regulation of the multidrug resistance (MDR1) gene expression. In Vivo 8: 835~841, 1994
12) ZHANG, X. P.; M. K. RITKE, J. C. YALOWICH, M. L. SLOVAK, J. P. HO, K. I. COLLINS, T. ANNABLE, R. J. ARCECI, P. E. DURR & L. M. GREENBERGER: P-glycoprotein mediated profound resistance to bisantrene. Oncology Research 6: 291~301, 1994