Therapeutic Cure against Human Tumor Xenografts in Nude Mice by a Microtubule Stabilization Agent, Fludelone, via Parenteral or Oral Route

Ting-Chao Chou,1 Huajin Dong,1 Xiuguo Zhang,1 William P. Tong,2 and Samuel J. Danishefsky3,4

1Preclinical Pharmacology and 2Analytical Chemistry Core Laboratories, and 3Bio-Organic Chemistry Laboratory, Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center; and 4Department of Chemistry, Columbia University, New York, New York

Abstract

Epothilones, 16-membered macrolides isolated from a myxobacterium in soil, exert their antitumor effect, like Taxol, by induction of microtubule polymerization and microtubule stabilization. They are effective against tumor cells that are resistant to Taxol or vinblastine. We recently designed, via molecular editing and total synthesis, a new class of epothilones represented by 26-trifluoro-(E)-9,10-dehydro-12,13-desoxy-epothilone B (Fludelone), which has emerged as a lead candidate for clinical development. Treatment of nude mice bearing MX-1 human mammary carcinoma xenografts (as large as 3.4% body weight) with Fludelone (6-hour i.v. infusion, 25 mg/kg, q3d × 5, q3d × 4) led to complete disappearance and de facto "cure" (i.e., remission without a relapse for over 15% of the average life span of 2 years). The toxicities induced by bolus i.v. injection could be avoided through prolonged i.v. infusion, which allowed for a 10-fold increase in maximal tolerated dose.

Complete remission of MX-1 xenografts was achieved with only one third of this maximal tolerated dose. Parallel studies with Taxol and Fludelone [20 mg/kg, 6-hour i.v. infusion (q2d × 4) × 3] against HCT-116 human colon carcinoma xenografts revealed that both drugs achieved significant regression. On the other hand, all Taxol-treated mice relapsed in ~1.3 months, whereas the Fludelone-treated mice were cured without any relapse for over 7 months. Furthermore, tumor remission was achieved by Fludelone against SK-OV-3 (ovary), PC-3 (prostate), and the Taxol-resistant CCRF-CEM/Taxol (leukemia) xenograft tumors. Most remarkably, p.o. administration of Fludelone (30 mg/kg, q2d × 7, q2d × 9, q2d × 5) against MX-1 xenografts achieved a nonrelapsing cure for as long as 8.4 months. The above results indicate that Fludelone is a highly promising compound for cancer chemotherapeutics. (Cancer Res 2005; 65(20): 9445-54)

Introduction

The epothilone family of macrolides originally isolated from the myxobacterium Sorangium cellulosum (1) is one of several classes of natural products that exert their antitumor activity via the mechanism of microtubule stabilization (2). Other natural products in these classes include the taxoids isolated from the yew plant Taxus brevifolia (3, 4), the discodermolides from the marine sponge Discodermia dissoluta (5), eleutherobins from the marine soft coral Eleutherobia albiflora (6), dictyostatin-1 from the marine sponge Spongia dicryptocerata (7), and laulimalides from the marine sponge Cacospongia mycophigienis (8).

Several hundred epothilone-related compounds have been synthesized or isolated during the past 7 years and subjected to biological testing and evaluation (9–16). Among them, epothilone B (EpoB, Epo-906), 15-desoxy-15-aza-EpoB (BMS-243550), 12,13-desoxy-EpoB (dEpoB, EpoD, NSC-703147, KOS-862), 21-amino-EpoB (BMS-310705), and 9,10-dehydro-dEpoB (KOS-1584) are in various phases of clinical trials in cancer patients.

We recently designed, through total synthesis, a new class of potent 12,13-desoxy-epothilones with 9,10-dehydro and 26-trifluoro structures. The preliminary observations highlighted several interesting chemical and pharmacologic properties for anticancer applications (17, 18). We now report in detail the completed experiments that led to particularly impressive therapeutic results obtained for one of our lead compounds, 26-trifluoro-9,10-dehydro-12,13-desoxy-EpoB or F2-deH-dEpoB (Fludelone; see Fig. 1A).

The "cure" of human cancer (e.g., complete remission of cancer without a relapse for over 15% of life span) through chemotherapy is not a rare occurrence. However, cure of xenograft tumors in nude mice or severe combined immunodeficient mice by chemotherapy is exceedingly difficult. This difficulty may be attributed to a number of factors. Thus, available compounds often fail to exhibit sufficient efficacy and selectivity. The rapidity of the growth of xenograft tumor (usually doubling size in 2-5 days) may be hard to suppress or to shrink. In addition, the immunodeficient nature of nude mice allows the regrowth of tumor from minute numbers of tumor cells remaining after therapy. In this study, we carried out experiments using Fludelone and used special strategies (such as 6-hour i.v. infusion techniques and extensive optimization of dose and schedule) to overcome these difficulties.

In earlier reports, we have applied the term "curative effect" to indicate the achievement of "complete remission" of xenograft tumors. Significant effort is still necessary to progress from demonstration of curative effect to documentable cures (15, 16). In this report, we advance from curative effects to de facto cures of xenograft tumors (e.g., complete remission without a relapse for over 4 months or >15% of the life span of the nude mice). Indeed, our longest xenograft experiment, including the follow-up, lasted 300 days.
We regard the findings of de facto cure for over 8 months (about one third of mouse life span) without a relapse to be extremely promising and, indeed, virtually unprecedented in experimental cancer chemotherapeutics. Among the remarkable features of Fludelone are as follows: (a) cures of extra large tumors with size as large as 3.4% of body weight; (b) over 6- to 8.4-month long-term complete tumor remission without a relapse for xenograft tumors from different human organ origins; (c) cure via p.o. route of administration; (d) complete remission in paclitaxel-resistant tumors; and (e) low toxicity and broad therapeutic safety margin, achieving complete tumor remission at a dose of one third of maximal tolerated dose; and (f) a new formulation based on ethanol-Tween 80 (i.e., Cremophor-free). For comparative purposes, concurrent studies on Taxol and on compounds closely related to Fludelone were conducted.

Materials and Methods

Chemicals. All epothilones were synthesized in-house as indicated in the text. Paclitaxel (Taxol) and vinblastine sulfate were purchased from Sigma (St. Louis, MO). All these compounds were dissolved in DMSO for the in vitro assays (except vinblastine in saline). For in vivo studies, all epothilones and paclitaxel were dissolved in ethanol/Cremophor (1:1) vehicle and then diluted with saline for the 6-hour i.v. infusion. For some experiments, Fludelone was dissolved in ethanol/Tween 80 (1:1) and diluted with saline for i.v. injection, i.v. infusion, or p.o. administration.

Tumor and cell lines. The CCRF-CEM human lymphoblastic leukemia cells and their vinblastine-resistant subline (CCRF-CEM/vinblastine100, 720-fold resistance in vitro) were obtained from Dr. William Beck (University of Illinois, Chicago, IL), and CCRF-CEM/Taxol cells (44-fold resistance in vitro) were obtained by exposing CCRF-CEM cells to increasing sublethal concentration (IC50-IC90) of paclitaxel for 6 months. The degrees of resistance are shown in Table 1. Human lung carcinoma cells (A549), human colon carcinoma (HCT-116), human ovarian adenocarcinoma (SK-OV-3), and human prostate adenocarcinoma (PC-3) were obtained from American Type Culture Collection (Rockville, MD). Human mammary carcinoma (MX-1) tumor cells were obtained from Memorial Sloan-Kettering Cancer Center cell bank.

Animals. Athymic nude mice bearing the nu/nu gene were obtained from National Cancer Institute (Frederick, MD) and used for all human tumor xenografts. Male or female nude mice 6 weeks weighing 20 to 24 g or more were used. Drugs were administered via the tail vein for 6 hours by i.v. infusion using a custom-designed infusion minicatheter and containment device (15, 16). A programmable Harvard PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) with multitrack was used for i.v. infusion. A typical 6-hour infusion volume for each drug in Cremophor/ethanol (1:1) was 100 μL in 2.0 mL saline. Tumor volume was assessed by measuring length × width × height (or width) by using a caliper. Figure 1. A, chemical structures of Taxol and some selected epothilones. B, microtubule stabilization activity of epothilones relative to Taxol at 37°C and 4°C. Columns, means of duplicate or triplicate experiments. C, plasma concentration of epothilone in nude mice following i.v. bolus injection or 6-hour i.v. infusion using a representative epothilone, 30 mg/kg dEpoB, for experimental illustration. Data on the left (i.e., 0-24 hours) are experimental results, whereas those in the middle (24-48 hours) and on the right (48-72 hours) are transposed from the 0 to 24-hour data. This is done to indicate the changes in susceptibility to neurotoxicity (e.g., paralysis in hind legs) or lethality following repeated bolus i.v. injections (e.g., qd or q2d). In contrast, the 6-hour i.v. infusion (30 mg/kg) can be repeated many times (e.g., 6-10 times q2d) without evoking toxicities and yet exerts marked therapeutic effects. Both dEpoB and Fludelone (at 30 mg/kg) induced similar neurotoxicity and lethal toxicities following repeated bolus i.v. injections, but these toxicities can be avoided by using repeated 6-hour i.v. infusions. Our results with dEpoB or Fludelone indicated that bolus i.v. injection for each compound is over 10-fold more toxic than the 6-hour i.v. infusion in terms of cumulative total doses (see text for details). The IC50 and IC95 values given are from in vitro data.
weight refers to total weight minus the weight of the tumor. Solutions of both Fludelone and Taxol for p.o. administration were prepared by dissolving the compound in ethanol and suspending with an equal volume of Tween 80. The suspension was diluted with 5 volumes of saline before administration to nude mice. Taxol solution should be used within 5 minutes to avoid precipitation. The gavage was carried out using a 1 ml syringe and a gage 22 ball-tipped animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY). All animal studies were conducted in accordance with the guidelines for the NIH Guide for the Care and Use of Animals and the protocol approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center.

Cytotoxicity assays. In preparation for in vitro cytotoxicity assays, cells were cultured at an initial density of 2 \( \times 10^{4} \) to 5 \( \times 10^{4} \) per milliliter. They were maintained in a 5% CO2-humidified atmosphere at 37 °C in RPMI 1640 ( Gibco BRL, Carlsbad, CA) containing penicillin (100 units/mL), streptomycin (100 µg/mL; Life Technologies), and 5% heat-inactivated fetal bovine serum. For cells grown in suspension (such as CCRF-CEM and its sublines), cytotoxicity was measured in duplicate by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) microculture method (19) in 96-well microtiter plates. For solid tumor cytotoxicity assays, cells growing in a monolayer (such as HCT-116 and A549), cytotoxicity of each well was measured with a microplate reader (Power Wave XS, Bio-Tek). Tubulin, 1 mg, in 200 µL of buffer [0.1 mol/L MES, 1 mmol/L EGTA, 0.5 mmol/L MgCl2, 0.1 mmol/L EDTA, and 2.5 mol/L glycerol (pH 6.5)] and with 10 µmol/L of drug (final concentration of 10 µmol/L) was used. For assembly, incubation was carried out at 35 °C for 40 minutes, and for disassembly of the same samples the incubation was carried out at 4 °C for 40 minutes. Absorbance at 350 nm was measured for the microtubule stabilization. Solvent blank (DMSO) was subtracted from the absorbance.

Stability of microtubule formation by epothilones and Taxol. Microtubules formed in the presence of 10 µmol/L Taxol were defined as 100%. Tubulin from bovine brain was a product of Sigma and the tubulin assembly assay was carried out according to the specifications of the manufacturer. Tubulin, 1 mg, in 200 µL was incubated with 790 µL of buffer [0.1 mol/L MES, 1 mmol/L EGTA, 0.5 mol/L MgCl2, 0.1 mol/L EDTA, and 2.5 mol/L glycerol (pH 6.5)] and with 10 µmol/L of drug (final concentration of 10 µmol/L). For assembly, incubation was carried out at 35 °C for 10 minutes, and for disassembly of the same samples the incubation was carried out at 4 °C for 40 minutes. Absorbance at 350 nm was measured for the microtubule stabilization.

### Table 1. Potency of epothilones against tumor cell growth in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nmol/L)</th>
<th>Human T-cell lymphoblastic leukemia sublines</th>
<th>Human lung carcinoma A549</th>
<th>Human colon carcinoma HCT-116</th>
<th>Human mammary carcinoma MX-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>dEpoB</td>
<td>5.6 ± 2.8</td>
<td>8.5 ± 5.6 (1.5×)</td>
<td>3.9 ± 0.4</td>
<td>6.8 ± 3.2</td>
<td>6.2 ± 1.7</td>
</tr>
<tr>
<td>dEpoF</td>
<td>1.5 ± 0.1</td>
<td>6.6 ± 3.1 (4.4×)</td>
<td>12 ± 4</td>
<td>3.4 ± 0.6</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>EpoB</td>
<td>0.53 ± 0.17</td>
<td>11.1 ± 0.08 (2.1×)</td>
<td>0.8 ± 0.5</td>
<td>0.38 ± 0.01</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>15-Aza-EpoB</td>
<td>2.4 ± 0.3</td>
<td>103 ± 68 (43×)</td>
<td>4.0 ± 3.5</td>
<td>1.4 ± 0.09</td>
<td>49.8 ± 3.0</td>
</tr>
<tr>
<td>deH-dEpoB</td>
<td>0.9 ± 0.4</td>
<td>1.2 ± 0.6 (1.3×)</td>
<td>0.89 ± 0.64</td>
<td>0.94 ± 0.35</td>
<td>19 ± 0.1</td>
</tr>
<tr>
<td>deH-dEpoF</td>
<td>0.51 ± 0.09</td>
<td>1.7 ± 1.0 (3.3×)</td>
<td>0.91 ± 0.06</td>
<td>0.56 ± 0.06</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>15-Aza-deH-dEpoB</td>
<td>0.23 ± 0.02</td>
<td>0.8 ± 0.64 (4.2×)</td>
<td>0.01 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.67 ± 0.11</td>
</tr>
<tr>
<td>F10-deH-EpoB</td>
<td>3.2 ± 0.3</td>
<td>4.7 ± 1.0 (1.5×)</td>
<td>3.7 ± 2.4</td>
<td>5.6 ± 1.0</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>16(z)-F2-deH-EpoB</td>
<td>2.040 ± 0.200</td>
<td>4.850 ± 1.560 (2.4×)</td>
<td>1.930 ± 210</td>
<td>2.370 ± 410</td>
<td>3.590 ± 530</td>
</tr>
<tr>
<td>F2-deEpoF</td>
<td>1.3 ± 0.3</td>
<td>0.6 ± 0.7 (5×)</td>
<td>0.9 ± 0.01</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>F2-deEpoB</td>
<td>9.3 ± 5.2</td>
<td>18 ± 1 (1.9×)</td>
<td>15 ± 4</td>
<td>12 ± 1</td>
<td>22.9 ± 0.9</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.8 ± 0.5</td>
<td>7.9 ± 29 (43.9×)</td>
<td>2.9 ± 0.3</td>
<td>2.6 ± 0.9</td>
<td>28.6 ± 4.2</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.54 ± 0.09</td>
<td>19.6 ± 11 (36.3×)</td>
<td>9.9 ± 1.8</td>
<td>8.7 ± 1.8</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>

*Cell growth inhibition for leukemia cells and solid tumor cells were measured by XTT tetrazolium assay and by sulfurhodamine B method, respectively, after 72-hour incubation for cell growth as described previously (15, 16). IC50 values were determined from dose-effect relationship at six or seven concentrations of each drug in duplicate by using a computer program (23) as described earlier (13). Value ranges given are the mean ± SE for two to five experiments except when otherwise indicated for a single experiment.

†The numbers in parentheses are fold of resistance based on the IC50 ratio when compared with the corresponding parent cell lines. CCRF-CEM/Vinblastine and CCRF-CEM/Taxol are the CCRF-CEM leukemic cells resistant to vinblastine and paclitaxel, 720-fold and 44-fold, respectively.
2.6 minutes, and the retention times (dEpoF, Fludelone, dEpoB, and F3-dEpoB, in this order, have IC50 11.13, 14.87, 19.68, and 20.78 minutes, respectively. The increased by trifluorination (Table 2). Reduced the potency. However, metabolic stability is greatly increased by trifluorination (Table 2). (b) Most epothilones are not cross-resistant with vinblastine, a typical substrate for the P-glycoprotein of multidrug resistance (MDR), nor are they cross-resistant with Taxol, a substrate for the MDR phenotype (Table 1), but Taxol resistance may also be generated by mutation in the \textit{tubulin} gene. The exceptions are 15-aza-EpoB and 15-aza-deH-dEpoB, which show considerable cross-resistance to both vinblastine and paclitaxel (Table 1). dEpoF and its derivative showed some cross-resistance with vinblastine but not with paclitaxel. (c) Fludelone, deH-dEpoB, and deH-EpoB are only slightly cross-resistant to vinblastine and virtually not cross-resistant to paclitaxel (Table 1).

At the present time, dEpoB, deH-dEpoB, and Fludelone have been selected to be the lead compounds for clinical developments. They are not among the most potent epothilones and none of them have an epoxide moiety at the C12-13 position.

**Results**

Structure-activity relationships \textit{in vitro} against human leukemic, Taxol- and vinblastine-resistant leukemic cells, and solid tumor cells. The potencies presented in terms of IC50 (in nmol/L) of 12 representative epothilones against the growth of human leukemic CCRF-CEM cells and their sublines resistant to vinblastine (CCRF-CEM/vinblastine) and resistant to paclitaxel (CCRF-CEM/Taxol) are shown in Table 1. Also listed are the IC50 values against three human solid tumor cell lines—lung carcinoma A549, colon carcinoma HCT-116, and mammary carcinoma MX-1. In \textit{vivo}, dEpoB, deH-dEpoF, EpoB, and deH-deEpoB, in this order, have subnanomolar IC50 values, whereas F3-deH-dEpoB, dEpoF, Fludelone, deH-dEpoB, and F3-dEpoB, in this order, have IC50 values ranging from 1.3 to 9.3 nmol/L against CCRF-CEM (Table 1). The following results are also shown: (a) 9,10-Dehydro modification on dEpoB, dEpoF, or F3-dEpoF always resulted in marked increase in potency, whereas trifluorination on the C-26 position somewhat reduced the potency. However, metabolic stability is greatly increased by trifluorination (Table 2). (b) Most epothilones are not cross-resistant with vinblastine, a typical substrate for the P-glycoprotein of multidrug resistance (MDR), nor are they cross-resistant with Taxol. Taxol is a substrate for the MDR phenotype (Table 1), but Taxol resistance may also be generated by mutation in the \textit{tubulin} gene. The exceptions are 15-aza-EpoB and 15-aza-deH-dEpoB, which show considerable cross-resistance to both vinblastine and paclitaxel (Table 1). dEpoF and its derivative showed some cross-resistance with vinblastine but not with paclitaxel. (c) Fludelone, deH-dEpoB, and deH-EpoB are only slightly cross-resistant to vinblastine and virtually not cross-resistant to paclitaxel (Table 1).

The experimental procedures for epothilones are against human xenografts in nude mice, such as MX-1 (see Fig. 2). The potencies presented in terms of IC50 values against three human solid tumor cell lines—lung carcinoma A549, colon carcinoma HCT-116, and mammary carcinoma MX-1. In \textit{vivo}, dEpoB, deH-dEpoF, EpoB, and deH-deEpoB, in this order, have subnanomolar IC50 values, whereas F3-deH-dEpoB, dEpoF, Fludelone, deH-dEpoB, and F3-dEpoB, in this order, have IC50 values ranging from 1.3 to 9.3 nmol/L against CCRF-CEM (Table 1). The following results are also shown: (a) 9,10-Dehydro modification on dEpoB, dEpoF, or F3-dEpoF always resulted in marked increase in potency, whereas trifluorination on the C-26 position somewhat reduced the potency. However, metabolic stability is greatly increased by trifluorination (Table 2). (b) Most epothilones are not cross-resistant with vinblastine, a typical substrate for the P-glycoprotein of multidrug resistance (MDR), nor are they cross-resistant with Taxol. Taxol is a substrate for the MDR phenotype (Table 1), but Taxol resistance may also be generated by mutation in the \textit{tubulin} gene. The exceptions are 15-aza-EpoB and 15-aza-deH-dEpoB, which show considerable cross-resistance to both vinblastine and paclitaxel (Table 1). dEpoF and its derivative showed some cross-resistance with vinblastine but not with paclitaxel. (c) Fludelone, deH-dEpoB, and deH-EpoB are only slightly cross-resistant to vinblastine and virtually not cross-resistant to paclitaxel (Table 1).

At the present time, dEpoB, deH-dEpoB, and Fludelone have been selected to be the lead compounds for clinical developments. They are not among the most potent epothilones and none of them have an epoxide moiety at the C12-13 position.

**Physicochemical, metabolic, and pharmacologic properties and therapeutic results of epothilone derivatives.** Interrelating the property profiles of a series of selected nine epothilones facilitates the understanding of factors that contribute to the therapeutic end results (Table 2). The microtubule stabilization potency of these nine compounds are quite similar to Taxol, except F3-deH-dEpoB, which is considerably weaker than Taxol (Fig. 1B). It is notable that our lead compounds, Fludelone, deH-dEpoB, and dEpoB all have high lipophilicity as indicated by the octanol-water partition. Water solubility and lipophilicity play varied roles in mitigating the observed therapeutic effect and are important for the design of formulation.

EpoB and 9,10-deH-EpoB are the most potent epothilones known in \textit{vivo} (Table 1) and also in \textit{vivo} (Table 2), but they do not yield the best therapeutic safety window (Table 2). Apparently, epoxide moieties at C12-C13 of EpoB and deH-EpoB contribute

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**Table 2.** Physicochemical and metabolic properties, and pharmacologic and therapeutic profiles of epothilone derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxic efficacy IC50* (nmol/L)</th>
<th>Microtubule stabilization potency at 37°C (Taxol as 100%)</th>
<th>Stability t1/2 in mouse plasma (^1) (min)</th>
<th>Stability t1/2 in human liver S9 fraction (^1) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpoB</td>
<td>0.53 ± 0.2</td>
<td>105</td>
<td>57</td>
<td>15.8</td>
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<tr>
<td>deH-EpoB</td>
<td>0.23 ± 0.02</td>
<td>95.1</td>
<td>38 ± 20</td>
<td>12.0</td>
</tr>
<tr>
<td>dEpoB</td>
<td>5.6 ± 2.8</td>
<td>106 ± 6.6</td>
<td>46 ± 7</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>deH-dEpoB</td>
<td>0.90 ± 0.40</td>
<td>105 ± 0.9</td>
<td>84 ± 6</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td>F3-dEpoB</td>
<td>9.3 ± 5.2</td>
<td>84.4</td>
<td>66 ± 7</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>F3-deH-dEpoB</td>
<td>3.2 ± 0.3</td>
<td>121</td>
<td>212 ± 88</td>
<td>10.5 ± 2.3</td>
</tr>
<tr>
<td>dEpoF</td>
<td>1.5 ± 0.1</td>
<td>97.3</td>
<td>48 ± 3</td>
<td>6.9 ± 0.8</td>
</tr>
<tr>
<td>deH-dEpoF</td>
<td>0.51 ± 0.09</td>
<td>96 ± 0.9</td>
<td>185 ± 15</td>
<td>14.5 ± 0.6</td>
</tr>
<tr>
<td>F3-deH-dEpoF</td>
<td>1.3 ± 0.3</td>
<td>97.8</td>
<td>403 ± 27</td>
<td>18.5 ± 0.2</td>
</tr>
</tbody>
</table>

Note: Measurements are described in Materials and Methods. Data are for single determination and the ranges shown are averages of two experiments or mean ± SD for n = 3 to 5.

*IC50 values are for CCRF-CEM leukemic cells. All values are obtained from eight data points based on the median effect plot (21) using a CalcuSyn software (23).

1 Plasma was diluted 2-fold with PBS.

2 Human liver microsomal S9 fraction was diluted 15-fold with PBS.

Graded relative therapeutic efficacy index at maximal tolerated dose: +, tumor growth suppressed by 25% to 50%; ++, tumor growth suppressed by 50% to 100%; +++, tumor shrinkage but no tumor disappearance; +++++, tumor disappearance in some or all nude mice with relapse in some mice within 4 months after tumor remission; ++++++, tumor disappearance in all nude mice with rapid body weight recovery and without relapse for over 4 months. The experimental procedures for epothilones are against human xenografts in nude mice, such as MX-1 (see Fig. 2).
greatly to the host toxicity as evidenced by the lower value in the decrease in maximal body weight percentage without death (Table 2). By contrast, Fludelone and dEpoB tolerated the highest values of body weight loss and rapid recovery, suggesting that the observed toxicity is not associated with damage to the vital organs. These drugs exhibited excellent therapeutic results, such as complete tumor remission or nonrelapsing real “cure.” Of special interest is that Fludelone exhibits a most impressive curative therapeutic dose range (10-30 mg/kg; Fig. 2A; ref. 18) and excellent metabolic stability and provides the best overall therapeutic results among the epothilones evaluated (Table 2). Furthermore, the finding that Fludelone provides a de facto cure via p.o. route of administration (see Fig. 4A) is highly encouraging. In general, we surmise that the epothilones lacking the 12,13-epoxide linkage, although less potent, seem to be displaying the more exploitable therapeutic indices. Of these, Fludelone is a particularly promising candidate for further development.

Dose, schedule, and route of administration in relation with toxicity and therapeutic effect. In the present studies, therapeutic effects were evaluated at or below the maximal tolerated dose. Even at these dosages, toxic effects, such as body weight decreases in the nude mice, can be reversed. Optimization in terms of schedule and route of administration played critical roles in achieving optimum therapeutic effects (see Fig. 1C). For both dEpoB and Fludelone, we found that bolus i.v. injection was undesirable because it led to a high spike in plasma concentration. Although single doses led to no significant toxicity, repeated bolus dosing (q2d × 2 or q4d × 2) led to delayed/aggravated neurotoxicity, such as paralysis of hind legs (in 3-5 days) or even death (in 4-10 days). These results suggest a decreasing threshold for toxicities following the first bolus injection. There was no evidence to suggest that the increased toxicity was due to drug accumulation (Fig. 1C). Toxic effects can be completely avoided through the use of 6-hour i.v. infusion via the tail vein (e.g., short term treatments (<10 doses) or cyclized treatments with resting periods). The plasma concentration spike at 2 to 3 minutes after 30 mg/kg bolus injection (~48,000 nmol/L) is ~53-fold higher than the steady-state concentration of 900 nmol/L during the 6-hour i.v. infusion (Fig. 1C). The 900 nmol/L concentration is ~160- to 230-fold of IC50 and 20- to 40-fold of IC5 for dEpoB and Fludelone in vitro. The high spike of 48,000 nmol/L might induce injuries on nervous and/or other organs that aggravate the toxicities during the subsequent bolus i.v. injections. The IC50 shown in Fig. 1C is based on the MX-1, HCT-116, and CCRF-CEM cell growth/cell kill data in vitro given in Table 1, whereas IC50 in vitro is determined by the median-effect equation and plot (21, 22) using a computer software (23). It will be noted that dEpoB and Fludelone (Tables 1 and 2) exhibit a similar range of property parameters in terms of the following: (a) in vitro cytotoxicity (e.g., IC50 values of 6.2 versus 3.8 nmol/L against MX-1 cells and 6.8 nmol/L versus 5.6 nmol/L against HCT-116 cells), (b) microtubule stabilization activity (106% versus 121% relative to Taxol), (c) solubility in water (10.7 versus 19 μg/mL), (d) lipophilicity (4.4 versus 3.3 octanol-water partition), (e) optimal therapeutic dose for 6-hour i.v. infusion (30 versus 10-30 mg/kg), and (f) maximal decrease in body weight during therapy without death (32% versus 33%; see Table 2). On the other hand, important differences between dEpoB and Fludelone are as follows: (a) stability in 2-fold diluted nude mouse plasma in vitro (t1/2 of 46 minutes and 212 minutes), (b) stability in liver microsomal S8 fraction (1 versus 10.5 hours), (c) therapeutic results against nude mice xenografts (many cases of complete remissions versus many cases of de facto cure, i.e., complete remission without a relapse for over 15% of life span; see Table 2), and (d) oral administration of dEpoB had little effect, whereas Fludelone led to the real cure. Based on the above observations, all the therapeutic evaluations for the epothilones in nude mice were carried out with 6-hour i.v. infusion schedules. A few lead compounds that showed stability in plasma and S8 fraction were also evaluated via p.o. administration. Overall, i.v. bolus injection of dEpoB or Fludelone is ~10- to 10-fold more neurotoxic or lethal than the 6-hour i.v. infusion in terms of cumulative total dose. Six-hour i.v. infusion of Fludelone led to complete “tumor remission” or “real cure” in many xenograft tumors, including those that are resistant to Taxol, as shown in Figs. 2 and 3. The cure by p.o. administration (see Fig. 4A), and the complete remission by 6-hour i.v. infusion of Fludelone in Cremophor-free formulation (see Fig. 2B) certainly serve to highlight potential important advantages of Fludelone as an anticancer agent.

### Table 2. Physicochemical and metabolic properties, and pharmacologic and therapeutic profiles of epothilone derivatives (Cont’d)

<table>
<thead>
<tr>
<th>Solubility in water (μg/mL)</th>
<th>Lipophilicity octanol-water partition</th>
<th>Therapeutic profile in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Maximal body weight percentage decrease without death (%)</td>
</tr>
<tr>
<td>353</td>
<td>2.1</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td>410</td>
<td>2.0</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>10.7 ± 1.3</td>
<td>4.4 ± 0.1</td>
<td>25-30</td>
</tr>
<tr>
<td>26.5 ± 0.5</td>
<td>3.4 ± 0.1</td>
<td>3-4</td>
</tr>
<tr>
<td>8</td>
<td>4.1 ± 0.1</td>
<td>15-20</td>
</tr>
<tr>
<td>19 ± 1.0</td>
<td>3.3 ± 0.1</td>
<td>10-30</td>
</tr>
<tr>
<td>27</td>
<td>2.4</td>
<td>30</td>
</tr>
<tr>
<td>200</td>
<td>2.0 ± 0.1</td>
<td>2-4</td>
</tr>
<tr>
<td>110</td>
<td>2.0 ± 0.1</td>
<td>25-30</td>
</tr>
</tbody>
</table>

Cure of extra large MX-1 tumor xenografts by Fludelone. As shown in Fig. 2A (top), treatment of MX-1 xenografts as large as 3.4% of body weight with Fludelone (25 mg/kg, 6-hour i.v. infusion q3d × 5 beginning at day 22 after tumor implantation) led to marked tumor shrinkage (>97.4%). During resting periods as long as
as 9 days without treatment (days 34-43), tumor sizes continued to shrink (>99.3%) and the tumor disappeared in two of five mice studied, whereas body weight of the treated group recovered to the pretreatment level during the same resting period (Fig. 2, middle). Resumption of treatment on day 43, q3d/C2,4, led to tumor disappearance in the remaining three mice on days 50, 50, and 51, respectively. After day 52, the date of the last doses, the animals were evaluated every 3 days until day 240 when the experiment was terminated. There was no tumor relapse in any of the five animals on day 240, which was over 6.3 months after cessation of treatment.

Photographs of the nude mice of this experiment taken on days 25, 31, 37, 43, and 52 of one mouse each from the control and treated groups are shown in Fig. 2A (bottom).

Complete remission or marked suppression of human ovarian SK-OV-3 and prostate PC-3 adenocarcinoma xenografts by Fludelone. We previously reported that Taxol was superior to many other anticancer agents in treating two xenografts, SK-OV-3 and PC-3 (16). In the present studies, both xenografts were treated with Taxol (25 mg/kg), Fludelone (25 mg/kg), and deH-dEpoB (6 mg/kg), q3d, 6-hour i.v. infusion beginning at days 10 and 11 after tumor implantation (Figs. 2B and C). For Fig. 2B, Fludelone used ethanol/Cremophor (1:1) formulation in three mice and ethanol/Tween 80 (1:1) formulation in two mice. Both Taxol [all used ethanol/Cremophor (1:1) formulation] and Fludelone [some used ethanol/Tween 80 (1:1) formulation] achieved complete remission virtually at the same time. For SK-OV-3, remission occurred on day 33.3 and 31.8, respectively; and for PC-3 on day 45.2 for both drugs, respectively. Fludelone and Taxol treatments were suspended immediately after complete remission for SK-OV-3 xenografts and 12 days before the complete remission of all five mice for PC-3 xenografts. For SK-OV-3 xenografts, neither the Taxol- nor the Fludelone-treated group had relapsed on day 136. For PC-3 xenografts, two of the five Fludelone-treated mice had relapsed on days 86 and 97 (i.e., 1.7 months after tumor remission). By contrast, Taxol-treated mice had relapsed on day 86 (i.e., 39 days after remission for that specific nude mouse). No further relapse was observed for either treated group on day 123. Clearly, complete remission does not necessarily mean cure. However, if consolidation treatments were made shortly after remission (such as those in Fig. 4A), cure would likely have occurred. The treatments with deH-dEpoB, 6 mg/kg, q3d, 6-hour i.v. infusion, showed much more...
toxicity than Taxol or Fludelone as indicated by body weight loses at much reduced doses (6 mg/kg) but with only moderate tumor suppression (Figs. 2B and C). It was found later that the optimal treatments for deH-dEpoB 6-hour i.v. infusion were best at q6d or q8d, at somewhat higher doses (e.g., 8-10 mg/kg) than at 6 mg, q3d schedule of 6-hour i.v. infusion. It is of interest to note that as PC-3 and HCT-116 xenografts grow, body weight decreases in the control group (Figs. 2C, bottom and D, bottom). These body weight decreases were not observed with MX-1, SK-OV-3, CCRF-CEM/Taxol, or A549/Taxol xenografts.

Experiments shown in Fig. 2B (for SK-OV-3 xenografts) represent the most intensive treatments in this paper for both Taxol and Fludelone (i.e., 25 mg/kg, 6-hour i.v. infusion q3d for a total of 12 doses without a rest). Prolonged follow-up observations for over 3 months following the cessation of treatments revealed a chronic toxicity (e.g., weakness or paralysis of hind legs) for both Taxol (on days 70 and 112) and Fludelone (on days 88 and 136) in two of five nude mice in each group. These results suggest that the prolonged intensive treatments may lead to delayed chronic toxicities. The injuries caused by the prolonged intensive treatments may be difficult to repair and may last a long period of time. All other experiments in this paper with long period of follow-up, including those achieving cure (Figs. 2A, 2D and 4A), did not show any delayed chronic neurotoxicity.

Cure of human colon carcinoma HCT-116 xenografts by Fludelone. As shown in Fig. 2D (top), treatment of nude mice bearing HCT-116 xenografts with Fludelone (20 mg/kg and 30 mg/kg) or Taxol at 20 mg/kg, q2d × 4, 6-hour i.v. infusion for three cycles led to tumor disappearance in four of four mice. The treatment started on day 9 after tumor implantation. There was a one-dose skip on day 17 between the first and second cycles and there was a two-dose skip on days 27 and 29 between the second and third cycles. Days 9 to 37 were the three-cycle therapy period; days 37 to 200 were the follow-up period. The experiment lasted 200 days, which constitutes more than a quarter of the average life span of the mice. For Fludelone at 30 mg/kg, tumors disappeared on days 21, 23, 33, and 41 (29.5 ± 8.0), and at 20 mg/kg, tumor disappeared on days 31, 35, 41, and 45 (38.0 ± 5.4). For both Fludelone doses, there was no tumor relapse in four of four mice on day 200 when the experiment was terminated. For Taxol at 20 mg/kg, tumor disappeared on days 33, 33, 41, and 45 (38.0 ± 5.2), which was

\(^5\) T-C. Chou et al., unpublished results.

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**Figure 2 Continued.** C, PC-3 (prostate, n = 4). D, HCT-116 (colon, n = 4). Arrows, Fludelone infusions. Doses and schedules are shown. Data of tumor disappearance and relapse (if any) are indicated. The length of experiments or follow-up (if any) in days are as shown. Points, mean; bars, SD. The control group, nude mice with excessive tumor burden (e.g., tumor size >10% body weight or tumor necrosis), was sacrificed. For side-by-side comparisons, Taxol was also used in (B) to (D), and 9,10-dEpoB was also used in (B) and (C).
similar to the observation with Fludelone at 20 mg/kg. However, in the Taxol-treated group, tumor relapse occurred on days 71, 75, 81, and 81 (77.0 ± 4.2). These results indicate that the HCT-116 tumor can relapse even 1.3 months after the tumor remission.

The schedule of the treatment, including the rest period, is dictated by the body weight decreases and the physical conditions of the mice. For the Fludelone treatment at 30 mg/kg dose, the maximal body weight loss was 30%, which occurred at the end of the second cycle of treatment (i.e., day 29; Fig. 2D, bottom). The large magnitude of body weight decrease without death is regarded as an important and favorable feature because vital organs were apparently not affected at or near the maximal tolerated dose. The body weight decreases and recoveries were similar to the 20 mg/kg Taxol and 20 mg/kg Fludelone in both pattern and magnitude.

Complete remission induced by Fludelone against Taxol-resistant CCRF-CEM/Taxol and marked suppression of A549/Taxol xenografts. As shown in Fig. 3A, we evaluated the therapeutic effect of Fludelone against paclitaxel-resistant xenograft, CCRF-CEM/Taxol (44-fold resistance to paclitaxel in vitro). Fludelone at 30 mg/kg q2d × 7, 6-hour i.v. infusion, lead to tumor disappearance in two of four mice. Additional q2d × 5 (after skipping one dose) lead to tumor disappearance in three of four mice, with the final tumor suppression of 99.8% (Fig. 3A, top). At the reduced dose of 15 mg/kg, tumor disappearance occurred in only one of four mice on the fifth day after the two cycle treatments. The final tumor suppression on day 34 was 98.8%. Parallel experiments with Taxol at 20 mg/kg yielded tumor growth suppression but with little or no tumor shrinkage. The final tumor suppression on day 34 was 75.6%.

Both Fludelone (at 15 and 30 mg/kg) and Taxol (20 mg/kg) treatment beginning day 8 persistently reduced the body weight during the first cycle of seven treatments via 6-hour i.v. infusion every other day. Skipping one treatment on day 22 led to regaining of body weight in all mice. The second cycle of treatment of q2d × 5 again led to persistent reduction of the body weight but without lethality to all mice tested (Fig. 3A, bottom).

The Taxol-resistant human lung carcinoma xenografts (A549/Taxol, 5-fold resistant in vitro) were treated with Fludelone (25 mg/kg), deH-dEpoB (5 mg/kg), and Taxol (25 mg/kg), q3d × 11, 6-hour i.v. infusion, beginning at day 18 after tumor implantation (Fig. 3B). At the end of the treatments (i.e., day 48), tumor growth was suppressed by 93%, 88%, and 86%, respectively. These results in persistent marked suppression but did not yield tumor shrinkage or disappearance (Fig. 3B, top). At the end of treatments (day 48), body weight decreased by 23%, 13%, and 18%, respectively. Upon cessation of treatment for 9 days (day 57), decreases in body weight were reduced to 6%, 1%, and 14%, respectively (Fig. 3B, bottom). These results indicate that the body weight recovery performance is better in deH-dEpoB and Fludelone than for Taxol.

Cures in MX-1 xenografts by Fludelone via p.o. administration. As shown in Fig. 4A, Fludelone given p.o. at 30 mg/kg every 2 days for seven times, skipped one dose, followed by nine more doses, led to shrinkage and tumor disappearances of MX-1 tumors in three of three mice. Additional five doses for consolidation treatments were given 10 days later. Follow-up observations up to day 300 revealed no tumor relapse. By contrast, p.o. treatment of Taxol at the same dose and the same schedule suppressed MX-1 tumor growth only moderately and did not lead to any tumor shrinkage. Fludelone treatment induced moderate yet persistent decreases in body weight with maximal decrease of 17% of the body weight (Fig. 4A, bottom). Taxol treatment induced only small changes in body weight, suggesting that p.o. administration of Taxol was not a suitable treatment, apparently due to drug metabolic inactivation or poor bioavailability.

Figure 3. Tumor size and body weight changes in nude mice bearing Taxol-resistant human tumor xenografts following 6-hour i.v. infusion with Fludelone. A, CCRF-CEM/Taxol (human T-cell lymphoblastic leukemia 44-fold resistant to Taxol in vitro, n = 3-4). B, A549/Taxol (human lung carcinoma, 5-fold resistant to Taxol in vitro, n = 4). See Fig. 2 for applicable legends. For side-by-side comparisons, Taxol was also used in (A) and (B) and 9,10-deH-dEpoB was also used in (B).
For comparison purposes, MX-1 xenografts were p.o. treated q2d with deH-dEpoB (8 mg/kg/C2, 6 mg/kg/C2, 40 mg/kg/C2), F3-deH-dEpoF (8 mg/kg/C2, 6 mg/kg/C2), and Capecitabine (Xeloda; 8 mg/kg/C2, 6 mg/kg/C2) beginning at day 17 after tumor implantation, as shown in Fig. 4B. Three days after cessation of treatments (day 33), tumor growth suppressions were only 48%, 27%, and 52%, for deH-dEpoB, F3-deH-dEpoF, and Capecitabine, respectively. There was no tumor shrinkage. Body weight decreases on day 33 were 24%, 16%, and 12%, respectively, from the pretreatment control.

Discussion

In this study, we showed that the structurally designed 16-membered macrolide microtubule stabilization agent, Fludelone, shrinks tumors, accomplishes tumor disappearance, and achieves complete remission or de facto cure when given by 6-hour i.v. infusion or p.o. as single-agent monotherapy. Its remarkable therapeutic spectrum encompasses leukemia as well as breast, colon, lung carcinomas, and ovary, prostate adenocarcinomas, including Taxol-resistant tumors and extra large tumors (Figs. 2-4).

The term complete remission should not necessarily be interpreted as absolute. In reality, minute amounts of tumor cells remaining after therapy are not necessarily detectable by gross inspection or microscopy. The longest remission with a relapse that we observed was 1.7 months (for PC-3 xenograft in Fig. 2C).

In this case, treatments were suspended on day 12 before the complete remission for a group of five mice.

For the HCT-116 experiment (Fig. 2D), both paclitaxel and Fludelone at 20 mg/kg were used and both achieved tumor disappearance. However, the paclitaxel-treated group relapsed at 1.3 months after tumor remission, whereas Fludelone-treated animals were tumor-free for over 7.3 months.

We propose that the log cell kill is approximately estimated by the following formula: log (cell kill) = log [2 (tumor remission in days/Tumor doubling time in days)].

Assuming the tumor doubling time of 4 days (based on the vehicle-treated control), the paclitaxel treatment of HCT-116 tumor resulted in 2.94-log cell kill (i.e., log [239/4] = 2.94), whereas the cell kill by Fludelone in MX-1 (log [240/45.8] = 14.6; Fig. 2A) and HCT-116 experiments (log [2260/380] = 16.7; Fig. 2D) would be >14.6 log and >16.7 log, respectively. Because 1 g of tumor tissue is usually composed of <10^9 cells, it is reasonable to expect that a >12 log cell kill would result in a cure. Our observations that Fludelone kills the last cancer cells of tumor as evidenced by the de facto cure suggest that Fludelone may be able to cure metastasized tumors. This possibility is currently under investigation.

The finding with Fludelone wherein tumor disappearance in all mice with no relapse for as long as >7.3 months (Fig. 2D) and the p.o. treatment without a relapse for over 8.4 months (Fig. 4A), represents, to our knowledge, the longest complete remission without a relapse that has been reported either with parenteral...
or p.o. administration that fit the definition of cure. Earlier studies on halichondrin B analogues (24) indicated that ER-086526 treatments against LOX tumor growth led to complete tumor remission (i.e., tumor-free) for 7 months in 3 of 10 mice. These results are closely comparable with our results presented here.

The long-term experiments on Fludelone, 240 to 300 days in the present studies (Figs. 2A, D and 4A), indicate that neither chronic or delayed toxicity nor carcinogenic effect had occurred. This information is not likely available from short-term experimentation.

The achievement of tumor disappearance and cure by p.o. treatment of Fludelone is of great significance because it suggests the possibility of outpatient or at-home usage in the future. Moreover, p.o. therapy allows for avoidance of the Cremophor formulation, which, in itself, can cause severe allergic reactions. It is also well known that the use of Cremophor in the formulation in Taxol, desoxy-EpoB, and 15-aza-Epo13 induced troublesome allergic reactions. To counter these effects, pretreatment with antihistamine and/or steroid can be required. However, our results also indicate that ethanol/Tween 80 formulation for Fludelone can successfully achieved remission of xenograft tumors without using any Cremophor (Fig. 2F).

The p.o. effectiveness of Fludelone is consistent with its remarkable metabolic stability in mouse plasma and in human liver microsomal S9 fraction in vitro. This metabolic stability is apparently the consequence of trifluorination at the C-26 position also increased metabolic stability (Table 2) of the epothilones. The closeness of the optimal doses of Fludelone for i.v. infusion (20-30 mg/kg, q2d) and for p.o. administration (30 mg/kg q2d) suggests that Fludelone is well absorbed and has excellent bioavailability in vivo.

It is worth noting that many of our in vivo therapeutic studies on Fludelone (Figs. 2B-D, 3A, B, and 4A) against xenografts were carried out in parallel with Taxol, which is one of the most important cancer therapeutic agents currently in use. The superior therapeutic efficacy of Fludelone, in comparison with Taxol, further underscores the promising potential of this compound for the treatment of cancer.

Prolonged i.v. infusion (e.g., 6 hours) has the advantage of maintaining high steady-state plasma concentrations of dEpoB or Fludelone (e.g., ~900 nmol/L), which are well above the antitumor threshold but far below the toxicity threshold (Fig. 1C). By contrast, bolus i.v. injection produced a very high (e.g., ~48,000 nmol/L) but relatively transient spike of plasma concentration of the drug. The sustained steady-state concentration and the high spike concentration of dEpoB or Fludelone each produced strong antitumor effects, but the former avoided toxicities and the latter induced toxicities. The sensitization of toxicities (i.e., decreasing toxicity threshold) following bolus i.v. injection (q2d or q3d) with dEpoB or Fludelone is manifested by the observation that the divided doses induced much more toxicity than a single bolus injection within a given time frame, such as 1 week. Repeated 6-hour i.v. infusions (q2d or q3d), due to their low steady-state concentration, avoided neurotoxicity and did not appear to induce the sensitization of toxicity threshold. The 6-hour i.v. infusion, although highly demanding technically, plays an important role in the successful cure of xenograft tumors in the present studies.

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References


Therapeutic Cure against Human Tumor Xenografts in Nude Mice by a Microtubule Stabilization Agent, Fludelone, via Parenteral or Oral Route

Ting-Chao Chou, Huajin Dong, Xiuguo Zhang, et al.


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