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Design and semisynthesis of novel fredericamycin A derivatives with an improved antitumor profile

Ulrich Abel,[a] Werner Simon,[b] Peter Eckard[b] and Friedrich G. Hansske[b,*]

[a]*Santhera Pharmaceuticals, Im Neuenheimer Feld 518-519, 69120 Heidelberg, Germany*
[b]*Discovery Partners International GmbH, Waldhoferstrasse 104, 69123 Heidelberg, Germany*

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Abstract—We report the design, semisynthesis, and biological activity of a series of fredericamycin (**1**) derivatives. Within this series compound **1e** combines low nanomolar cytotoxic potency in vitro, increased tumor cell line selectivity, and in vivo activity in a human xenograft model.

Despite the recent successes of targeted antitumor therapies, the more classic cytotoxic compounds either alone or in combination with other compound classes are still an important element of today's therapies and represent a valuable component of the antitumor product portfolio of modern pharmaceutical industry. The natural product fredericamycin A (**1**, FMA, Fig. 1) is a cytotoxic antitumor lead compound with interesting properties, which never reached clinical development. This was probably due to limited accessibility of sufficient quantities for lead optimization and a general trend away from natural products in the 1990s.

FMA is a structurally unique[1] antitumor antibiotic which was isolated from the fermentation broth of a *Streptomyces griseus* strain.[2] It is active in vitro against fungi, gram-positive bacteria, and tumor cell lines, and shows in vivo activity against P388 leukemia, CD8F mammary, and DU-145 prostate tumor xenografts in mice.[3] FMA is a low micromolar inhibitor of both topoisomerases I and II, and inhibits the peptidyl-prolyl *cis–trans* isomerase Pin1 with a K_i of 0.82 μM.[4,3b] The unprecedented architecture along with the biological activity of FMA gave rise to numerous efforts toward the total synthesis of racemic[5] as well as of enantiopure (*S*)-**1**.[6] Nevertheless, there are few reports of synthetic approaches being utilized for the generation of FMA analogues and to establish a structure–activity relationship (SAR). Kelly et al., for example, used their total synthesis approach to produce fredericamycin derivatives exhibiting antiproliferative activity.[7] A series of derivatives was generated starting from a synthetic quinone precursor lacking the F-ring. Introducing various substituted amines into the A-ring yielded compounds with IC_{50}s ranging from 30 to 60 ng/mL in a microdilution assay using six different tumor cell lines. A set of 63 A-ring modified derivatives was produced by reacting synthetic fredericamycin (**1**) with a diverse set of primary and secondary amines. However, no biological activity was given for this series. Kouichi et al. published the acylation of B/E/F-ring hydroxy groups as well as the hydrogenation of the F-ring pentadiene sidechain starting from fermentation derived fredericamycin (**1**) resulting in derivatives with an improved antibacterial activity.[8]

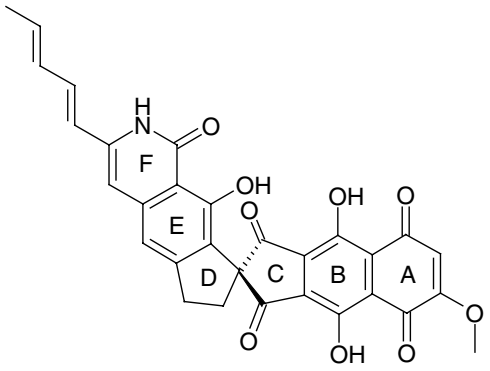

Figure 1. Fredericamycin A (**1**).

Keywords: Fredericamycin; Antitumor; Spiro quinone; Natural product; Semisynthesis.
* Corresponding author. Tel.: +49 6221 8204 0; fax: +49 6221 8204 50; e-mail: fhansske@hd.discoverypartners.com

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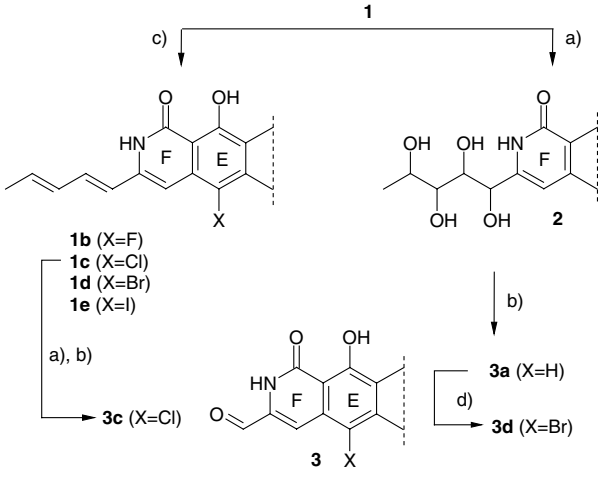
Herein we describe a semisynthetic strategy aiming at FMA derivatives with improved potency and tumor selectivity.[9] Since only limited SAR was available from literature data, the design of derivatives was initially driven by chemical feasibility and for some of the compounds by the overall goal to introduce polar and small pharmacophoric substituents to increase polarity and solubility. Iterative synthetic and testing cycles resulted in the compounds described below.

Multigram quantities of pure **1** could be obtained using an optimized process for the isolation and purification from the fermentation broth of *Streptomyces griseus* strain ATCC49344 mutants without the need for chromatographic purification steps.[10]

Initially, we focused on E- and F-ring modifications of the western portion and envisaged to degrade the F-ring diene sidechain to an aldehyde functionality in order to lower molecular weight and to gain a reactive and versatile functional group for subsequent synthetic transformations. Bis-dihydroxylation of the pentadiene sidechain of **1** resulted in the formation of tetrahydroxylated FMA (**2**) in 87% yield,[11] which was then successfully subjected to diole cleavage using sodium periodate in a mixture of dichloromethane, methanol, and water to generate aldehyde **3a** (Scheme 1). When wet DMSO was used as a solvent for periodate cleavage, we observed the formation of iodinated side-products in LC–MS. This led us to investigate phenolic halogenation using various halogen sources despite being aware that E-ring halogenation would not necessarily increase product polarity and solubility. Conversion of **1** was easily achieved using either Selectfluor® or halosuccinimides yielding E-ring halogenated products **1b** to **1e**.

We attempted to synthesize various E-ring halogenated aldehydes as building blocks for compounds combining E-ring halogenation with replacements of the F-ring



Scheme 1. E- and F-ring modifications. Reagents: (a) NMO, OsO_4, DCM, MeOH, H_2O, 87% from **1**, 75% from **1c**; (b) $NaIO_4$, DMF, H_2O, 100% yield for **3a**, 90% yield for **3c**; (c) for X = F: Selectfluor®, DMF, 14% yield for **1b**; for X = Cl: NCS, DMF, 30% yield for **1c**; for X = Br: NBS, DMF, 32% yield for **1d**; for X = I: NIS, DMF, 17% yield for **1e**; (d) Br_2, DMF, 83% yield.

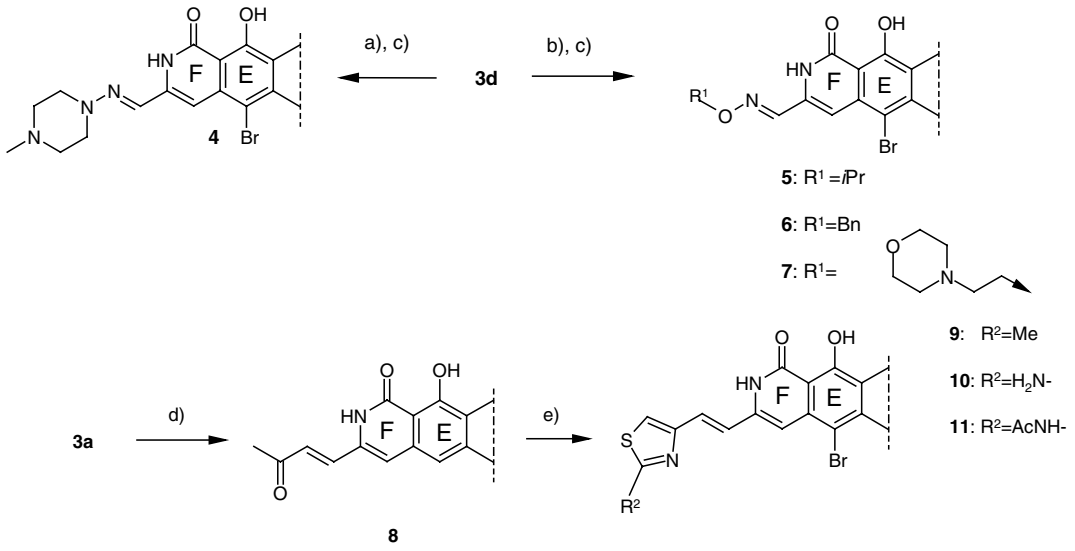
pentadiene sidechain. Direct halogenation of fredericamycin-aldehyde **3a** was successful when using bromine in DMF to give bromo-aldehyde product **3d**, whereas treatment of **3a** with *N*-chlorosuccinimide led to decomposition of starting material. Nevertheless, chloro-aldehyde **3c** was finally accessible from chloro-fredericamycin **1c** by bis-dihydroxylation and subsequent diole cleavage. We then started to search for chemoselective conversions of the aldehyde group of halogenated and unhalogenated FMA derivatives leading to derivatives having the FMA pentadiene sidechain replaced by more polar substituents. Treatment of bromo-aldehyde **3d** with a slight excess of various commercially available hydrazines or *O*-alkylhydroxylamines under acidic conditions led to the formation of hydrazones and oximes as exemplified by compounds **4**–**7** (Scheme 2). Clean conversions were observed in all cases and unreacted reagent and/or starting material could be easily removed using Wang-aldehyde polystyrene and sulfonylhydrazide polystyrene scavenger resin to give products that were typically >95% pure by LC–MS.

We then envisaged to introduce F-ring sidechain thiazoles which were thought to be accessible by a short chemoselective synthesis sequence and would eventually undergo additional interactions with the biochemical target. Wittig–Horner olefination toward F-ring butenone **8** was achieved by treating aldehyde **3a** with diethyl 2-oxopropylphosphonate and excess tetramethylguanidine as a base. When **8** was subsequently treated with bromine, bromination of phenolic E-ring as well as of the terminal methyl group occurred. The intermediate α-bromoketone was further converted into thiazole-substituted bromo-fredericamycines **9**–**11** by reaction with substituted or unsubstituted thioamides.

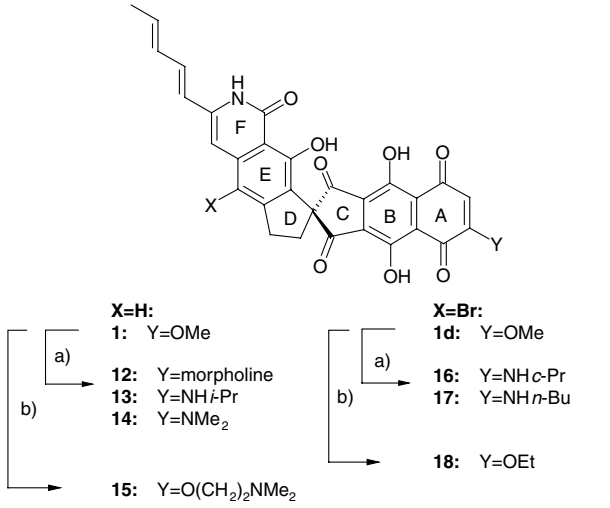
Inspired by Kelly's work,[7] we then started replacing the A-ring methoxy group of FMA (**1**) and the more potent bromo-FMA (**1d**) by nucleophiles such as primary and secondary amines and by alcohols. This might eventually lead to compounds with increased potency and/or an increased stability toward biogenic nucleophiles in vivo. A-ring modifications were possible by exchanging the methoxy group with alcohols (compounds **15** and **18**) or amines (compounds **12**–**14**, **16**, and **17**) as outlined in Scheme 3. Conversion with amines proceeded smoothly at room temperature in DMF whereas reaction with alcohols required the use of excess reagent, elevated temperatures, and prolonged reaction times.

Attempts to purify synthesis products by either flash chromatography on silica gel or by preparative RP-HPLC resulted in generally low recovery rates. However, we found column chromatography using Sephadex® LH-20 gel (Amersham) and dichloromethane–methanol mixtures as eluent to be the method of choice if purification was required.

Fredericamycin A (**1**) inhibits topoisomerases and Pin1 with low micromolar activity.[4,3b] The published data however do not provide clear evidence that the observed antitumor activity is caused by topoisomerase or Pin1 inhibition. Other targets may contribute



Scheme 2. Sidechain functionalization of aldehydes **3**. Reagents: (a) 4-Methylpiperazin-1-amine, TFA, DMF, (b) $RONH_2$, TFA, DMF, (c) polystyrene-CHO, polystyrene-SO_2NNH_2; 90–95% (d) $MeC(O)CH_2P(O)OEt_2$, 1,1,3,3-tetramethylguanidine, 69% yield; (e) 1—Br_2, DMF, 87% yield, 2—$R^2C(S)NH_2$, 62% yield for **9**; 93% yield for **10**; 39% yield for **11**.



Scheme 3. Nucleophilic replacement of A-ring methoxy group. Reagents and conditions: (a) Amine, DMF room temperature; each 99% yield; (b) KOAc, ROH as solvent, 80 °C, 36% yield for **15**, 71% yield for **18**.

to the antitumor effect of fredericamycin A (**1**) and its derivatives. We therefore based the evaluation of the antitumor properties on in vitro cell viability assays using a panel of 10–12 human tumor cell lines and two main parameters: cytotoxic potency and differential cytotoxicity. Potency was expressed as mean of the IC_{70}s of each cell line tested (mean IC_{70}). Differential cytotoxicity was calculated as the fold difference in IC_{70} between the least sensitive and the most sensitive cell line. A selective antitumor compound should display preferential killing of certain cell types. An unspecific membrane disruptor, for example, would kill all cells, whereas a compound targeting a certain tumorigenic pathway would show preferential cytotoxic activity against cell lines, which depend on the respective pathway. A high differential cytotoxicity in a tumor cell line panel is therefore indicative of tumor selectivity and represents an efficient strategy of evaluating the antitumor potential of compound sets in vitro.

Table 1. Cytotoxic activity of FMA derivatives

Compound	Inhibition[a] (%)	Mean IC_{70}[b] (nM)	Differential cytotoxicity[c] (fold)
Paclitaxel	nd[d]	0.7	7847
Camptothecin	nd	8.6	199
Adriamycin	91	39	894
1	82	517	14
1b	91	20	104
1c	91	14	98
1d	92	8.1	100
1e	93	11	837
2	85	186	53
3a	31	nd	nd
3c	25	nd	nd
3d	42	nd	nd
4	73	658	9
5	91	74	47
6	91	50	15
7	88	101	24
8	59	nd	nd
9	85	124	12
10	77	612	7
11	83	512	14
12	98	77	153
13	91	634	21
14	96	306	18
15	94	1019	3
16	97	96	104
17	94	302	20
18	97	160	17

[a] Inhibition: mean inhibition of cell viability of the 10 cell lines at 1 μg/mL.

[b] Mean IC_{70}: cytotoxic potency expressed as mean of the IC_{70}s of the 12 cell lines tested.

[c] Differential cytotoxicity: calculated as the fold difference in IC_{70} between the least sensitive and the most sensitive cell line.

[d] nd, not determined.

Cytotoxic activity[12] of the derivatives was initially evaluated in a panel of 10 tumor cell lines[13] at a fixed compound concentration of 1 μg/mL (Table 1). Compounds exhibiting an inhibition of >70% (mean of the inhibition values of all cell lines) at 1 μg/mL were subjected to dose–response studies using a slightly broader cell line panel[14] and the cytotoxic potency (mean IC_{70}) and differential cytotoxicity values were determined (Table 1).

Halogenation of the E-ring resulted in considerably enhanced potency in the low nM range (**1b**, **1c**, **1d**, and **1e**). Selectivity increased with the size of the substituent (H < F ~ Cl ~ Br < I).

Replacement of the F-ring pentadiene with an aldehyde group resulted in drastically reduced potency (compounds **3a**, **3c**, and **3d**). Other F-ring replacements containing polar groups, such as a hydrazide (**4**) or a carbonyl (**8**) reduced potency as well.

Replacement of the A-ring methoxy group by a morpholine group (**12**) was favorable with respect to selectivity and potency. Other A-ring amines (**13**, **14**, and **15**) showed similar potency and selectivity compared to fredericamycin (**1**). The A-ring vinylogous ester (Michael system) may react covalently with biogenic nucleophiles. Similar or increased potency of the amines (**12**–**15**) with reduced reactivity of the Michael system compared to **1** suggests that fredericamycin's cytotoxic activity is not the result of such a covalent reaction with a potential target.

Additional work and the identification of molecular targets of fredericamycin (**1**) would be required to decide whether the new functionalities effect potency and selectivity by changes in cellular permeation, stability, and/or interaction with the target.

Figure 2 summarizes the cellular data and compares the cytotoxic potency and the differential cytotoxicity of the novel derivatives with fredericamycin (**1**) and reference compounds. Compound **1e** is outstanding due to its high potency of 11 nM and a differential cytotoxicity of 837-fold compared to fredericamycin (**1**) with 517 nM and 14-fold, respectively. Other compounds showing considerable improvements are the E-ring halogens **1b**, **1c**, and **1d**, the A-ring amines **12** and **16**, and the oxime **5**.

Figure 3 visualizes the cellular activity of fredericamycin (**1**) and two halogenated derivatives (**1d** and **1e**) as mean graphs and demonstrates the improvement in differential cytotoxicity and tumor cell selectivity. Both compounds show pronounced activity against the breast cancer cell line MCF7 and the uterus cancer cell line 1138L. Compounds **1d** and **1e** show similar patterns of cytotoxicity in the cell line panel indicating a similar mode of action.

Based on the available data derivatives **1d** and **1e** are excellent candidates for follow-up testing in in vivo tu-

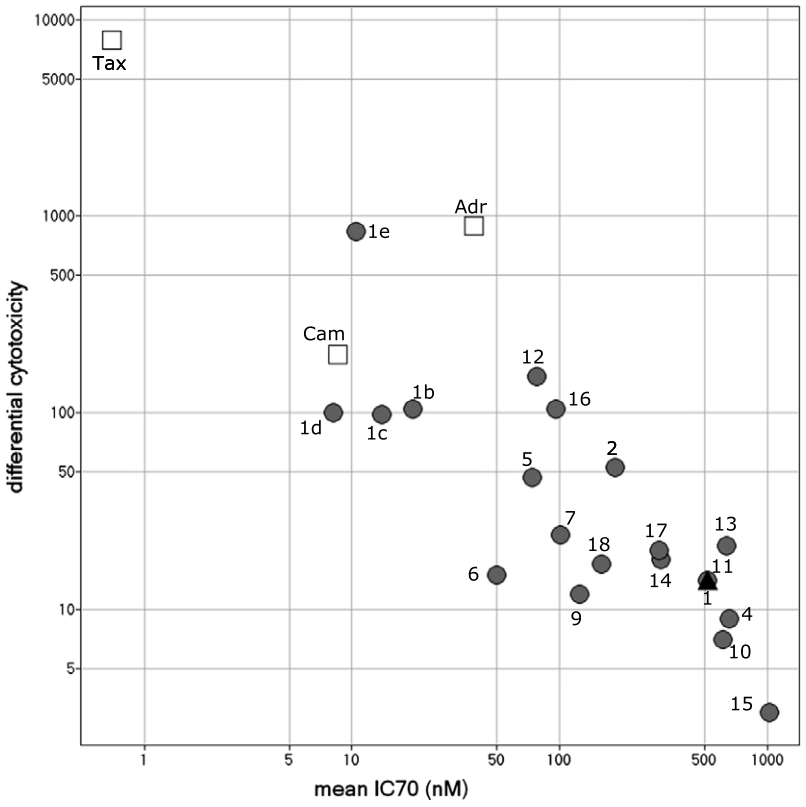


Figure 2. In vitro cytotoxic potency (IC_{70}) and differential cytotoxicity of FMA derivatives. Cell viability and the plotted parameters were determined as described in the text. Mean IC_{70} is plotted against differential cytotoxicity for the lead compound (**1**, black triangle), the reference compounds paclitaxel (Tax, open square), camptothecin (Cam, open square), adriamycin (Adr, open square), and the fredericamycin derivatives (gray circles).

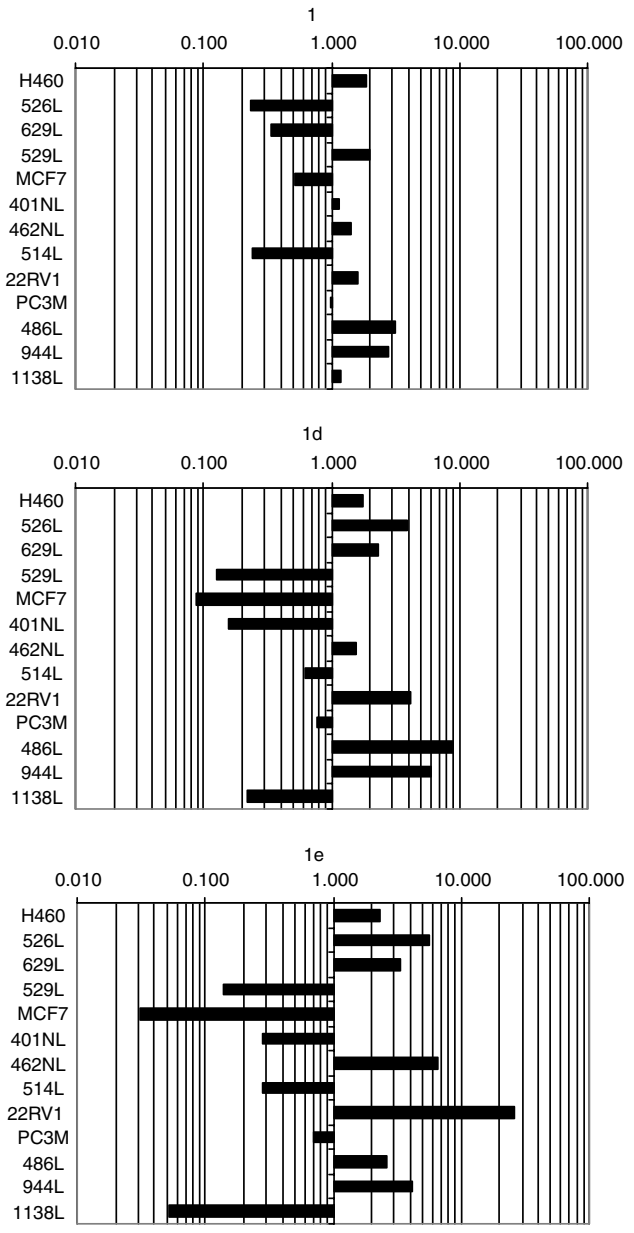


Figure 3. Cellular activity of FMA (**1**) and the halogenated derivatives **1d** and **1e**. Cell viability was determined as described in the text. Variations of individual IC_{70}s from the mean IC_{70} value are plotted as bars on a logarithmic scale. Each bar represents the median of 3–4 (22RV1:2) individual experiments. The mean IC_{70} (517 nM for **1**, 8.1 nM for **1d**, and 11 nM for **1e**) was set to 1. Bars to the left represent lower IC_{70}s, bars to the right higher IC_{70}s compared to the mean. Human tumor cell line panel: non-small cell lung: H460, 526L, 629L, 529L; breast: MCF7, 401NL; melanoma: 462NL, 514L; prostate 22RV1, PC3M; renal: 486L, 944L; uterus: 1138L.

mor xenograft models. At this stage we decided to select compound **1e** for a proof-of-concept experiment in a human xenograft model in nude mice using the human uterine cancer cell line UXF 1138.

The maximum tolerated dose (MTD) was determined in tumor-free nude mice (data not shown). Compound **1e** was toxic at doses of 3 and 1 mg/kg/day. A dose of 0.3 mg/kg/day given on days 0, 4, and 8 resulted in a body weight change of −11% and was considered as the MTD. A dose below the MTD of 0.15 mg/kg/day was chosen for the xenograft experiments. **1e** at 0.15 mg/kg/day given on days 0, 4, and 8 was well tolerated but not effective (data not shown). Given instead on five consecutive days (0–4) compound **1e** produced an antitumor effect with a maximum T/C value (tested group relative to control group) of 51% at day 7 (Fig. 4A). **1e** was well tolerated and resulted only in a slight loss of body weight (−3.1% at day 7, Fig. 4B).

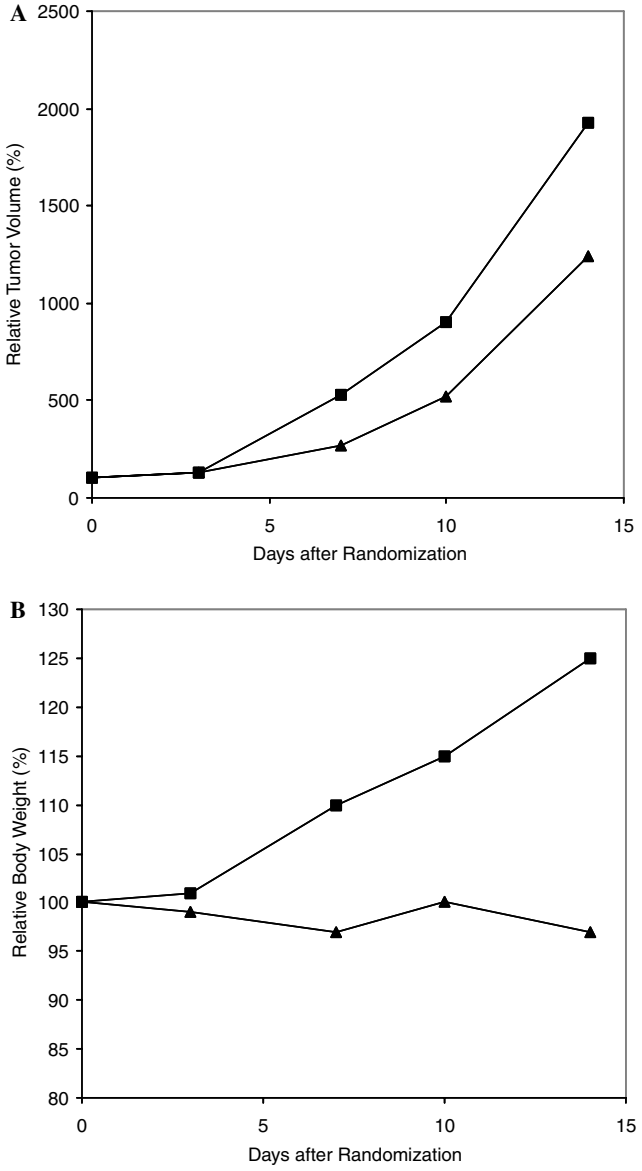


Figure 4. In vivo antitumor activity of derivative **1e** in a human uterine cancer (UXF 1138) xenograft in nude mice. Tumor fragments of about 20 mg were implanted subcutaneously in both flanks of athymic nude mice. Mice were randomly assigned to the treatment and control group with 4–6 mice in each group. The compound was administered ip (3 mL/kg in pure DMSO) at a dose of 0.15 mg/kg/day at days 0–4. Animal weights and tumor diameters were measured twice weekly and tumor volumes calculated. (A) Relative tumor volume, control: black squares, treated: black triangles. (B) Relative body weight, control: black squares, treated: black triangles. Experiments were performed by Oncotest GmbH, Freiburg, Germany.

These first in vivo results are promising but need to be extended to additional FMA derivatives, other tumor cell lines, extended dosing, and optimized administration routes.

In summary, starting from natural fredericamycin (**1**), a variety of semisynthetic derivatives have been synthesized with modifications of A- and E-rings and of the F-ring pentadiene sidechain. This resulted in compounds with increased, low nanomolar cytotoxic activity in vitro, and increased tumor selectivity. A first set of in vivo experiments revealed promising antitumor activity in an uterine xenograft model.

The results exemplify the power of semisynthetic natural product derivatization for the optimization of natural product drug leads.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.029.

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11. The relative and absolute stereochemistry of the tetrol sidechain could not clearly be assigned by ^{1}H NMR. However ^{1}H NMR and LC–MS analysis indicate the existence of a single stereoisomer.
12. Human tumor cells were grown at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany) and 0.1% gentamicin (Invitrogen). Cells are routinely passaged once or twice weekly, split at a ratio of 1:3–6, and maintained no longer than 20 passages in vitro. A propidium iodide assay was used to assess the effects of the compounds. Cells were harvested from exponential phase cultures by trypsination and plated in 96-well microplates at a density of 5000–12,000 cells/well. After 24 h 20 μL of culture medium (control) or culture medium containing the test compounds was added to the wells. For IC_{70} determination test compounds were applied at 5 concentrations ranging from 0.1 ng/mL to 1 μg/mL. Following 4 days of continuous drug exposure, medium or medium with drug was replaced by 200 μL of an aqueous propidium iodide (PI) solution (7 μg/mL) and the first measurement was performed (excitation 530 nm, emission 620 nm) giving the number of dead cells. The total number of cells is quantified after permeabilizing the cells by freezing/thawing followed by the second measurement. The number of living cells compared to controls is used to calculate % inhibition of cell viability and IC_{70} values.
13. LCL H460 lung, MACL MCF7 mammary, LXFL 529L lung, LXFA 629L lung, MEXF 462NL melanoma, MEXF 514L melanoma, MAXF 401NL breast, RXF 944L renal, RXF 486L renal, and UXF 1138L uterus. The H460 and MCF7 cell lines were from the National Cancer Institute (Bethesda, USA). The other cell lines were established by Oncotest GmbH (Freiburg, Germany).
14. Cell lines as in Reference 13, supplemented by PR PC3M prostate (from the National Cancer Institute) and PRCL 22RV1 prostate (from the American Type Culture Collection, Rockville, MD).