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ORIGINAL ARTICLE

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Preclinical pharmacology of epothilone D, a novel tubulin-stabilizing antitumor agent

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Abstract *Purpose*: To determine, for various species, the pharmacological and biochemical properties of epothilone D (EpoD) that are relevant in establishing an appropriate animal model for further evaluation of this promising antitumor agent. *Methods*: A method involving high-performance liquid chromatography (HPLC) was developed and used to assess the stability and protein binding of EpoD in plasma from various species, its metabolism by various S9 fractions, and its pharmacokinetics in mice. *Results*: EpoD was stable in dog and human plasma. In plasma from other species, stability decreased in the order: hamster > mouse > guinea pig > rat. EpoD was highly bound to proteins in dog and human plasma. In an evaluation of S9 fractions from mouse, rat, guinea pig, dog, and human, mouse S9 was most efficient in metabolizing EpoD. Following administration to CD2F1 mice, the initial half-lives for plasma elimination of EpoD were < 5 min for an intravenous dose and < 20 min for an intraperitoneal dose. *Conclusions*: The species differences in EpoD biostability and metabolism may have implications in assessing its antitumor activity and pharmacologic and toxicologic profiles in humans. Relative to humans, the mouse is not a good model for disposition of EpoD; the dog would be more appropriate.

Keywords Epothilone D · HPLC · Metabolism · Protein binding · Pharmacokinetics

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Introduction

Cancer poses a major human health problem, and chemotherapy represents an important approach in its treatment. Although substantial progress has been made, there is still a need to search for new chemotherapeutic agents. An important class of anticancer drugs consists of the microtubule inhibitors, which exert their therapeutic effects mainly through interference with mitosis and cell division [1]. For instance, paclitaxel and docetaxel have potent chemotherapeutic effects against a variety of malignancies in the clinic [2]. In contrast to the older drugs, vincristine and vinblastine, which are inhibitors of tubulin polymerization, these agents promote polymerization of tubulin heterodimers into microtubules and stabilize preformed microtubules, resulting in arrest of cell growth or apoptosis [3].

Recently, a novel class of depolymerization inhibitors, the epothilones, which are secondary metabolites produced by the myxobacterium *Sorangium cellulosum*, have been identified [4]. Exposure of human cancer cells to epothilones leads to aberrant spindle formation during mitosis, to mitotic arrest at the metaphase/anaphase boundary, and eventually to apoptosis [5, 6]. More importantly, the epothilone analogs inhibit the growth of cancer cells that overexpress the P-glycoprotein efflux pump and are resistant to various other chemotherapeutic agents through the MDR-mediated drug resistance mechanism [5]. Another advantage of epothilones, compared to paclitaxel and docetaxel, is their greater water solubility, a favorable characteristic for pharmaceutical formulation [7, 8]. Further, relative to paclitaxel and docetaxel, the structures of epothilones are less complex; total chemical syntheses of several epothilones have been achieved [9–12] and some have been generated by biotransformation [13]. Based on their activity against experimental tumors in mice, epothilone B and two of its derivatives, CGP 85715 (a C26-fluoro derivative of epothilone B) and aza-epothilone B, have entered clinical trials [8, 14].

Another member of the epothilone class is epothilone D (EpoD, NSC-703147, previously known as 12,13-desoxyepothilone B, dEpoB; see Fig. 1 for its structure). As an inducer of tubulin polymerization, EpoD is equivalent to other epothilones and more potent than paclitaxel [8], although its antiproliferative activity is somewhat less than that of other epothilones. In contrast to paclitaxel, EpoD demonstrates efficacy in a wide variety of tumors, including those resistant to paclitaxel, vinblastine, and doxorubicin [15, 16]. Perhaps one of the major advantages of EpoD is that it is less toxic than the other epothilone analogs [15]. In the present study, aimed at investigating the in vitro and in vivo biostability and metabolism of EpoD as well as pharmacokinetics in experimental animals, we set the emphasis on potential species differences, providing a basis for selection of an appropriate animal model for future studies.

Materials and methods

Test compounds, chemicals, reagents, and animals

All chemicals and solvents used for sample preparation and high-performance liquid chromatography (HPLC) analysis were of analytical grade. EpoD was provided by the National Cancer Institute. Paclitaxel was purchased from Sigma (St. Louis, Mo.). Samples of plasma from CD2F1 mice, *Nu*/*Nu* mice, and Sprague-Dawley rats (provided by NCI) were prepared from blood collected in our laboratory. Southern Research Institute (Birmingham, Ala.) kindly provided dog plasma. Plasma from HsdPrc:DH guinea pigs was purchased from Lampire Biological Laboratories (Pipersville, Pa.) and hamster plasma from Harlan Sera-Lab (Belton, Loughborough, UK). Hepatic S9 fractions from male ICR-CD-1 mice, male Sprague-Dawley rats, male Dunkin-Hartley guinea pigs, male beagle dogs, and male humans were purchased from In Vitro Technologies (Baltimore, Md.).

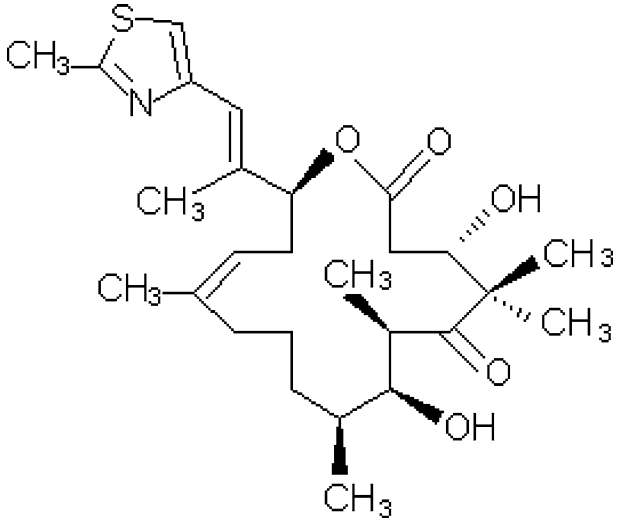

Fig. 1 Structure of EpoD

Analytical method

EpoD in biological samples was analyzed by an analytical procedure involving liquid–liquid extraction and reversed-phase HPLC. An internal standard solution of paclitaxel (final concentration, 10 μg/ml) was added to plasma samples prior to extraction. Following addition of four volumes of acetonitrile/water (62.5/37.5, v/v) to biological samples, each preparation was mixed and then centrifuged for 10 min at 3000 *g*. The resulting supernatant was mixed with 3 ml of chloroform and then centrifuged at 3000 *g* for 5 min. The resulting organic phase was dried with a stream of air. The residue was dissolved in 200 μl methanol/0.3% orthophosphoric acid (70/30, v/v) prior to analysis by HPLC (100 μl/injection). Extraction efficiency was in the range of 90–105%.

The HPLC system consisted of a Beckman Gold module 406 with a computer-controlled solvent delivery system and a UV detector. The analytical column was a Zorbax SB-phenyl (250×4.6 mm i.d.; Agilent Technologies, Palo Alto, Calif.) coupled with an in-line guard column (RP-18, 5 μm, Lichrosphere 100; EM Sciences, Gibbstown, N.J.). The mobile phase was acetonitrile/0.3% orthophosphoric acid (50/50, v/v), pumped at a flow rate of 1.5 ml/min. The eluent was monitored at 250 nm. The ratios of the peak heights of EpoD to those of internal standard were used to establish standard curves and for quantitative computations of samples. Relative to peak areas, peak heights provided a more accurate assessment of concentrations of EpoD. The retention times for paclitaxel and EpoD were approximately 8 and 9 min, respectively.

Stability in plasma

By use of the validated HPLC method, we performed in vitro stability studies of EpoD at 37°C in plasma from rats, CD2F1 mice, *Nu*/*Nu* mice, hamsters, guinea pigs, dogs, and humans. There were three initial concentrations of EpoD (1, 10, and 100 μg/ml), except for plasma from hamsters and guinea pigs, for which there were only the two lower concentrations. At the designated time points, samples were removed and then extracted by the above procedure. The EpoD concentrations at various times were quantified by HPLC and, to illustrate the in vitro stability, expressed as percentage of initial concentration.

Binding to plasma proteins

The plasma protein binding of EpoD was assessed using a previously described procedure involving a

microultrafiltration system [17]. Pooled samples of human or dog plasma containing EpoD at concentrations of 10 or 100 μg/ml were maintained at 37°C for 1 h. From each of these preparations, portions were loaded into the reservoirs of Centrifree tubes. These devices were centrifuged at 3000 *g* for 2 h at 35°C. The EpoD concentrations of the filtrate and unfiltered plasma preparations were analyzed by HPLC. The percent of EpoD bound was calculated as follows:

$$\text{Percent Bound} = [(T - F)/T] \times 100$$

where F denotes the concentration of free drug in the filtrate and T is the concentration of the unfiltered plasma preparation.

S9 metabolism

The in vitro metabolism of EpoD was determined using hepatic microsomal S9 preparations from mice, rats, guinea pigs, dogs, and humans. The reaction mixture contained Tris buffer (120 m *M*, pH 7.4) and EpoD (2.4 μg/ml, experiment A, or 1.2 μg/ml, experiment B). Controls lacked the substrate. The reactions, accomplished at 37°C, were initiated by adding the appropriate S9 fractions (1.2 mg/ml, experiment A, or 2.4 mg/ml, experiment B). At designated times, triplicate portions of the incubation mixtures were taken for HPLC analysis.

Pharmacokinetic studies with mice

We determined the plasma pharmacokinetics of EpoD in male CD2F1 mice (4–6 weeks old) following intravenous (via a tail vein) or intraperitoneal administration. The University of Alabama at Birmingham's Institutional Animal Care and Use Committee approved the protocol of animal use and care. The dose, 20 mg/kg, was administered in dimethylsulfoxide (2 and 3 μl/g body weight, respectively). At selected times after dosing, blood samples were collected from three mice by periorbital bleeding. Plasma was prepared by centrifugation. Pharmacokinetic values were derived with WinNonlin 2.1 software (Mountain View, Calif.).

Results

Method validation

In the investigated concentration range of 0.1–20 μg/ml, the calibration curves were linear. The mean correlation coefficients (r^2) for daily calibration curves were >0.999. The validated lower limit of quantitation (LLQ) was 0.1 μg/ml; the detection limit was 0.05 μg/ml. Intraday and interday variations were acceptable (Table 1). Values for both precision and accuracy were within the acceptable range of 85–115%; for the LLQ, the range was 80–120%. Samples with concentrations >20 μg/ml were diluted so that the amount measured was between 0.1 and 20 μg/ml.

Table 1 Intraday and interday variation for analysis of EpoD in rat plasma (*RSD* relative standard deviation for five samples)

Concentration (μg/ml)	Intraday		Interday	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
0.1	5.12	96.7	10.1	106.2
0.5	2.77	100.3	4.1	97.7
2.5	4.79	96.5	4.6	97.8
10	2.87	101.7	1.5	101.8

Stability in plasma

We performed in vitro stability studies of EpoD at 37°C in plasma from CD2F1 mice, *Nu*/*Nu* mice, rats, hamsters, guinea pigs, dogs, and humans. The data are summarized in Table 2. EpoD was more stable in mouse plasma than in rat plasma, but there was no substantial difference in stability for plasma from CD2F1 and *Nu*/*Nu* mice. Stability in guinea pig plasma was similar to that in rat plasma. EpoD was more stable in hamster plasma than in plasma from any of the other rodent species. Over a period of 8 h, EpoD was stable in dog and human plasma. In rat plasma, EpoD was more stable at room temperature than at 37°C. In rat and human plasma, EpoD was stable at −80°C over a period of 8 weeks. Further, there was no degradation of the compound in plasma preparations from which proteins had been removed by precipitation. In the present study, no attempts were made to determine the cause of EpoD degradation in plasma or to identify the products.

Protein binding

Protein binding studies (Table 3) demonstrated that EpoD was highly bound ($>93\%$) to proteins in dog and human plasma. Because EpoD was not stable in the

Table 2 In vitro plasma stability of EpoD in plasma from different species (*ND* not done)

Species	Temperature	$t_{1/2}$ (min, at concentration of)		
		1 μg/ml	10 μg/ml	100 μg/ml
Guinea pig	37°C	7.2	5.7	ND
Rat	37°C	5.7	6.9	34.9
Rat	Ambient	8	13.4	89.7
Nu/*Nu* mouse	37°C	16.3	20.4	47.9
CD2F1 mouse	37°C	17.1	19.6	52.6
Hamster	37°C	51.8	51.6	ND
Dog	37°C	>500	>500	>500
Human	37°C	>500	>500	>500

Table 3 Protein binding of EpoD (10 and 100 μg/ml) in the plasma of humans and dogs. Values are means ± SD

Species	Total[a] (μg/ml)	Free[b] (μg/ml)	Bound[c] (μg/ml)	Percent bound
Human	9.1 ± 0.5	0.6 ± 0.1	8.5 ± 0.5	93.6 ± 0.6
	98.0 ± 1.6	1.3 ± 0.1	96.7 ± 1.5	96.7 ± 0.1
Dog	10.0 ± 0.5	0.6 ± 0.1	9.5 ± 0.5	94.4 ± 0.3
	95.6 ± 2.3	0.6 ± 0.0	95.1 ± 2.3	99.4 ± 0.0

[a]Initial concentration in plasma.
[b]Concentration in filtrate.
[c]Difference between total and free concentrations.

plasma of other species, protein binding could not be determined using this method.

S9 metabolism

With the lower concentration of S9 (1 mg/ml), the amounts of EpoD remaining after 1 h of incubation were as follows: control, 86%; guinea pig, 86%; human, 76%; dog, 62%; rat, 60%; and mouse, 8% (Fig. 2). With the higher concentration of S9 (2 mg/ml), the amounts of EpoD remaining after 1 h of incubation were as follows: control, 85%; guinea pig, 89%; human, 68%; dog, 59%; rat, 53%; and mouse, 4%. Corresponding values for the higher concentration of S9 after 120 min of incubation were as follows: control, 85%; guinea pig, 76%; human, 51%; dog, 38%; rat, 25%; and mouse, 0% (Fig. 2). The higher concentration of S9 did not greatly increase the rate of metabolism of EpoD, but extending the time of incubation to 120 min moderately increased the extent of metabolism (data not shown). At 1 h of incubation (or longer), a single metabolite with a retention time of 2.9 min was visible in all reaction mixtures, except the control. The greatest amounts of this metabolite were formed in reaction mixtures containing S9 from mouse liver.

Pharmacokinetics

Representative chromatograms derived from analysis of plasma from mice dosed with EpoD are shown in Fig. 3. Following intravenous or intraperitoneal doses of 20 mg/kg, the initial half-lives for elimination of EpoD were < 5 and < 20 min, respectively (Fig. 4, Table 4). These values are consistent with the observed instability of EpoD in mouse plasma and in the presence of S9 fractions of mouse liver. With intravenous dosing, values for C_{max} were greatest at the initial sampling time. For intraperitoneal dosing, these values were greatest at 5 min after dosing, indicating that EpoD was rapidly absorbed from the peritoneal cavity. Bioavailability of the intraperitoneal doses was 0.68.

Discussion

Significant species differences in plasma stability were demonstrated in the present experiments, which complement those reported previously in abstract form [18]. O'Connor et al. have shown that EpoD is rapidly degraded in mouse plasma, with a half-life of about 10 min, but is stable in human, dog, and cat plasma [18]. Although in vitro results should be cautiously extrapolated to predict in vivo situations, species differences in biostability of EpoD may be relevant to both its therapeutic and its toxic effects. A model such as dog may be better used to predict pharmacologic and toxicologic profiles in humans.

Traditionally, in pharmacokinetic studies, total plasma drug concentrations are used to describe the distribution and elimination of tested drugs and their metabolites. For many drugs, however, their therapeutic (and toxic) effects correlate better with the concentration of diffusible, unbound drug than with that of the total drug. Thus, in anticipation of future pharmacokinetic and clinical studies, it is important to evaluate the binding to plasma proteins. In the present study,

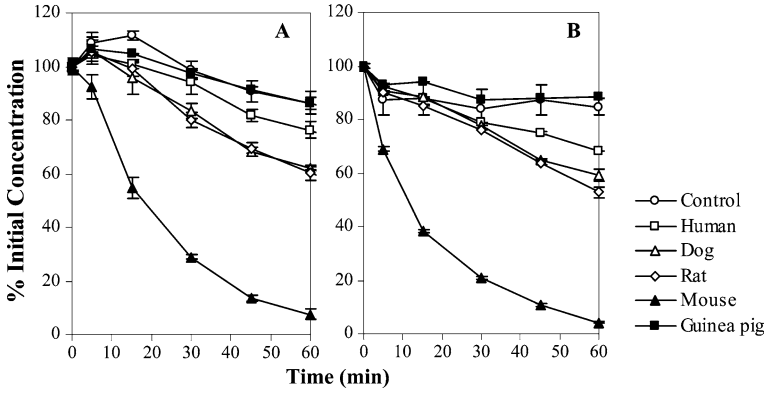

Fig. 2 In vitro metabolism of EpoD by S9 fractions from various species: **a** initial EpoD concentration 2 μg/ml, S9 concentration 1 mg/ml; **b** initial EpoD concentration 1 μg/ml, S9 concentration 2 mg/ml. Values are means ± SD

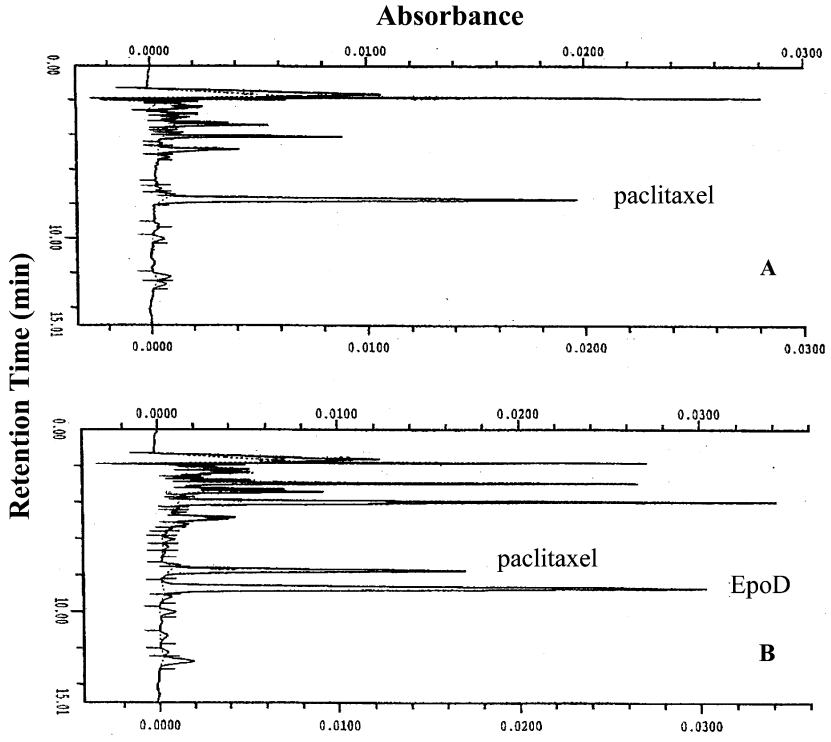

Fig. 3 Chromatograms derived from analysis of plasma from mice: **a** control; **b** 5 min after dosing with EpoD. The peak eluting at 7.8 min is the internal standard, paclitaxel, and that eluting at 8.7 min is EpoD

extensive protein binding of EpoD was found in plasma from humans and dogs. Such binding may have a role in the disposition and therapeutic effects of EpoD. Displacement of such binding by other drugs may modify its pharmacokinetics, resulting in increases in plasma concentration of free unbound drug, increases in uptake and accumulation of drug in targeted and non-targeted tissues, and, therefore, changes in pharmacological and toxicological responses.

Results from our metabolic study indicate that, of the fractions evaluated, mouse S9 was most efficient in metabolizing EpoD. A recent report, in abstract form, indicates that liver esterases and members of the P450 family CYP3A are involved in the metabolism of EpoD [19]. Although epothilone B and EpoD are rapidly degraded in mouse plasma, the compounds possess considerable anticancer activity [15, 16]. It is possible that metabolites of these compounds, formed by the action of esterases or P450, are the active forms of the agents.

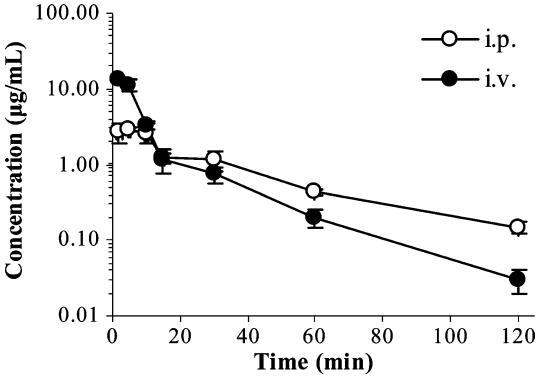

Fig. 4 Time–concentration curves for EpoD in plasma from CD2F1 mice dosed intravenously (*filled circles*) or intraperitoneally (*open circles*). The dose was 20 mg/kg. Values are means ± SE for three mice

It is likely that the observed species differences in in vitro biostability are related to in vivo pharmacokinetics. In the present study, a short plasma elimination half-life was observed in mice, regardless of the route of administration. An early study involving dogs given doses of EpoD (2, 4, or 6 mg/kg) revealed a terminal half-life of about 4 h [18]. Another brief report states that, in rats dosed with 5, 10, or 15 mg/kg, EpoD has a plasma half-life of 89–112 min [19]. The longer half-life in dogs, relative to that in mice and rats, likely relates to the fact that EpoD is more stable in dog plasma.

Evaluations of plasma stability, protein binding, S9 metabolism, and pharmacokinetics for different species can be important in the selection of an appropriate animal model for preclinical testing of antitumor drugs. Although EpoD has anticancer activity in mice bearing human tumor xenografts [15, 16], the present results

Table 4 Pharmacokinetic parameters of EpoD in CD2F1 mice dosed by intravenous or intraperitoneal injection with 20 mg/kg. There were three mice for each of seven time points

Parameter	Intravenous	Intraperitoneal
AUC (µg h/ml)	2.14	1.45
$t_{1/2}$ (h)	0.08	0.32
C_{max} (µg/ml)	18.64	3.15
CL (l/kg/h)	9.35	9.39[a]
MRT (h)	0.12	0.46
V_{ss} (l/kg)	1.07	6.35
F (bioavailability)	N/A	0.68

[a]CL = F×dose/AUC.

indicate that, relative to humans, the mouse is not a good model for studying disposition and toxicology of EpoD. Because the similarity of stability of EpoD in dog and human plasma and similar metabolism by S9 fractions from these species, the dog would be more appropriate. Since greater stability of EpoD in human plasma and slow metabolism with human S9 fractions were observed in the present study, greater in vivo stability of this drug is expected in humans, which may translate into substantial antitumor activity. Based on the same reasoning, since higher drug concentrations and better bioavailability are expected, precaution should be taken when designing the starting doses for humans based on animal data, in order to avoid severe toxicity. In summary, the present study indicates that, in further evaluation of EpoD as a new chemotherapeutic agent for the treatment of human cancer, rodents do not provide an appropriate model for pharmacokinetic and toxicity studies.

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References

1. Lu MC (1995) In: Foye WO (ed) Cancer chemotherapeutic agents. American Chemical Society, Washington, DC, pp 345–368
2. Rowinsky EK (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48:353–374
3. Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci U S A 77:1561–1565
4. Gerth K, Bedorf N, Höfle G, Irschik H, Reichenbach H (1996) Epothilons A and B: antifungal and cytotoxic compounds from Sorangium cellulosum (Myxobacteria). Production, physicochemical and biological properties. J Antibiot 49:560–563
5. Bollag DM, McQueney PA, Zhu J, Hensens O, Koupal L, Liesch J, Goetz M, Lazarides E, Woods CM (1995) Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. Cancer Res 55:2325–2333
6. Kowalski RJ, Giannakakou P, Hamel E (1997) Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol). J Biol Chem 272:2534–2541
7. Höfle G, Bedorf N, Steinmetz H, Schomberg D, Gerth K, Reichenbach H (1996) Epothilone A and B—novel 16-membered macrolides with cytotoxic activity: isolation, crystal structure, and conformation in solution. Angew Chem Int Ed Engl 35:1567–1569
8. Altmann KH, Wartmann M, O'Reilly T (2000) Epothilones and related structures—a new class of microtubule inhibitors with potent in vivo antitumor activity. Biochim Biophys Acta 1470:M79–M91
9. Meng D, Bertinato P, Balog A, Su D-S, Kamenecka T, Sorensen EJ, Danishefsky SJ (1997) Total syntheses of epothilones A and B. J Am Chem Soc 119:10073–10092
10. Kalesse M, Quitschalle M, Eckhard C, Gerlach K, Pahl A, Meyer HH (1999) The formal total synthesis of epothilone A. Eur J Org Chem 11:2817–2823
11. Harris CR, Kuduk SD, Balog A, Savin K, Glunz P, Danishefsky SJ (1999) New chemical synthesis of the promising cancer chemotherapeutic agent 12,13-desoxyepothilone B: discovery of a surprising long-range effect on the diastereoselectivity of an aldol condensation. J Am Chem Soc 121:7050–7062
12. Stachel SJ, Lee CB, Spassova M, Chappell MD, Bornmann WG, Danishefsky SJ, Chou TC, Guan Y (2001) On the interactivity of complex synthesis and tumor pharmacology in the drug discovery process: total synthesis and comparative in vivo evaluations of the 15-aza epothilones. J Org Chem 66:4369–4378
13. Tang L, Qiu RG, Li Y, Katz L (2003) Generation of novel epothilone analogs with cytotoxic activity by biotransformation. J Antibiot 56:16–23
14. Goodin S, Kane MP, Rubin EH (2004) Epothilones: mechanism of action and biologic activity. J Clin Oncol 22:2015–2025
15. Chou TC, Zhang XG, Balog A, Su DS, Meng D, Savin K, Bertino JR, Danishefsky SJ (1998) Desoxyepothilone B: an efficacious microtubule-targeter antitumor agent with a promising in vivo profile relative to epothilone B. Proc Natl Acad Sci U S A 95:9642–9647
16. Chou TC, Zhang XG, Harris CR, Kuduk SD, Balog A, Savin K, Bertino JR, Danishefsky SJ (1998) Desoxyepothilone B is curative against human tumor xenografts that are refractive to paclitaxel. Proc Natl Acad Sci U S A 95:15798–15802
17. Agrawal S, Zhang X, Cai Q, Zhao H, Tan W, Yu D, Zhang R (1998) Effect of aspirin on pharmacokinetics of antisense oligonucleotides in rats. J Drug Target 5:303–312
18. O'Connor O, Bergman P, Hohenhaus A, Tong WPY, Bertino J, Stachel S, Danishefsky S (2001) Stability and toxicity of 12,13-desoxyepothilone (dEpoD) in dogs with spontaneous tumors (abstract). Proc Am Assoc Cancer Res 42:368–369
19. Hoch U, Carney JR, Johnson RG Jr, Hannah A (2002) Metabolism and pharmacokinetics of KOS-862 (epothilone D) in the rat and dog (abstract). Proc Am Assoc Cancer Res 43:426