# FREDERICAMYCIN A, A NEW ANTITUMOR ANTIBIOTIC

## II. BIOLOGICAL PROPERTIES*

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Fredericamycin A is a novel antibiotic produced by a soil isolate of *Streptomyces griseus* (FCRC-48). *In vitro*, fredericamycin A exhibits antibacterial, antifungal, and cytotoxic activities. *In vivo*, fredericamycin A exhibits very good antitumor activity against P388 mouse leukemia as well as the CD8F mammary tumor and marginal activity against $B_{16}$ melanoma. Fredericamycin A failed to demonstrate any interaction with DNA and inhibited protein and RNA synthesis preferentially to DNA synthesis in *Bacillus subtilis* and P388 cells.

Fredericamycin A is the major biologically active component present in the fermentation broth of a strain of *Streptomyces griseus* (FCRC-48), isolated from a soil sample collected at Frederick, Maryland. The production, isolation, and physicochemical properties of fredericamycin A are described in the preceding paper[1]. This paper describes fredericamycin A's antimicrobial spectrum, *in vitro* antitumor activities, potential interaction with DNA, and effects on macromolecular synthesis.

## Materials and Methods

### Determination of Minimal Inhibitory Concentrations

Minimal inhibitory concentrations (MIC's) were determined using the agar plate dilution method. Dilutions into appropriate agars were made from a stock solution of fredericamycin A in DMSO - $H_2O$ (1: 1). All microorganisms were used at a concentration of $2\times10^6$ organisms/ml and spotted (5 $\mu$l) onto nutrient agar or SABOURAUD dextrose agar plates. Endpoints were scored after 24 hours for bacteria and 48 hours for yeast and fungi.

### Determination of Bactericidal Activity

Fredericamycin A was serially diluted in minimal medium ($K_2HPO_4$, 6.45 g; $KH_2PO_4$, 3.55 g; $MgSO_4\cdot7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; $Na_3C_6H_5O_7\cdot2H_2O$, 0.5 g; $H_2O$ 1 liter) supplemented with 4.0 g/liter glucose and 8.0 g/liter casein. Logarithmically growing *Bacillus subtilis* cells were added to give a final concentration of $10^6$ organisms/ml. Subsequent to overnight incubation, viable cell counts were performed.

### Cytotoxicity Determinations

The cytotoxicity against the KB, P388, and L1210 cell lines was determined under contracted accessory testing services provided through the National Cancer Institute, U.S.A.[2,3].

The cytotoxicity agaist the Glioma cell line was determined according to the procedure of KORNBLITH and SZYPKO[4].

The cytotoxicity against the spectrum of primary human tumors was assessed by Dr. VON HOFF according to the HAMBURGER-SALMON technique[5,6].

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Antitumor Determinations

The antitumor activity of fredericamycin A was determined by contracted accessory testing services according to the National Cancer Institute (NCI) guidelines for natural products[2,3].

Spectral Difference Determinations

The assessment of both the metachromatic and hyperchromic effects was made on a GCA/McPherson Model EU-700-32 spectrophotometer. In the metachromatic determination, 1.5 ml of antibiotic solution (22.5 $\mu$g fredericamycin A in 0.3 ml DMSO subsequently diluted to 1.5 ml with distilled $H_2O$, or 22.5 $\mu$g daunorubicin in 1.5 ml distilled $H_2O$) was added to 1.5 ml buffer (0.01 M tris-HCl, pH 7.6, and 0.01 M NaCl) with or without DNA (50 $\mu$g highly polymerized salmon DNA per ml buffer) and read against a buffer blank. In the hyperchromic determination, 2.0 ml of Sigma Type I calf thymus DNA (0.4 mg per ml 0.1 M sodium acetate, pH 5.0, and 0.004 M $MgSO_4$ buffer) was mixed with 1.0 ml of Sigma deoxyribonuclease (0.01 mg per ml 0.15 M NaCl) or fredericamycin A (prepared the same as for the metachromatic analysis) and monitored for increase in absorbance as a function of time for five minutes.

Effect of Exogenous DNA on Antibacterial Activity

Herring sperm DNA (4 mg DNA per ml 0.2 M potassium phosphate buffer, pH 7.7) was added at a final concentration of 0.1% to serial dilutions of fredericamycin A. Then all tubes were inoculated with logarithmically growing *Bacillus subtilis* at a final concentration of $10^6$ organisms per ml. After overnight incubation at 37°C, endpoints were scored according to the presence or absence of visual turbidity.

Ames Mutagenicity Test and Biochemical Induction Assay (BIA)

The Ames tests were performed according to standard protocol[7]. The BIA, a $\beta$-galactosidase prophage induction assay for possible DNA interaction was performed according to the method of ELESPURU and YARMOLINSKY[8].

Procaryotic Macromolecular Synthesis Inhibition

*B. subtilis* was grown to early logarithmic phase in 300 ml side-arm shake flasks containing 60 ml of supplemented minimal medium. Additions of 240 $\mu$l of 50 $\mu$Ci/ml [$^{14}$C]uridine (52 mCi/mmole) and 1.0 ml of 0.01 M cold uridine; 13 $\mu$l of 1.0 mCi/ml [$^3$H]thymidine (43 Ci/mmole); or 25 $\mu$l of 1.0 mCi/ml [$^3$H]valine (29 Ci/mmole) were made to the medium. After 11 minutes, fredericamycin A (at a final concentration of 0.05 $\mu$g/ml) or tetracycline (at a final concentration of 0.18 $\mu$g/ml) was added to test flasks One-ml samples were mixed with equal volumes of 10% ice-cold trichloroacetic acid (TCA). The insoluble materials were collected on glass fiber filters (Whatman GF/A), washed twice with cold 5% TCA, once with 95% ethanol, and dried. The radioactivity was determined with a Searle isocap/300 liquid scintillation counter.

Eucaryotic Macromolecular Synthesis Inhibition

Suspension cultures of P388 mouse leukemia cells were grown to early log phase ($\sim 1.6 \times 10^5$ cells/ml) using medium compositions and roller bottle techniques established by KLEIN and RICKETTS[9]. Five microliters of 1.0 mCi/ml [$^3$H]uridine (27 Ci/mmole), 5 $\mu$l of 1.0 mCi/ml [$^3$H]thymidine (43 Ci/mmole) or 50 $\mu$l of 0.1 mCi/ml [$^{14}$C]valine (286 mCi/mmole) was added to 10 ml roller tube cultures, incubated 1 hour at 37°C and sampled. Fredericamycin A, actinomycin D, or puromycin was added and after an additional 1-hour incubation at 37°C, a second sample was taken. All samples were precipitated using 5% cold TCA, centrifuged, washed, and redissolved in protosol. Radioactivity was determined in a Searle isocap/300 liquid scintillation counter.

## Results

### Antimicrobial Spectrum

Minimal inhibitory concentrations (MIC's) of fredericamycin A against a spectrum of microorganisms are shown in Table 1. Fredericamycin A shows no activity against the Gram-negative bacteria and relatively good activity against both the fungi and Gram-positive bacteria.

Table 1. Antimicrobial spectra of fredericamycin A.

| Test organisms | ATCC # | Minimal inhibitory concentration* |
|---|---|---|
| *Bacillus subtilis* | 6633 | 0.025 |
| *Penicillium notatum* | 9478 | 0.1 |
| *Candida albicans* | 10231 | 1.5 |
| *Staphylococcus aureus* | 6538P | 1.5 |
| *Micrococcus luteus* | 9341 | 3.0 |
| *Saccharomyces cerevisiae* | 2601 | 3.0 |
| *Escherichia coli* | 10536 | >100.0 |
| *Klebsiella pneumoniae* | 10031 | >100.0 |
| *Pseudomonas aeruginosa* | 27853 | >100.0 |

* Determined by agar dilution method.

Table 2. *In vitro* cytotoxicity of fredericamycin A against established cell lines.

| Cell line tested | Activity measurement *(μg/ml) |
|---|---|
| KB | $7\times10^{-1}$ |
| P388 | $5\times10^{-4}$ |
| L1210 | $2\times10^{-4}$ |
| Glioblastoma | $1\times10^{-1}$ |

* Endpoints for the KB, P388, and L1210 cells represent 50% effective dose determinations; the endpoint for the glioblastoma cells represents a 44% kill ratio.

Against *B. subtilis* fredericamycin A is bactericidal. After 24 hours, fredericamycin A, at a dose of 0.312 μg/ml, reduced the initial inoculum viability of $10^6$ organisms/ml to $10^2$ organisms/ml.

### *In Vitro* Cytotoxicity

As shown in Table 2, fredericamycin A is a very cytotoxic compound. Its $ED_{50}$ against mouse leukemias P388 and L1210 compares closely to reported figures for actinomycin D and adriamycin[10]. Its activity against glioblastoma cells is comparable to 1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU). Preliminary testing of fredericamycin A at 10 μg/ml in a human stem cell assay consisting of 21 tumors of 13 different types has also given very encouraging results[11].

### *In Vivo* Antitumor Activity

The *in vivo* activity of fredericamycin A is shown in Table 3. According to these data, fredericamycin A is effective in extending the life span of mice inoculated with P388 leukemic cells and in reducing the

Table 3. *In vivo* antitumor activity of fredericamycin A.

| Tumor tested | Drug dose (mg/kg) | % T/C* | Tumor tested | Drug dose (mg/kg) | % T/C* |
|---|---|---|---|---|---|
| P388 lymph leukemia (DMSO: i.p.)** | 0.06 | 122 | CD8F mammary fragment (DMSO: i.p.) | 0.31 | 68 |
| | 0.12 | 146 | | 0.62 | 53 |
| | 0.25 | 134 | | 1.25 | 7 |
| | 0.50 | 200 | Lewis lung carcinoma (DMSO: i.p.) | 0.06 | 103 |
| | 1.00 | 166 | | 0.12 | 109 |
| | >1.00 | toxic | | 0.25 | 106 |
| L1210 lymph leukemia (DMSO: i.p.) | 0.06 | 108 | C38 colon fragment (DMSO: i.p.) | 0.15 | 81 |
| | 0.12 | 113 | | 0.30 | 85 |
| | 0.25 | 118 | | 0.60 | 87 |
| | 0.50 | 94 | MX-1 breast xenograft | 2.00 | 116 |
| | 1.00 | 104 | | 4.00 | 107 |
| | 2.00 | 98 | | 8.00 | 115 |
| B16 melanoma (Klucel: i.p.) | 0.75 | 133 | | 16.00 | 92 |
| | 1.50 | 133 | LX-1 lung xenograft | 0.50 | 151 |
| | 3.00 | 128 | | 1.00 | 151 |
| | 6.00 | 107 | | 2.00 | 103 |
| | 12.00 | 114 | | 4.00 | 109 |

* T/C (test *vs.* controls) endpoints for P388, B16 melanoma and lewis lung=median survival time; T/C endpoint for L1210=average survival time; T/C endpoints for CD8F mammary and C38 colon=median tumor weight; T/C endpoints for MX-1 breast xenograft and LX-1 breast xenograft=average tumor weight.

** (Solvent: intraperitoneal route of injection)

median tumor weight of the CD8F mouse mammary tumor. Under the conditions tested, fredericamycin A is ineffective against L1210 leukemia, Lewis lung carcinoma, C38 colon tumor, MX-1 breast xenograft, and LX-1 lung xenograft. Fredericamycin A is marginally active against $B_{16}$ melanoma.

## Assessment of Interaction of Fredericamycin A with DNA

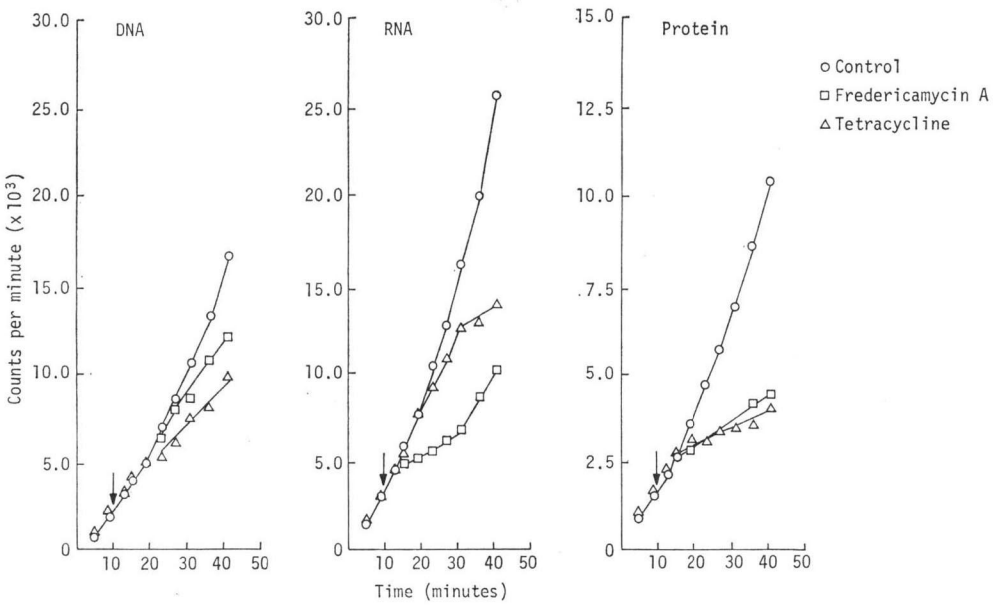
The potential of fredericamycin A to interact with DNA was monitored by spectral difference studies and by reversal of antibacterial activity. Spectroscopically, fredericamycin A failed to exhibit a metachromatic shift in the presence of exogenous DNA, or cause a hyperchromic effect upon DNA. An inhibitory dose (0.07 $\mu$g/ml) of fredericamycin A against *B. subtilis* was not affected by the presence of exogenous DNA at 1 mg/ml. In a control experiment with actinomycin D, this concentration of DNA under the same conditions changed the MIC from 1.25 $\mu$g/ml to greater than 5 $\mu$g/ml.

In the Ames mutagenicity assay, fredericamycin A was tested at a concentration of 100 $\mu$g/ml against each of five strains (98, 100, 1535, 1537, 1538) with and without S9 activation. In none of these strains did fredericamycin A show mutagenicity. Furthermore, fredericamycin A failed to show induction in the BIA at concentrations ranging from 4 to 500 $\mu$g/ml. There was no indication of toxicity (antibacterial activity) at the higher concentrations tested in either the BIA or Ames assays.

## Inhibition of Macromolecular Synthesis by Fredericamycin A

The effect of fredericamycin A on both procaryotic and eucaryotic macromolecular synthesis was assessed. The results for procaryotic protein, RNA, and DNA syntheses are presented in Fig. 1. RNA and protein syntheses are inhibited earlier and to a greater extent than DNA synthesis. There is a slight propensity for fredericamycin A to shut down RNA synthesis prior to protein synthesis. The effect of fredericamycin A on protein synthesis is similar to that of tetracycline.

Fig. 1. Inhibition of *B. subtilis* macromolecular synthesis by fredericamycin A. Inhibitors (fredericamycin A or tetracycline) were added at 11 minutes.



The effect of fredericamycin A on macromolecular synthesis in P388 mouse leukemia cells was monitored using actinomycin D and puromycin as reference inhibitors of transcription and translation, respectively. Within this system, fredericamycin A exhibited a preferential inhibition of protein synthesis (Table 4).

Table 4. *In vitro* P388 mouse leukemia macromolecular synthesis inhibition.

| Compound | DNA (%) | RNA (%) | Protein (%) |
|---|---|---|---|
| Fredericamycin A (0.25 μg/ml) | 0 | 15 | 34 |
| (0.1 μg/ml) | 0 | 10 | 37 |
| Actinomycin D (0.05 μg/ml) | 6 | 32 | 0 |
| Puromycin (8.0 μg/ml) | 16 | 16 | 36 |

The MIC of fredericamycin A against P388 is 1.0 μg/ml.

## Discussion

Fredericamycin A exhibits antibacterial, antifungal, cytotoxic, and antitumor activities. Based upon the select spectrum of bacteria tested, fredericamycin A appears not to be active against Gram-negative bacteria. In the case of *B. subtilis*, it has been shown that its effect is bactericidal.

*In vitro*, fredericamycin A is very cytotoxic against P388 and L1210 mouse leukemia cells and human glioblastoma cells. In addition, fredericamycin A has shown very good initial results in the human primary stem cell assay, and dose range studies could further reveal the potential for clinical efficacy.

*In vivo*, fredericamycin A has shown antitumor activity against the P388 ascites tumor and the mammary CD8F tumor. In addition, fredericamycin A has demonstrated marginal activity against the B16 melanoma tumor. In other NCI tumor models, fredericamycin A has failed to be effective under the conditions tested. However, due to solubility problems[1], further pharmacological studies could serve to broaden the range of activity of fredericamycin A.

Although fredericamycin A was not mutagenic in the Ames assay and did not induce β-galactosidase in the BIA, no toxicity was evident in either test. Therefore, the negative results in both assays could have resulted from a restricted permeability towards the antibiotic (it was inactive against all Gram-negative strains tested) rather than indicating the absence of any interaction with DNA. Further studies designed to test for interaction between fredericamycin A and DNA lent support to the negative Ames and BIA results. There was no demonstrable metachromatic nor hyperchromic effect on mixing the antibiotic with DNA, and exogenous DNA failed to reverse the antibacterial activity.

Inhibition studies of procaryotic macromolecular synthesis clearly showed that RNA and protein synthesis were affected earlier and to a greater extent than DNA synthesis. When the same studies were repeated in P388 leukemia cells, protein synthesis was inhibited more completely than RNA, while DNA synthesis remained virtually unaffected. The propinquity of the RNA and protein synthesis inhibition led to a study designed to test the effect of fredericamycin A on *Escherichia coli* RNA polymerase. No inhibition could be demonstrated in this test (data not shown). The procaryotic data combined with fredericamycin A's proclivity to inhibit protein synthesis in P388 eucaryotic cells suggest that its cellular action is exerted within the complexities of protein synthesis.

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