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# In vitro and in vivo antitumor effects of novel actinomycin D analogs with amino acid substituted in the cyclic depsipeptides

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ABSTRACT

The actinomycin D (AMD) analogs in which the D-valine residues (the second amino acid residue in the cyclic depsipeptide of AMD) and the N-methyl-L-valine residues (the fifth amino acid residue in the cyclic depsipeptide of AMD) were replaced with D-Phe or L- and D-forms N-methylvalines, N-methylisoleucine, N-methylleucine, N-methylphenylalanine, N-methylalanine, and sarcosine were synthesized. The antimicrobial activity and cytotoxic activities of these compounds in vitro were investigated. The results showed that most D-valine substituted analogs had much lower antimicrobial activity and cytotoxic activities in vitro than AMD itself, but three N-methyl-L-valine substituted analogs had comparable or even more remarkable cytotoxic activities in vitro than AMD. Acute toxicities and antitumor effects of the N-methyl-L-valine substituted analogs in mice were also examined. The result showed that the acute toxicity of compound 4 L-methylleucine$^5$-AMD analog is comparable to AMD itself and that of compound 3(L-Methylisoleucine$^5$-AMD analog) is slightly more toxic, about 1.25-fold than AMD. However, the acute toxicity of compound 5 D-methylleucine5-AMD analog is about 2-fold lower than AMD. This suggested that the N-methyl-D-amino acid replacement in the cyclic ring might play a vital role in their decreased acute toxicities, and perhaps the N-methyl-D-leucine substituent is more favorable, though there may be a slight loss of antitumor activity. This finding may be helpful for the design and development of more potent antitumor agents together with low acute toxicity, and suggests that the N-methyl-D-leucine substituent has the potential to be used as antitumor drug lead.


## 1. Introduction

Actinomycin D (AMD), a well known peptide antibiotic produced by *Streptomyces melanochromogenes* No. 1779 or *Streptomyces parvullus* , consists of a planar 2-aminophenoxazin-3-one chromophore and two bulky cyclic pentapeptide lactones (Fig. 1). It has been clinically used as an anticancer agent for treatment of highly malignant cancers such as Wilm's tumor [3] and gestational choricaucinaoma [10,20]. Currently AMD is used with combinations of other antitumor drugs to treat high-risk tumors [19,11,18]. In addition, because AMD binds noncovalently to DNA and strongly inhibits the transcription of DNA to RNA [23], it has become a powerful tool in biochemistry, molecular and cell biology research.

Analogs of AMD have been produced by directed biosynthesis, partial synthesis, and total synthesis [17,15,12], in order to reduce its cytotoxity while retaining most of its antitumor activity. Many previous reports focused on replacement of amino acid residues in the cyclic depsipeptides of AMD. However, the replacements have been found to render such analogs inactive or less active except a few analogs shown very promising results. For example, the replacement of N-methylvalines by N-methylalanine produces a substantial reduction in antimicrobial activity, and a loss of antitumor activity [13]. On the other hand, the replacement by N-methylleucine displays higher antitumor activity in vitro [14].

In the crystal structures of DNA–AMD complexes, the i-propyl groups of the D-valine residues at Aa2 and Aa2′ and N-methyl-L-valine residues at Aa5 and Aa5′ in the depsipeptides are pointed to the outside of the complex, and thus these groups are considered not to participate in interaction with DNA [6]. Therefore, in the present study, to elucidate the possible effects of the replacements on their bioactivity, the water solubility, in vitro cytotoxcity and

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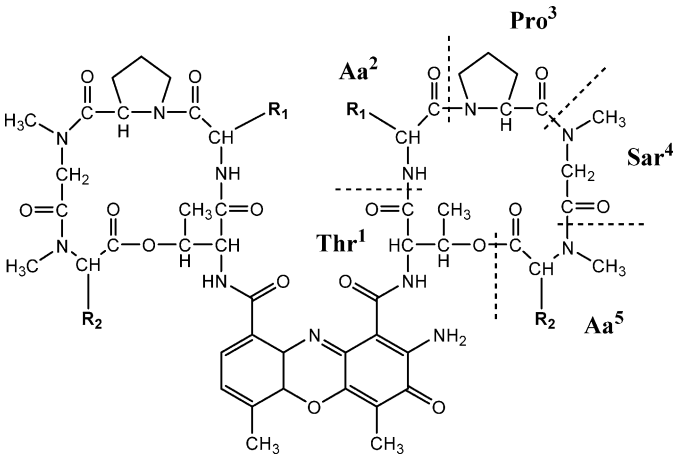


**Fig. 1.** The structure of AMD and its analogs. (1) [D-Val$^2$, L-MeVal$^5$]$_2$AMD, $R_1 = CH(CH_3)_2$ and $R_2 = CH(CH_3)_2$. (2) [D-Val$^2$, Sar$^5$]$_2$AMD, $R_1 = CH(CH_3)_2$ and $R_2 = H$. (3) [D-Val$^2$, L-Melle$^5$]$_2$AMD, $R_1 = CH(CH_3)_2$ and $R_2 = CH(CH_3)CH_2CH_3$. (4) [D-Val$^2$, L-MeLeu$^5$]$_2$AMD, $R_1 = CH(CH_3)_2$ and $R_2 = CH_2CH(CH_3)_2$. (5) [D-Val$^2$, D-MeLeu$^5$]$_2$AMD, $R_1 = CH(CH_3)_2$ and $R_2 = CH_2CH(CH_3)_2$. (6) [D-Phe$^2$, Sar$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = H$. (7) [D-Phe$^2$, D-MeAla$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH_3$. (8) [D-Phe$^2$, D-MeVal$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH(CH_3)_2$. (9) [D-Phe$^2$, L-Melle$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH(CH_3)CH_2CH_3$. (10) [D-Phe$^2$, L-MeLeu$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH_2CH(CH_3)_2$. (11) [D-Phe$^2$, D-MeLeu$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH_2CH(CH_3)_2$. (12) [D-Phe$^2$, L-MePhe$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH_2C_6H_5$. (13) [D-Phe$^2$, D-MePhe$^5$]$_2$AMD, $R_1 = CH_2C_6H_5$ and $R_2 = CH_2C_6H_5$.

the antimicrobial activity of all the analogs of AMD in which the D-valine residues at Aa2 and Aa2′ and the N-methyl-L-valine residues at Aa5 and Aa5′ both were replaced by other L/D-amino acids, or only the N-methyl-L-valine residues at Aa5 and Aa5′ were replaced by other N-methyl-L/D-amino acid respectively were examined. On the basis of their in vitro cytotoxicity and antibacterial activity, some analogs were selected for further evaluation of their bioactivity to explain possible effects of replacements on the mechanism and in vivo activity of AMD and its analogs.

## 2. Methods and materials

### 2.1. Chemistry

The AMD analogs examined were synthesized by the procedure described previously [14] with minor modification. The NMR spectra were obtained with 200 MHz $^1$H NMR and 400 MHz $^1$H NMR (Bruker). The Mass spectra were obtained with Fast-atom bombardment mass spectra (FABMS, VG-ZAB), ESI-MS (Marriner) and HR-MS (Bruker). The homogeneity of the products was checked by thin-layer chromatography on silica-gel plates. The intermediates and the final products of the AMD analogs were confirmed by $^1$H NMR and mass analysis. The structure of AMD and its analogs were illustrated in Fig. 1.

### 2.2. Water solubility measurements

The water solubility of AMD and its analogs was measured as described before [21] with a minor modification. In briefly, AMD and its analogs were dissolved in the binding buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM KCl) until the compounds were saturated. After centrifugation, the concentrations of drugs were determined spectrophotometrically at 25 °C.

### 2.3. Cell lines and culture conditions

The human tumor cell lines, prostate carcinoma (PC-3), bladder carcinoma (Biu-87 and EJ), and cervical carcinoma (Hela) were obtained from Shanghai Cell Institute, Chinese Academy of Science. All the cell lines were grown in RPMI 1640 medium (Gibico-BRL, USA). Cells were maintained at 37 °C in humidified air with 5% $CO_2$. Media were supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS) (Minhai biotech, China).

### 2.4. Animals

Kunming mice (Grade II, certificate No. 9700047) were provided by the Animal Center of Lanzhou University (Lanzhou, China). The animals (20 ± 1 g, 8–10 weeks old) were housed at a room temperature of approximately 22 ± 1 °C and 50–60% relative humidity with circadian light rhythm of 12 h, and given standard sterile diet pellets and tap water according to institutional guidelines.

### 2.5. In vitro cytotoxicity

MTT assay was employed to evaluate the in vitro cytotoxicity of the AMD analogs as described previously [25] with a slight modification. Drug stock solutions were prepared in DMSO. The final concentration of DMSO in the growth medium was <1‰ (v/v) or lower concentration without effects on cell replication. In all of these experiments, three replicate wells were used to determine each point.

### 2.6. Antimicrobial activity

Antimicrobial activities of AMD and its analogs against selected microorganisms, including two gram-positive, one gram-negative bacteria, and one fungi were determined using the broth micro-dilution assay. Briefly, single colonies of bacteria and fungi were inoculated into the medium (LB broth for bacteria, Sabouraud's Medium for fungi) and cultured overnight at 37 °C. An aliquot of the culture was transferred to 10 ml fresh medium and incubated for an additional 3–5 h at 37 °C until mid-logarithmic phase. A 2-fold dilution series of peptides in 1% peptone was prepared. Serial dilutions (100 μl) were added to 100 μl cells ($2 \times 10^6$ CFU/ml) in 96-well microtiter plates (Costar) and incubated at 37 °C for 16 h. The lowest concentration of peptide that completely inhibited growth was defined as the minimal inhibitory concentration (MIC). MIC values were obtained as an average from triplicate measurements in three independent assays.

### 2.7. Analysis of DNA fragmentation by agarose gel electrophoresis

Apoptotic degradation of DNA into the oligonucleosomal fragments was analyzed by agarose gel electrophoresis as described previously [5]. Briefly, PC-3 cells treated with AMD and its analogs, $1 \times 10^6$ cells were washed twice with ice-cold PBS. After centrifugation, the cell pellet was suspended in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 7.4) and incubated for 1 h at 50 °C. RNase A (2 mg/ml) was added and the mixture was incubated for 2 h at 37 °C. Then proteinase K (0.5 mg/ml) was added and incubated for additional 1 h at 37 °C. Next, NaCl was added to a concentration of 5 mM, and 3-fold of volumes of ice-cold ethanol was added. Finally the mixture was incubated at −20 °C overnight to precipitate the DNA. After centrifugation (14,000 rpm for 20 min at 4 °C), the entire pellet was dissolved in 20 ml of TE buffer (10 mM Tris–base, 1 mM EDTA, pH 8.0). The DNA samples were run on 1.5% agarose gel electrophoresis (5 V/cm 2–3 h in TAE buffer: 40 mM Tris–base, 20 mM NaAC, 2 mM EDTA) and visualized by ethidium bromide staining and photographed under ultraviolet illumination.