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Review Article

Second-generation epothilones: discovery of fludelone and its extraordinary antitumor properties

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We dedicate this paper to the accomplishments of Professor E.J. Corey in the interfacing of total synthesis with drug discovery (*cf. inter alia* prostanoids, eicosanoids, omuralides, and ET-743).

CONTENTS

Abstract

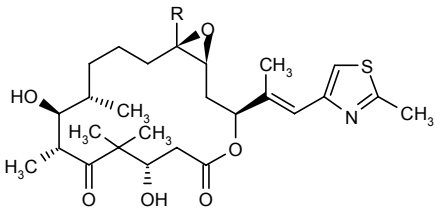
Herein, we describe the development of the second generation of epothilone derivatives, which possess impressive preclinical antitumor properties. In particular, we focus on the discovery and biological evaluation of a trifluoromethyl-9,10-dehydroepothilone derivative, termed fludelone, to which we gained access through diverted total synthesis. This review serves to highlight the role of natural product-based research combined with the power of chemical synthesis in the discovery and development of valuable drug candidates.

Introduction

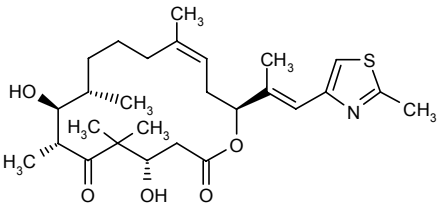
The synthesis of complex small-molecule natural products presents a unique set of challenges to the organic chemist. Such endeavors can enrich both chemistry and biology. At the level of chemistry, natural product total synthesis provides a setting in which to evaluate the scope of current methodology, to identify shortcomings in the available methodological menu, and to develop creative solutions to challenges in synthesis as they arise. Ideally, such undertakings often provide a platform upon which to demonstrate valuable new methodological advances (1-3).

The implications of total synthesis in biology can be felt in several ways. It is often difficult to obtain required quantities of natural products through the traditional methods of isolation and purification. Thus, total synthesis can play a critical role in providing access to sufficient quantities of material for extensive *in vitro* and *in vivo* biological evaluation. Furthermore, total synthesis allows entrance to the exploration of structure-activity relationships in molecular "space" that may not be accessible through partial synthesis from the natural product itself. Thus, through the process of diverted total synthesis (4), it is possible to systematically "edit" a natural product, deleting sites of potential nonspecific toxicity and manipulating functionality to improve potency and pharmacokinetic profiles.

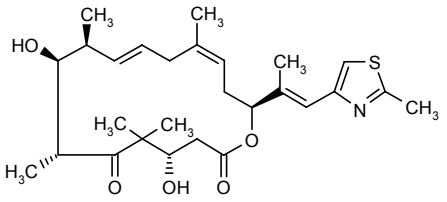
Scientists have long attempted to learn from the "wisdom" of nature in their quest to discover novel agents of medicinal value. Indeed, nature has shown itself to be an excellent resource, harboring a rich array of structurally diverse, biologically active molecular agents that serve as valuable lead candidates in drug discovery and development. The role that natural products can play in the drug discovery process is, perhaps, not surprising, when one considers that these structures may have been biosynthesized to accommodate (or optimize) the very features that are sought in medicinal agents themselves. First, although the developmental justifications for the biosynthesis of small-molecule natural product structures (SMNPS) are often mysterious, it seems likely that many such structures have evolved to possess optimal ability to selectively bind to strategic protein targets. The activation or inhibition of key proteins through selective binding is a hallmark feature of virtually every drug. Furthermore, these SMNPS have the added advantage that they have been successfully maintained in some viable host system. Clearly, it serves the medicinal chemist well to exploit the wisdom that has been accumulated by nature through countless cycles of evolution and structural optimization.



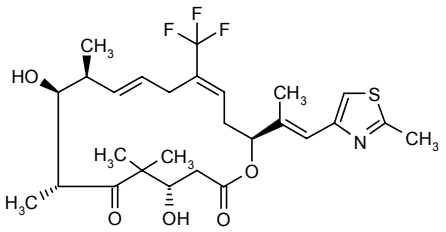
R = H, (**1**) Epothilone A (EpoA)
R = Me, (**2**) Epothilone B (EpoB)



dEpoB (**3**)



9,10-Dehydro-dEpoB (**4**)



Fludelone (**5**)

The natural product reservoir has been a particularly valuable source of antitumor agents (5). This may be explained, in part, by the fact that SMNPS possessing antitumor properties also tend to exhibit antibacterial activity. Although carcinostatic properties, per se, may not be of obvious utility for the survival of most organisms, the presence of an antibacterial agent certainly could well constitute a significant and universal evolutionary advantage. In fact, approximately 60% of the anticancer agents approved in the past 22 years have been of natural origin. This number is especially remarkable when one considers the declining level of resources invested in the development of naturally derived therapeutic agents, as many major pharmaceutical companies are increasingly directing their efforts toward high-throughput screening of massive chemical libraries. Although such standard diversity libraries may well be of some value in identifying lead therapeutic agents, the *de novo* discovery of a new molecular agent of medicinal value is a daunting task, the risks of which are virtually incalculable. The study of natural products, and the synthesis of small, intelligently designed “libraries” of natural product analogs may well prove to be of significantly more value than the random screening of huge stochastically inspired libraries in the hopes of finding a “hit” with the assistance of little or no guiding logic.

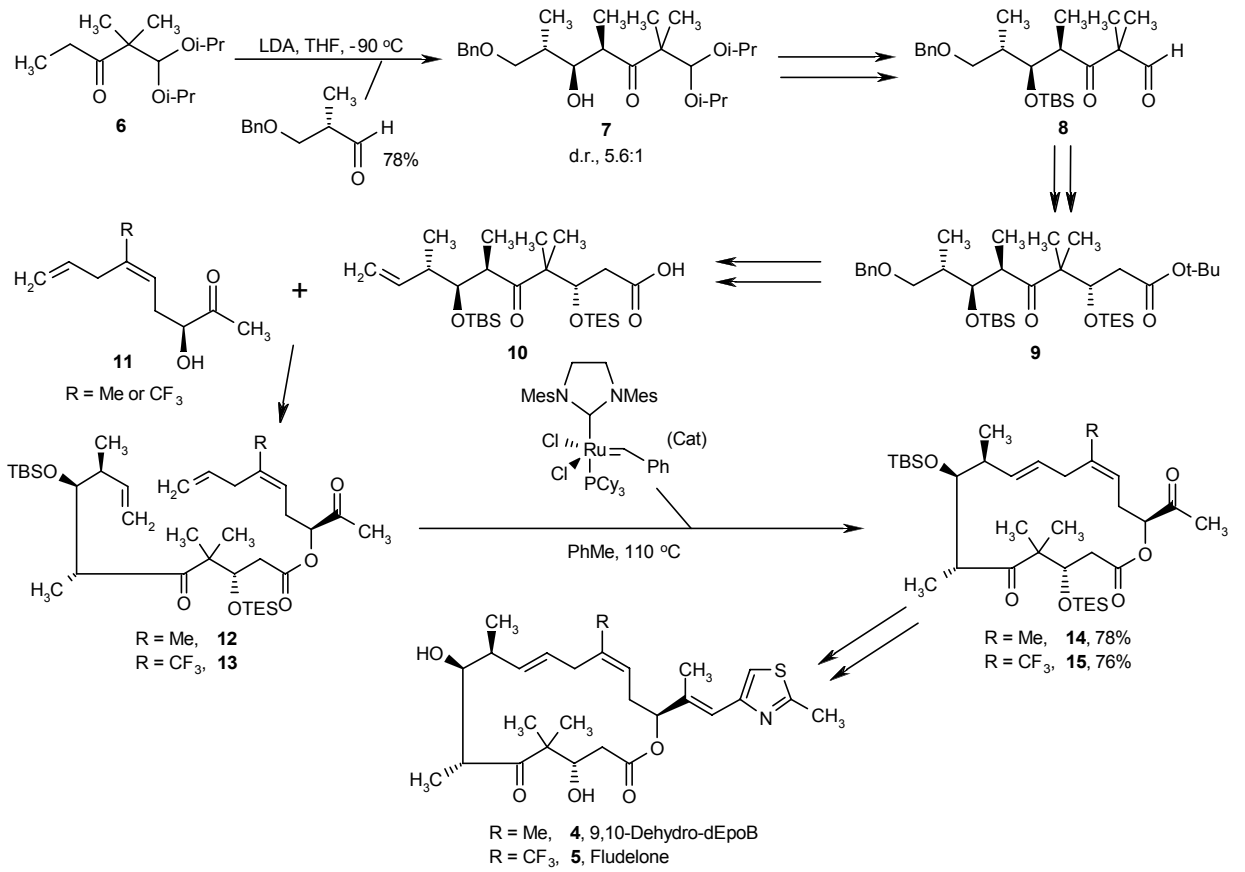
Fortunately, even in the context of the current trend favoring high-throughput screening of random sample collections, the potential of natural products in drug development has not been entirely overlooked by those interested in developing powerful new therapeutic agents. Some notable examples of naturally derived antitumor agents that are currently being evaluated in clinical settings include halichondrin (6), ET-743 (7) and the epothilone family (8).

Our interest in the epothilone family of natural products was piqued when we learned that this newly discovered class of anticancer agent appeared to function via a mechanism similar to that of paclitaxel, but retained its activity against various multidrug-resistant (MDR) cell lines (8-15). Paclitaxel, vinblastine, etoposide and adriamycin represent some of the most widely used and highly effective cancer fighting agents in clinical use. However, each of these drugs suffers in therapeutic settings from its considerable susceptibility to disablement by the onset of multidrug resistance. The epothilones, like paclitaxel, function by stabilizing the microtubules of dividing cancer cells, stalling the cell cycle in metaphase, and ultimately inducing apoptosis. However, unlike paclitaxel, the epothilones appear to be surprisingly robust as to the onset of MDR. Intrigued by both the biological opportunities and chemical challenges presented by the epothilones, we set out to design and execute synthetic routes to the natural products, epothilone A (EpoA) and epothilone B (EpoB).

First-generation epothilones

Happily, our synthetic efforts were successful. Inaugural total syntheses of EpoA (**1**) and EpoB (**2**) were reported by our laboratory in 1996 and 1997, respectively (16). Importantly, these first-generation syntheses allowed access to sufficient quantities of material for extensive *in vitro* and *in vivo* biological evaluations. The results of our *in vitro* investigations confirmed the earlier reported cytotoxicity of the epothilones, particularly EpoB, against both multidrug-sensitive and -resistant cell lines. However, despite its undeniable cytotoxicity, preliminary *in vivo* toxicology studies revealed a significant liability of EpoB. In our earliest *in vivo* investigations, doses as low as 0.6 mg/kg of EpoB administered to healthy nude mice led to significant weight loss and death (17, 18). A hypothesis emerged, to the effect that the 12,13-epoxide functionality common to the epothilones A and B might be responsible for the nonspecific toxicity of the natural product agents. This thinking led to a decision to “edit” out this 12,13-oxido linkage (19). Thus, 12,13-desoxy-epothilone B (dEpoB, **3**), an intermediate in the synthesis of EpoB

Scheme 1: Synthesis of 9,10-Dehydro-dEpoB and Fludelone



itself, was evaluated for its biological activity and *in vivo* toxicity. In fact, our suspicions apparently proved well-founded. Although dEpoB exhibits less *in vitro* potency than EpoB, it is also significantly less toxic. Most importantly, it exhibits a much broader therapeutic index in xenograft models than EpoB. In further studies, dEpoB was shown to have major advantages relative to paclitaxel (20). Notably, dEpoB retains the advantages of EpoB with respect to MDR disablement. Now available by the fermentation method, dEpoB, our first-generation drug candidate, has recently entered phase II human clinical trials (21).

Second-generation epothilones

In keeping with the prevailing working style of our laboratory, we have continued to pursue the discovery of effective epothilone-derived anticancer agents (22-27). Thus, using our technique of hypothesis-driven molecular editing, we hoped to identify a second-generation drug candidate in which some of the pharmacokinetic limitations of dEpoB would be ameliorated. In this second-generation drug candidate, we hoped to recover some of the loss of potency caused by the deletion of the oxido linkage from EpoB, to increase the metabolic stability of the drug, and to produce an agent with a clinically exploitable therapeutic index that could lead to more effective cures in xenograft models.

Toward such goals, we synthesized and evaluated a number of epothilone derivatives. From these investigations, we identified two structures with particularly promising *in vitro* and *in vivo* properties. They are 9,10-dehydro-dEpoB (**4**) and 26-trifluoro-(*E*)-9,10-dehydro-dEpoB (fludelone, **5**). Each of these epothilone analogues possesses an E-9,10 double bond, which seems to result in a marked increase in *in vitro* potency, possibly arising from its significantly improved metabolic stability. In addition, fludelone possesses a 26-trifluoro functionality, which can, conceivably, further impact metabolic stability, binding affinity and toxicity. The syntheses of 9,10-dehydro-dEpoB and fludelone are illustrated briefly in Scheme 1 (24).

Table I: In vitro cytotoxicity (IC_{50}) against tumor cell lines[a].

Compound	CCRF-CEM (nM)	CCRF-CEM/VBN_{100}(nM)	CCRF-CEM/Taxol (nM)
dEpoB	5.6 ± 2.8	16 ± 3	8.5 ± 5.5
9,10-Dehydro-dEpoB	0.9 ± 0.4	4.2 ± 2.2	1.2 ± 0.6
Fludelone	3.2 ± 0.3	23 ± 2	4.7 ± 1.0

[a]XTT assay following 72 h inhibition. CCRF-CEM is a human T-cell acute lymphoblastic leukemia cell line. CCRF-CEM/VBN_{100} cell line is resistant to vinblastine, and CCRF-CEM/Taxol is resistant to Taxol.

Table II: Profile of 9,10-dehydro-dEpoB and fludelone in xenograft models.

Parameter	dEpoB	9,10-Dehydro-dEpoB	Fludelone
IC_{50} (nM) against CCRF-CEM	5.6	0.90	3.2
Maximal drop in body weight[a] w/o death (%)	32	29	33
Half-life: Mouse plasma (min)	46	84	212
Half-life: Human liver S9 (h)	1.0	4.9	10.5
Solubility in water (μg/ml)	9.4	27	20
Therapeutic dose regimen (mg/kg)[b,d]	25-30	3-4	10-30
Relative therapeutic index at MTD[c,d]	++++	++++	+++++

[a]Body weight refers to total body weight minus tumor weight. [b]Therapeutic dose regimen for q2d 6h i.v. infusion. [c]Graded relative therapeutic index (TI) at MTD (maximal tolerated dose): + Tumor growth suppressed by 25-50%. ++ Tumor growth suppressed by 50-100%. +++ Tumor shrinkage but no tumor disappearance. ++++ Tumor disappearance in some or all nude mice with slow body weight recovery and/or with relapse in some mice 1 week after treatment was stopped. +++++ Tumor disappearance in all nude mice with rapid body weight recovery and/or without relapse. [d]Determined with the CCRF-CEM cell line.

9,10-Dehydro-dEpoB and fludelone were first evaluated for their *in vitro* cytotoxicity against several multidrug-resistant and -sensitive cell lines. In comparison with dEpoB, 9,10-dehydro-dEpoB exhibited enhanced inhibitory effects against vinblastine-resistant, paclitaxel-resistant and paclitaxel-sensitive cell lines. Similarly, fludelone, although somewhat less cytotoxic against the vinblastine-resistant cell line, showed improved inhibition against the paclitaxel-resistant and -sensitive cell lines (Table I).

The impressive cell growth inhibition data demonstrated by 9,10-dehydro-dEpoB and fludelone prompted us to investigate their pharmacokinetic and toxicological profiles in xenograft models. Each analogue, as well as dEpoB, was introduced to mice containing human T-cell acute lymphoblastic leukemia cell line CCRF-CEM xenografts, and the IC_{50} and body weight change were evaluated (Table II). In addition, we assessed mouse and human plasma stability and water solubility for each compound. An examination of the IC_{50} values reveals that some of the cytotoxicity lost upon deletion of the 12,13-oxido linkage (cf. dEpoB) is restored in the second-generation analogues, presumably as a result of the introduction of the 9,10-dehydro functionality. This improved potency is more marked in the 9,10-dehydro-dEpoB congener than in fludelone. Conversely, although both second-generation analogs exhibit improved plasma stability in both mice and humans, fludelone is dramatically more stable than either dEpoB or 9,10-dehydro-dEpoB. The second-generation analogues also exhibit significantly better water solubility than dEpoB. As will be shown, further studies ultimately revealed fludelone to be a particularly promising drug candidate. The loss of potency of fludelone relative to 9,10-dehydro-dEpoB is more than compensated for by its particularly exploitable therapeutic index.

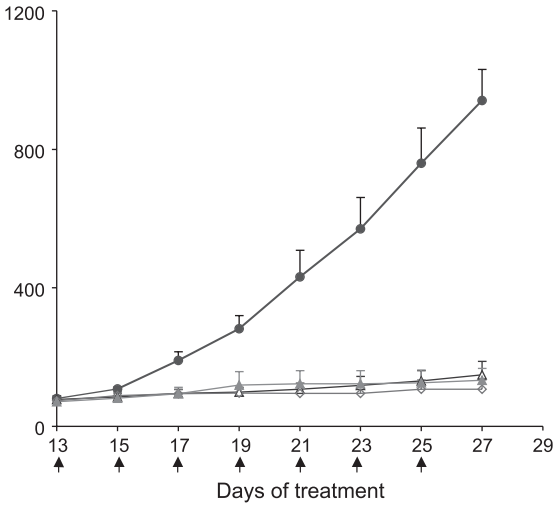


Fig. 1. Therapeutic effect of 9,10-dehydro-dEpoB in nude mice bearing HCT-116 xenograft. Control (l); 9,10-deH-dEpoB, 3 mg/kg (k); 9,10-deH-dEpoB, 4 mg/kg (n=4) (Δ); 9,10-deH-dEpoB, 5 mg/kg (s) as 6-h i.v. infusion (except 9,10-deH-dEpoB, 5 mg/kg i.v. injection) q2dx7, n=3. Arrows indicate drug administrations.

The treatment of various xenograft models, including HCT-116, with 9,10-dehydro-dEpoB resulted in growth suppression and tumor shrinkage (Fig. 1). Unfortunately, at this writing, we have been unable to achieve complete

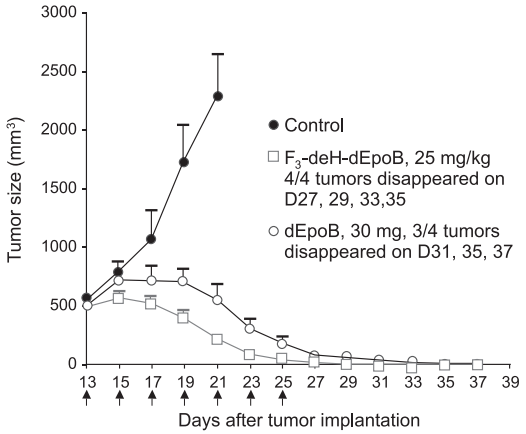


Fig. 2. Chemotherapeutic effect of fludelone and dEpoB against MX-1 xenografts in nude mice. Tumor tissue (40–50 mg) was implanted subcutaneously on day 0. Treatment was started when tumor size reached about 500 mm^3. All treatments, indicated by arrows, were carried out by a 6-h i.v. infusion into the tail vein with a minicatheter and programmable pump. Each dose group consisted of 4 mice.

tumor eradication with 9,10-dehydro-dEpoB against a subcutaneous tumor established from the HCT-116 cell line.

By contrast, fludelone seems to possess a significantly broader and more readily exploitable therapeutic index. Early comparisons were recorded when mice bearing the human mammary carcinoma MX-1 xenograft were treated with fludelone and with other epothilones (Fig. 2). In this study, treatment with either fludelone or the first-generation drug candidate, dEpoB, was commenced when the tumor size reached 0.5 g (~ 2.3% body weight). Each mouse was treated with either 25 mg/kg fludelone (q2dx7, every other day, 7 total doses) or 30 mg/kg dEpoB (q2dx8), administered through i.v. infusion. In each of the 4 fludelone-treated mice, the tumor disappeared by day 35. In comparison, only 3 of the 4 dEpoB-treated mice exhibited complete tumor disappearance.

Encouraged by these results, we set for ourselves the additional challenge of delaying treatment until the tumor size reached 0.96 g (~3.4% body weight), at which point 25 mg/kg fludelone (q3dx5, 9 days rest, q3dx4) was administered through i.v. infusion. Remarkably, in each of the 5 mice evaluated, this course of treatment led to complete disappearance of the extra-large tumors and the absence of any relapse for over 8 months following suspension of treatment (Fig. 3).

Although the paramount objective of this program is the development of a potent anticancer agent with a broad therapeutic index, we are also sensitive to the practical clinical considerations of drug administration. In this context, it would be of significant added benefit to be able to develop a drug that is orally active. We speculated that fludelone could be an excellent candidate for oral bioavailability, given its previously demonstrated plasma stability and water solubility. Thus, nude mice implanted with MX-1 xenografts were treated orally with 30 mg/kg fludelone in 3 cycles, beginning on day 16 following implantation. Remarkably, complete tumor disappearance was observed in each of the 3 fludelone-treated mice, and there was no relapse on day 115. For comparison, a similar study performed with paclitaxel showed that oral administration of the drug had almost no effect on tumor growth, even when the doses were increased to as much as 60 mg/kg (Fig. 4). These results are hardly

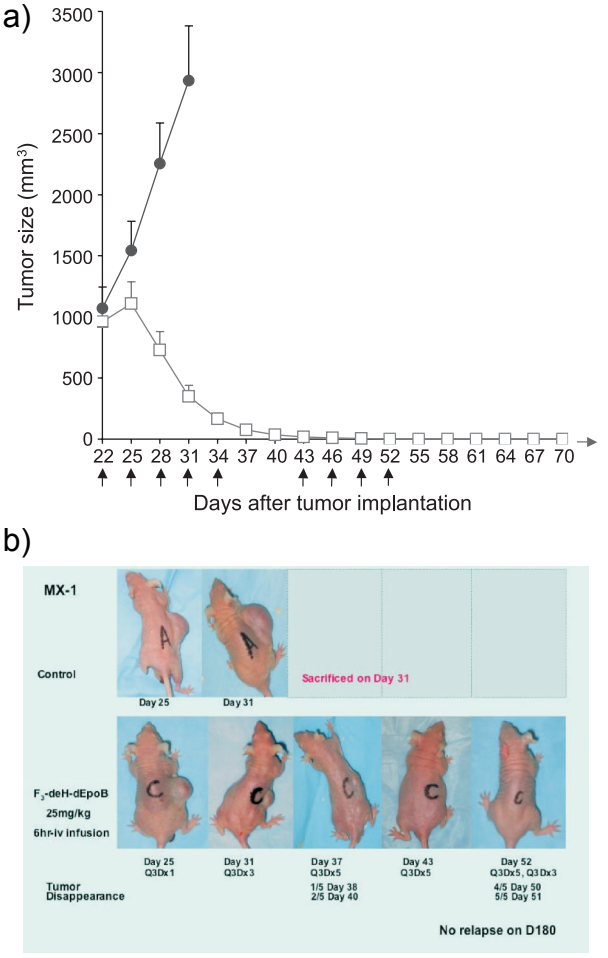


Fig. 3. Chemotherapeutic effect of fludelone against extra-large MX-1 xenografts in nude mice. (a) MX-1 tumor tissue (50 mg) was implanted s.c. on day 0. Tumor size changes in the vehicle-treated control (l) and fludelone-treated (25 mg/kg) group (k) (n=5 each) were observed. On day 22 (D22) when tumor size reached 960 ± 132 mg (about 3.4% of body weight), fludelone treatment was commenced. Fludelone, 25 mg/kg as a 6-h i.v. infusion q3dx5 was given on D22, 25, 28, 31 and 34, as indicated by the arrows. The second cycle of treatment, following a 9-day rest, was given on D43, 46, 49 and 52. For the fludelone-treated group, 5/5 tumors disappeared on D38, 40, 50, 50 and 51. Observation was continued q3d and no relapse was observed on day 180. (b) Photographs for the nude mice (one mouse each selected from the control group and the treated group) taken on D25, 31, 37, 43 and 52.

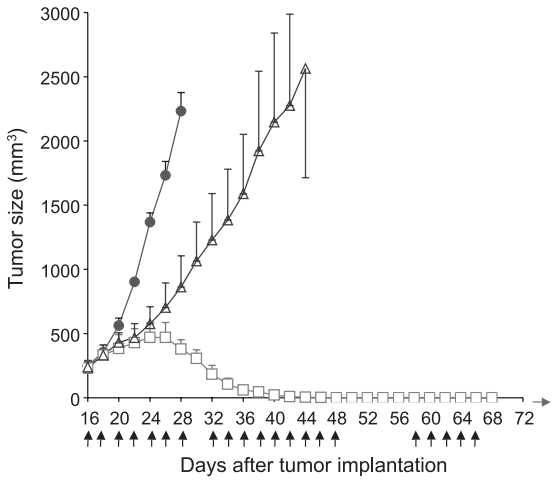


Fig. 4. Therapeutic effects against human mammary carcinoma MX-1 xenograft by fludelone or paclitaxel (Taxol®) via oral administration. Female nude mice were used. fludelone 30 mg/kg (cuadrado blanco) (n = 3) was given orally q2dx7 beginning D16 after tumor implantation and then q2dx9 on D32-48, as indicated by arrows. All three mice's tumor disappeared on D40, 45 and 48. For consolidation therapy, the third cycle of treatment was given q2dx5 from D58-66 when all mice were tumor free on D48. There was no relapse on D115 (49 days after stopping treatment). Control (l) (n=2) received vehicle only. Parallel comparative experiment was carried out with Taxol 30 mg/kg (s) (n=3), orally beginning on D16, q2dx3 and then the dose was increased to 40 mg/kg q2dx3 (D22-26) and then 60 mg/kg q2dx3 (D28-40).

surprising, as paclitaxel is not administered orally in a clinical setting; however, this comparative study does serve to highlight yet another potential clinical benefit of fludelone.

Fludelone has thus demonstrated itself to be a very promising second-generation drug candidate, based on its broader therapeutic index, its lower toxicity and its improved ability to eliminate tumors in an *in vivo* setting. We next sought to evaluate the breadth of the cytotoxicity of fludelone *in vitro* against a variety of human tumor cell lines. A number of cell lines were treated with dEpoB and with fludelone, and the IC_{50} values were evaluated (Table III). In comparison with dEpoB, fludelone exhibited equivalent or improved cytotoxicity against lung, colon and breast carcinoma cell lines and against embryonic lung fibroblasts. A more dramatic difference between the two compounds was observed in the treatment of various ovarian carcinoma and myeloma cell lines, which revealed the significantly improved growth inhibition of fludelone over the first-generation candidate, dEpoB. Importantly, both of the normal bone marrow stromal cell lines evaluated (HS-27A and HS-5) appeared to be relatively resistant to both dEpoB and fludelone, indicating a safe therapeutic window for each drug.

The broad-based *in vitro* activity of fludelone was generally found to translate to excellent *in vivo* efficacy in mice bearing various human tumor xenografts. For instance, mice implanted with slow-growing lung carcinoma A549 xenografts were treated with 25 mg/kg fludelone or 30 mg/kg dEpoB. Despite their similar *in vitro* IC_{50}values, fludelone and dEpoB behaved quite differently from each other in this *in vivo* setting. All 4 of the fludelone-treated mice experienced 99.5% tumor suppression, with eventual tumor eradication. The decrease in body weight (as much as 35%) observed during treatment did not cause any lethality, and rapid body weight recovery was observed after suspension of treatment. By contrast, although the dEpoB-treated mice experienced 97.6% tumor suppression, none exhibited tumor eradication (Fig. 5).

The extraordinary curative effects of fludelone were also observed in a series of long and thorough *in vivo* therapeutic studies of a variety of tumor cell lines, including paclitaxel-resistant human lung carcinoma (A549/Taxol), paclitaxel-resistant T-cell lymphoblastic leukemia (CCRF-CEM/Taxol) and human colon carcinoma (HCT-116).

We recently launched a program aimed at examining the *in vivo* effects of fludelone against human multiple myeloma cell lines. Paclitaxel has been used to treat multiple myeloma, but its utility is limited due to its high toxicity and the onset of multidrug resistance (28, 29). Our preliminary *in vitro* data (Table III) had suggested that fludelone could be a promising candidate for the treatment of multiple myeloma. We were able to quantify the tumor burden in subcutaneous, disseminated or metastatic tumor xenografts in live nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice through

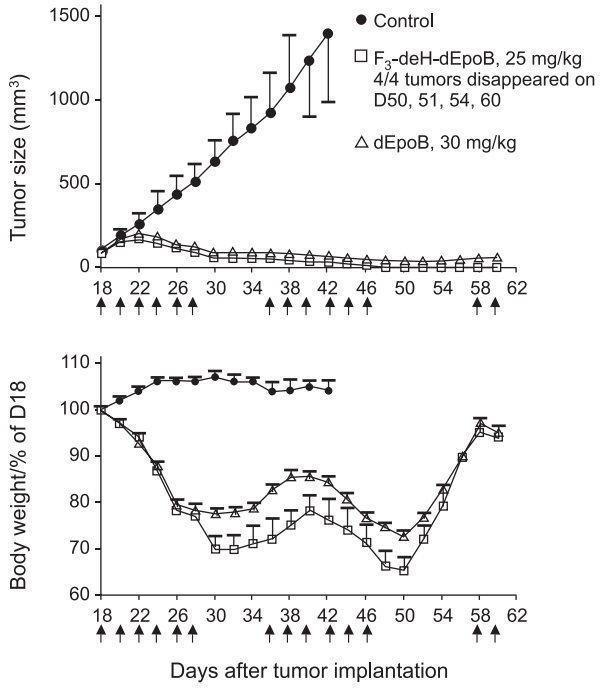


Fig. 5. Therapeutic effect of fludelone and dEpoB in nude mice bearing slow-growing A549 lung carcinoma xenograft (q2dx6, x6, x2, 6-h i.v. infusion, n=4).

Table III: Cell growth inhibition (IC_{50}, nM) of dEpoB and fludelone against a broad spectrum of human tumors and normal human cells.

Histology	Tumor cell line	dEpoB	Fludelone
Lung carcinoma	A549	3.9 ± 0.4	3.7 ± 2.4
Embryonic lung fibroblasts	MRC-5	8.2 ± 4.3	7.4 ± 2.7
Colon carcinoma	HT-29	7.2 ± 2.2	4 ± 1.7
	HCT-116	7.5 ± 3.1	3.6 ± 1.3
Breast carcinoma	MDA-MB-435	7.8 ± 4.2	5.8 ± 2.8
Ovarian carcinoma	IGROV	15 ± 3.8	2 ± 1.2
	SK-OV-3	13 ± 4.7	1.6 ± 0.5
	OVCAR-3	14 ± 3.6	1.1 ± 0.4
	OVCAR-4	16 ± 2.5	1.8 ± 0.7
Myeloma	RPMI-8226	36.7 ± 2.0	7.6 ± 1.2
	CAG	61.3 ± 4.2	12.0 ± 1.8
	OPM-2	38.9 ± 3.3	8.2 ± 2.2
	NCI-H929	42.7 ± 4.5	9.2 ± 1.9
	MOLP-5	68.6 ± 5.5	14.4 ± 2.6
Normal marrow stroma	HS-27a	100 ± 10	102 ± 8
	HS-5	100 ± 8	96 ± 7

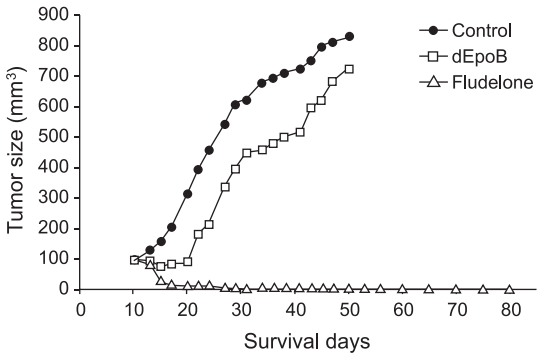


Fig. 6. Treatment of subcutaneous RPMI-8226 xenograft myeloma mice with fludelone and dEpoB.

the use of the imaging system of luciferase bioluminescence developed by Tjuvajev *et al.* (30). NOD/SCID mice were implanted with subcutaneous human myeloma RPMI8226 xenografts and treatment was commenced with either fludelone or dEpoB on day 10, when tumor size had reached approximately 100 mm^3. The results of this study are quite compelling. While the dEpoB-treated mice exhibited little improvement over the control, all 4 fludelone-treated mice experienced tumor disappearance on day 20, only 10 days after the initiation of treatment (Fig. 6). These dramatic results are further illustrated by images taken after 8 doses of dEpoB or fludelone (Fig. 7). While all control mice died within 50 days following tumor implantation, the fludelone-treated mice survived for at

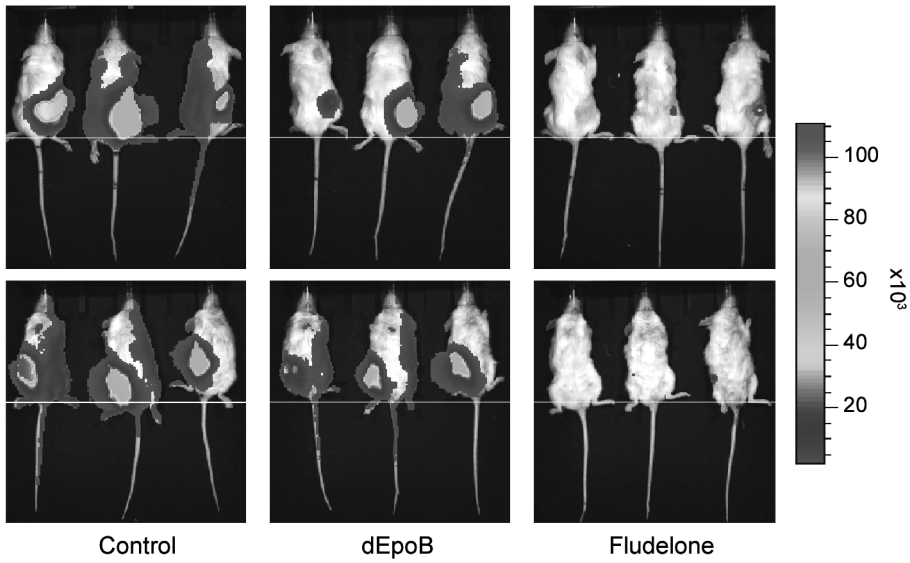


Fig. 7. Treatment of subcutaneous RPMI-8226 xenograft myeloma mice by dEpoB or fludelone. The bioluminescence images were taken at day 50 in both dorsal (upper panel) and ventral (lower panel).

least 100 days. We have expanded our investigation to a disseminated myeloma xenograft using CAG and MM.1S myeloma cells with excellent results, which will soon be reported in a full account (31).

Conclusions

In summary, we have described herein the discovery and evaluation of an extremely promising second-generation epothilone-derived drug candidate, fludelone. Throughout the course of the epothilone program, we have adhered to the guiding program of our laboratory. We first turned to nature to supply us with a valuable lead candidate. Through the power of chemical synthesis and reasoned scientific analysis, we were able to further expand upon the wisdom of nature by designing a series of analogues in which the therapeutic limitations of the natural product were mitigated through structural "editing". Through our own rather short time scale evolution process, we have identified a novel agent, fludelone, which could well be a breakthrough drug. Despite the excellent results obtained through extensive *in vitro* and *in vivo* studies, the true test of the strength of a drug is its performance in human clinical settings. While it remains to be seen whether these astounding preclinical results will translate into clinical success, we are hopeful that fludelone can eventually provide real patient benefit.

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