Drugs of the Future 2002, 27(12): 1149-1163

CCC: 0377-8282/2002

Review Article

Epothilones: new tubulin polymerization agents in preclinical and clinical development

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CONTENTS

Abstract

The epothilones are tubulin polymerization agents that have recently made their way into clinical development for the treatment of solid tumors. With a mode of action similar to Taxol® and the potential to overcome known mechanisms of drug resistance, these agents may offer advantages over existing therapies. This article describes the discovery of the polyketide-derived epothilone natural products and the ensuing drug discovery efforts to identify improved drug analogues. An overview of the preclinical biological properties of the more promising members of this drug class is provided. Accordingly, the compounds included in this article demonstrate significant *in vitro* effects on tubulin polymerization and tumor cell viability. In addition, the *in vivo* activities of 7 different epothilones in human tumor xenograft models has been disclosed and reviewed herein. To date, 2 natural products and 2 semisynthetic analogues have been evaluated in cancer patients (EPO-906, KOS-862, BMS-247550, and BMS-310705), and the preliminary safety and efficacy data from the respective phase I/II studies are discussed.

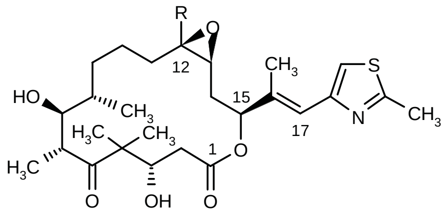
Introduction

In 1991, chemist Gerhard Höfle and microbiologist Hans Reichenbach made a discovery that launched one of the most intense anticancer drug discovery efforts in recent years. In their research laboratories at Gesellschaft für Biotechnologische Forschung (GBF), they isolated and identified a new class of cytotoxic natural products from fermentation of a common soil bacteria, *Sorangium cellulosum* (1). A particular strain of this myxobacterium, originating from a soil sample collected in South Africa, is unique in its production of these newly discovered natural products referred to as epothilones (the name being derived from molecular features: epoxide, thiazole, ketone). A full appreciation of the potential value of epothilones was realized following the pivotal contributions of Bollag and coworkers who demonstrated that the epothilones are microtubule stabilization agents and that they maintain their cytotoxic effects against tumor cell lines that are resistant to paclitaxel, the tubulin polymerizing agent in the anticancer drug Taxol® (2).

Since the mid-1990s, multidisciplinary epothilone research has produced a collective body of work described in more than 300 scientific articles and reviews. From a drug discovery perspective, it is estimated that nearly 1000 new molecular entities or drug analogues of the epothilones have been chemically synthesized during that same timeframe. Beyond the advancement of basic research in the field, the ultimate objective of this effort has been the development and commercialization of a clinically beneficial chemotherapeutic agent(s). This review will provide a brief background and then quickly focus on molecular entities that have biological data and structural features suggestive of progression towards clinical development. Consequently, a comprehensive account of structure-activity relationships for the epothilones is beyond the scope and outside the intent of this review.

Background

Although natural product efforts are declining at many large pharmaceutical companies, there is no question that nature provides a richness of structural diversity and complexity that is unparalleled in the chemical laboratory. The efforts of Höfle and Reichenbach at GBF and later by Bollag and coworkers at Merck, demonstrate the value of



(**1**) R = H epothilone A (Epo A)
(**2**) R = Me epothilone B (Epo B)

Fig. 1. Epothilones A and B.

pursuing natural product approaches when they are compatible with institutional philosophy and strategy. The epothilone natural products have engendered widespread interest and investigation from the scientific community. The questions of "How they work and how to make them work better?" have inspired many that have entered into this field.

Accordingly, Bollag and coworkers provided the first indication that epothilones A (Epo A, **1**) and B (Epo B, **2**) (Fig. 1) exert their antimitotic effects via microtubule stabilization (3). On a molecular level, there is evidence suggesting that the epothilones and taxanes interact at a common binding site on the β-subunit of the tubulin protein heterodimer. Competitive binding experiments involving displacement of [^{3}H]-paclitaxel from preformed, GTP-induced tubulin polymer support this assertion. Thus, an apparent K_i value of 0.71 μM has been obtained for Epo B inhibition of paclitaxel binding to polymerized bovine brain tubulin (4).

This strong biochemical evidence of a common binding site for the epothilones and taxanes would benefit from corroborating structural data. In fact, a detailed description of the binding interaction has been elucidated for the taxanes where a 3-dimensional image of docetaxel bound to zinc-induced α,β-tubulin sheets was obtained utilizing electron diffraction techniques (5). The resolution of the structure (3.7Å) was sufficient to allow a visual fit of docetaxel to the electron density and permit confident positioning of the rigid taxane skeleton and one side chain. The proposed binding site and atomic interactions are supported by previously reported photoaffinity labeling studies (6). An analogous, experimentally-derived structure of epothilone bound to β-tubulin has not been reported. Therefore, several groups are attempting to define the epothilone binding interaction using photoaffinity labeling approaches similar to those described for the taxanes, but successful epothilone labeling studies have not yet appeared in the literature.

While the two agents may share a common binding site on the tubulin protein, there is ample data to suggest that the taxane and epothilone molecular frameworks have distinct interactions with the amino acid residues that constitute the taxane/epothilone binding site. For instance, Giannakakou and coworkers demonstrated that taxane-resistant cell lines possessing mutated forms of the tubulin protein are not cross-resistant with epothilones (7). In these elegant studies, epothilone- and taxane-resistant cell lines were developed and shown to have unique tubulin mutational profiles. The taxane-resistant cell lines 1A9PTX10 and 1A9PTX22, derived from an ovarian cancer cell line A2780, have a phenylalanine to valine mutation at residue β-270 and an alanine to threonine mutation at residue β-364 in the most abundant M40 isotype of the protein, respectively. When compared to the parental cell line, these mutants are 24-fold resistant to the effects of paclitaxel, yet they retain most of their sensitivity to Epo B. Similarly generated epothilone-resistant cell lines 1A9/A8 and 1A9/B10 have mutations in the same region of the protein, comprising a threonine to isoleucine mutation at residue β-274 and an arginine to glutamine mutation at residue β-282, respectively (8). These latter cell lines are 24-fold resistant to Epo B and somewhat less resistant to paclitaxel (6- to 10-fold) when compared to the parental cell line. The absence of a strict taxane/epothilone cross-resistance in these cell lines does not preclude a common binding site, rather it suggests that the detailed binding interactions between the molecules and the protein side chains are weighted differently for the two agents.

Attempts to sort out these detailed binding interactions using computer modeling techniques have relied on the structural data for the docetaxel-tubulin complex, the mutational data generated for resistant cell lines and the structure-activity relationships derived for epothilone analogues. Nevertheless, there is no consensus on an encompassing pharmacophore model for retrospective understanding or predictive evaluation of epothilone-tubulin interactions (9).

The relative abundance of the natural product and the accessibility of this class of agents via chemical synthesis has enabled a spirited effort in the discovery of biologically active drug analogues. Many academic and industrial research groups have taken part, and they have obtained epothilones by one or a combination of the following methods: fermentation (1, 10-11), biosynthesis (12-17), biotransformation (18-19), total synthesis (20-35), semisynthesis (36-43) and combinatorial synthesis (44-46). As mentioned above, natural epothilones A and B were isolated from fermentation of *S. cellulosum* and represent the major secondary metabolites isolated by Höfle and Reichenbach (1, 10). Other members of the natural product family, specifically epothilones C-F, were obtained as minor components in the fermentation process, and like the parent congeners Epo A and Epo B, were eventually prepared by chemical synthesis.

While investigating the epothilone biosynthesis in different *S. cellulosum* strains, scientists at Novartis (12) and Kosan Biosciences (13) independently discovered that Epo A and Epo B were derived from the same polyketide synthase (PKS) gene cluster. During one of these pioneering studies (13), it was established and later confirmed by GBF (14-15) through radiolabeling experiments, that olefinic epothilones C and D were in fact the endproducts of the PKS process. A post-PKS modifying enzyme, cytochrome P450 monooxygenase, was responsible for the final epoxidation to afford Epo A and Epo B. From this work it became evident that the PKS technology could provide a means to alter the selectivity or improve the output of a specific epothilone molecule. Toward this end, Kosan Biosciences elegantly demonstrated the concept that heterologous expression of the epothilone gene cluster in *Streptomyces coelicolor* could provide epothilones A and B (13). A related study demonstrated that the epothilone PKS could be incorporated into a different myxobacteria host, *Myxococcus xanthus,* where further genetic manipulation facilitated the production of Epo D as the major constituent (16-17). A second and equally important facet of this work is the combinatorial biosynthesis of unnatural epothilone analogues via genetic engineering of the PKS. With respect to the production of other unnatural epothilone analogues, semisynthesis and total synthesis approaches have been quite successful. Reference to the generation of specific molecules will be provided in the following section describing the *in vitro* activity of select epothilone derivatives.

In vitro biological activity

As in the case of the taxanes, the intrinsic activities of epothilone analogues have been assessed using various tubulin polymerization assays with purified bovine or porcine brain microtubule proteins obtained from ultracentrifugation (3, 4, 47-49). The ability of a compound to induce the formation of hyperstable tubulin polymers is commonly measured spectrophotometrically as a function of changes in turbidity. The results from these biochemical assays are generally expressed as a percent tubulin polymerization at a given concentration of drug compared to Epo B (%TP) or the concentration of drug that induces an initial slope of 0.01 when plotting absorbance *versus* time ($EC_{0.01}$). More rigorous assays involve the quantitative measurement of unpolymerized or polymerized tubulin protein by standard methods and are reported as either %TP or the effective concentration of the drug that produces 50% polymerization of protein (EC_{50}). One of the latter methods has been adapted for high throughput screening of epothilone analogues (3).

The *in vitro* cytotoxic potencies exhibited by the epothilones have been generally obtained using tetrazolium or sulforhodamine-based assays following a 3-5 day exposure to a variety of paclitaxel-sensitive and paclitaxel-resistant human cancer cell lines. Results are expressed as concentrations required for 50% growth inhibition (IC_{50} values). It has been shown that the epothilones significantly inhibit growth in cell lines insensitive to paclitaxel and other conventional chemotherapeutic agents such as etoposide, teniposide, camptothecan, actinomycin and vinblastine, indicating that the epothilones overcome both the multidrug-resistant (MDR) phenotype (P-gp overexpression and/or multiple resistance mechanisms) and resistance due to specific β-tubulin mutations (22, 26).

In general, similar trends have been observed in epothilone structure-activity relationships (SAR) gathered from the primary tubulin polymerization assays and subsequent cytotoxicity studies. The discrepancy, however, between the nanomolar IC_{50} values obtained in cell culture *versus* the micromolar concentrations required for induction of microtubule polymerization, has been attributed to rapid accumulation and retention of epothilones or taxanes in cells at sufficient levels to affect microtubule stability (50). It should be noted that due to the different tubulin polymerization assays employed and the variety of human cancer cell lines used to evaluate epothilone analogues, it is difficult to directly compare the *in vitro* results reported by different research groups. Furthermore, many excellent comprehensive reviews have appeared in the literature describing the detailed SAR of the epothilones (20-21, 51-55), so the following truncated account highlights only a select group of biologically interesting molecules. This description of the *in vitro* SAR is organized and presented according to structural modifications to the 3 regions of the molecule defined in Figure 2.

As a point of reference, the natural epothilones A and B are 2-4 fold more potent than paclitaxel ($EC_{0.01}$ = 5 μM) in the tubulin polymerization assay used by Bristol-Myers Squibb (41). A number of groups have shown that Epo B is generally 5-25 times more effective (IC_{50}s: 0.1-0.6 nM) at inhibiting the cell growth of human lung (A549, NCI-H460), colon (HCT116, SW620), prostate (DU145, PC3), breast (MDA-MB-231, MCF-7, BT-20, ZR-75-1), bladder (T-24), epidermoid (A431, KB-3.1) and ovarian (1A9) carcinoma cell lines than both Epo A and paclitaxel (IC_{50}s for both: 1.3-5.7 nM) (22-23, 40-41, 52). In addition, Epo A and Epo B have also demonstrated activity in human leukemic cells. For example, the IC_{50} values for Epo A and Epo B are 2.7 and 0.35 nM in the T-cell acute lymphocytic CCRF-CEM line. Importantly, both epothilones maintained activity while paclitaxel was more than 2 orders of magnitude less potent in the MDR

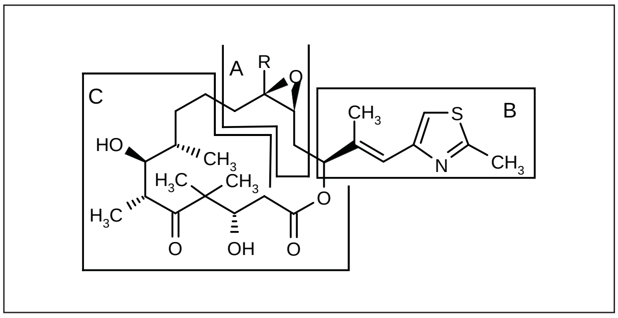


Fig. 2. Structure-activity relationships by region.

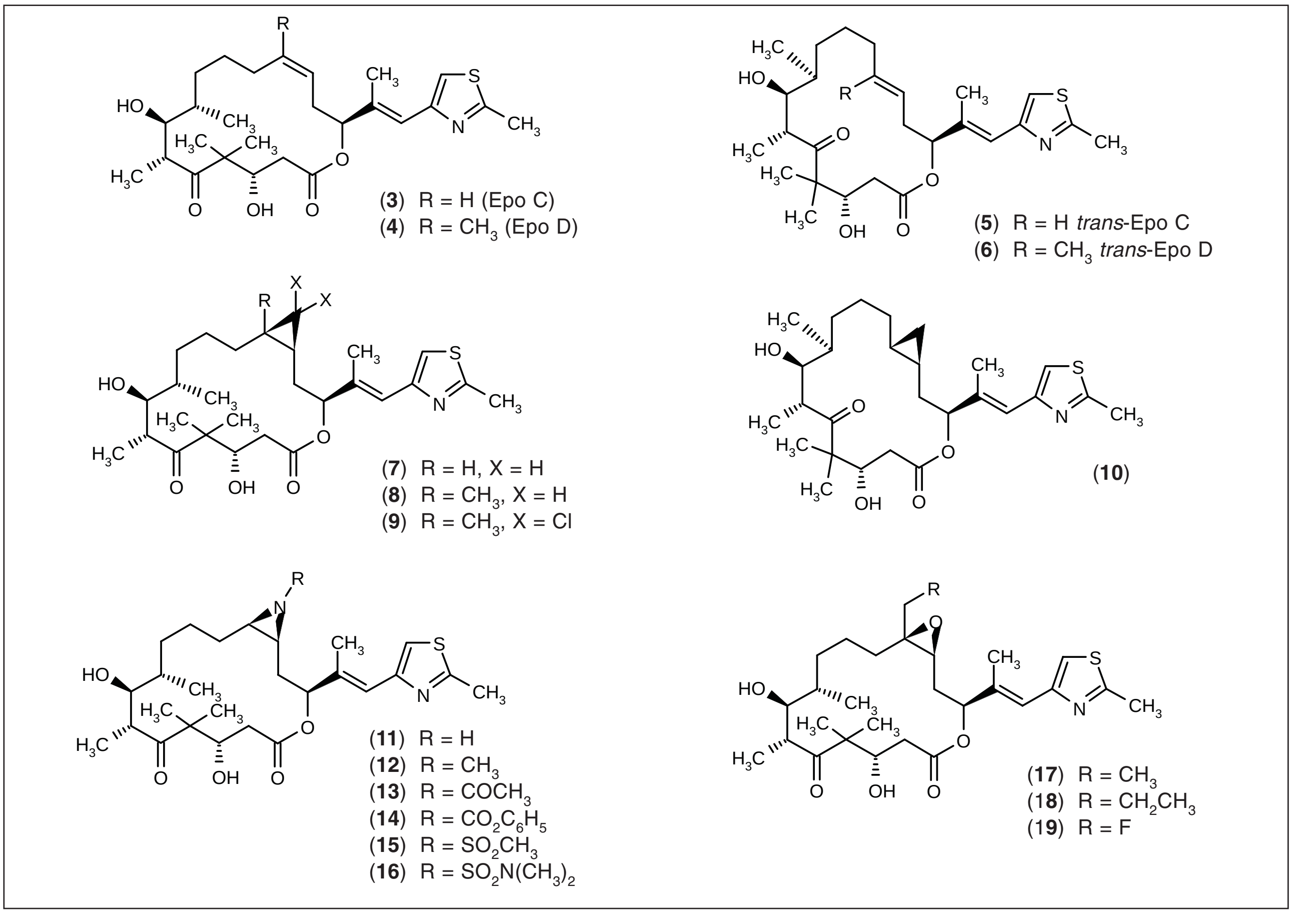


Fig. 3. Structural modifications of region A.

cancer cell lines KB-8511, SW620AD-300 (both involve P-gp overexpression) and 1A9PTX22 (β-tubulin mutation) compared to their parental lines (46, 52). Similarly, only marginal decreases (3-18-fold) in potency were observed for Epo A and Epo B in the CCRF-CEM/VBL$_{100}$, CCRF-CEM/VM$_1$ and MCF-7/ADR MDR cell lines (23, 52). Generation of epothilone resistance *in vitro* upon continuous drug exposure to cancer cells in culture was surprisingly more difficult than for paclitaxel (8, 52). Overall, the *in vitro* profile of Epo B is superior when compared to Epo A or paclitaxel. This trend was also manifested in subsequent *in vivo* studies.

Structural modifications and relative stereochemical changes within region A (Fig. 2) have been extensively explored and are well tolerated (Fig. 3) (20-21, 51-55). For example, the olefinic epothilones C (Epo C, **3**) and D (Epo D, **4**) which lack an epoxide group are potent tubulin polmerizeration agents with EC$_{0.01}$ values of 5.0 and 0.8 μM, respectively (36). The corresponding geometric isomers **5** and **6**, isolated as reaction side products during the total synthesis of the natural antipodes (20-21), are slightly less effective at inducing microtubule assembly (EC$_{0.01}$ = 21 and 11 μM) (41). Epo D is a 2-10-fold more potent cytotoxic agent than Epo C with IC$_{50}$ values of 6.0, 20 and 24 nM in the HCT116, A549 and KB-3.1 carcinoma cell lines, respectively (40-41). Although the antiproliferative effects of both Epo C and Epo D are several times less than that of the parent epothilones A and B, Danishefsky and coworkers at Memorial Sloan-Kettering Institute for Cancer Research carried out extensive *in vitro* and *in vivo* evaluation of Epo D based on the potential for reduced toxicity of this agent (22-24, 26). For example, cell culture studies with Epo D showed IC$_{50}$ values of 2-22 nM in the following paclitaxel-sensitive leukemia (CCRF-CEM, K-562), prostate (PC3), colon (HT-29), mammary (MCF-7, MX-1), ovarian (SK-OV-3, UL-3-C) and neuroblastoma (SK-N-As) human tumor cell lines. Equally impressive, Epo D displayed minimal resistance effects (2-4-fold) in vinblastine-, teniposide- and paclitaxel-resistant leukemia; adriamycin-resistant mammary and neuroblastoma; and paclitaxel-resistant ovarian sublines as compared to the parental lines.

The Bristol-Myers Squibb and Novartis/Scripps groups have shown that epothilone A and B cyclopropanes **7-10** maintain biological activity *in vitro* (Fig. 3) (27-28, 36). The *cis*-12,13-cyclopropyl analogues **7-9** obtained from direct cyclopropanation of olefinic epothilones (*i.e.*, Epo C and Epo D derivatives) are equipotent in the tubulin polymerization ($EC_{0.01}$ = 1.4-2.1 μM) and HCT116 cell line cytotoxicity (IC_{50} = 0.7-1.9 nM) assays (36). Similarly, a representative *trans*-12,13-cyclopropyl analogue **10** prepared via total synthesis, has IC_{50} values of less than 1 nM in the paclitaxel-sensitive KB-3.1 and paclitaxel-resistant KB-8511 (P-gp overexpression) epidermoid cell lines (28). Unlike the parent epoxide-containing epothilones, where Epo B is 10-fold more potent than Epo A in cell culture, a 12-methyl substituent does not improve the activity of either the *cis*- or *trans*-cyclopropane systems.

Utilizing a semisynthesis approach, scientists at Bristol-Myers Squibb also discovered that aziridines **11-16** serve as viable bioisosteric replacements of the epoxide ring (Fig. 3) (37). While the parent aziridine **11** and the N-sulfonamide derivative **15** ($EC_{0.01}$ = 14 μM) are 7-fold less active than Epo A and Epo B in the tubulin polymerization assay, related aziridines **12-14** and **16** are comparable to the parent macrolides ($EC_{0.01}$ = 1-2.6 μM). However, all of these aziridines are more potent than Epo A in the HCT116 cytotoxicity assay, and in fact, the N-methyl and N-sulfonylureido derivatives **12** and **16** have IC_{50} values of 0.13-0.2 nM which are 4-6 times more potent than Epo B. Since conformationally constrained cyclopropanes, aziridines and olefins are active *in vitro*, the role of the epoxide ring in the context of tubulin binding appears to be largely conformational, and evidently the presence of the epoxide is not critical for cellular activity.

The Danishefsky (Memorial Sloan-Kettering) and Nicolaou (Scripps Research Institute) laboratories have shown that elaboration of the 12-methyl substituent of Epo B provides analogues with potent cell killing effects (22, 29-30). Epothilone B homologues **17** and **18** are active against sensitive (CCRF-CEM) and resistant (CCRF-CEM/VBL) lymphoblastic leukemia cell lines with IC_{50} values of 1-7 nM (Fig. 3) (22). Although less toxic than Epo B, the 26-fluoro analogue **19** possesses promising growth inhibitory capabilities (IC_{50} = 0.15-2.8 nM) against human prostate (PC3, LNCaP and clinically derived, androgen-independent MDA PCa2a and 2b) and ovarian (1A9) cancer cell lines (29-30).

Structural permutations of the 15-thiazolylethenyl side-chain (Region B, Fig. 2) of the epothilone skeleton have been extensively investigated. While numerous analogues were found to be active *in vitro*, a few representative examples with significant potential for further development are depicted in Figure 4. Since epothilone F (Epo F, **20**) was initially isolated in relatively low abundance from fermentation, GBF and Bristol-Myers Squibb pursued alternative routes such as semisynthesis (38-40) and biotransformation (18-19) to secure sufficient amounts of Epo F for in-depth preclinical evaluation. Epo F was found to be equipotent to Epo B in the tubulin polymerization assay ($EC_{0.01}$ = 1.8 μM) and against human colon (HCT116), cervix (KB-3.1) and lung (A549) carcinoma cell lines (IC_{50}s 0.2-1 nM) (38, 40). The interesting N-oxide **23**, isolated as an intermediate in the semisynthesis of Epo F, also performed well in the KB-3.1 and A549 lines (40); however, a loss of activity in the MDR-positive cell line NCI/ADR was reported by Schering AG (55). Cyano-substituted analogue **21** is slightly more effective at inducing microtubule assembly than amine **22** ($EC_{0.01}$s 2.9 *vs.* 7.1 μM), yet **22** maintains activity in both MDR HCT116/VM46 and β-tubulin mutated A2780/TAX-R cell lines (39).

In order to address the physicochemical and pharmacokinetic properties of the epothilones, the Scripps/ Novartis team explored 6-membered ring heterocycles (*i.e.*, pyridines and pyrimidines) as potential replacements for the 2-methylthiazolyl ring (31, 54). For example, epothilone B pyridyl analogues **24-26** are all active in the tubulin polymerization assay (80-92% TP) and essentially indistinguishable from each other and Epo B against the parental (1A9) and paclitaxel-resistant (β-tubulin mutations, PTX10, PTX22, B10) ovarian carcinoma cell lines (31). Further cytotoxicty profiling against additional human paclitaxel-sensitive (KB-3.1, A549, HCT116, PC3, MCF-7) and paclitaxel-resistant (KB-8511, HCT-15, MCF-7/ADR) cancer cell lines revealed that the 4- and 5-methyl substituted pyridyl analogues **25** and **26** are essentially equivalent to Epo B.

Novartis (32) and Schering AG (33, 55) independently discovered that replacing the thiazolyl side chain with planar benzo-heterocyclic ring systems provided analogues with enhanced inhibitory activity against human cancer cell lines. Toward this end, benzothiazole **27** and quinoline **28** (Fig. 4) are more potent than Epo B against parental KB-3.1 and MCF-7 cancer cell lines, as well as the corresponding P-gp overexpressing MDR sublines KB-8511 and MCF-7/ADR (IC_{50}s 0.09-0.13 nM) (32-33). Interestingly, quinolinyl derivative **28** is more potent than the corresponding isosteric pyridyl analogue **24**.

Reports of biologically active epothilone derivatives prepared from modification of the polypropionate-derived segment of the macrocycle (Region C, Fig. 2) have been scarce. One important example prepared via a highly efficient semisynthesis approach by Bristol-Myers Squibb, was the esterase-resistant lactam analogue of epothilone B (**29**, BMS-247550) (Fig. 5) (41). Although **29** ($EC_{0.01}$ = 3.8 μM) is 1.5-3 fold less potent than Epo A and Epo B at inducing tubulin polymerization, the semisynthetic analogue is comparable to paclitaxel in this assay. Like Epo B, **29** was found to possess broad spectrum cytotoxic activity (IC_{50}s = 1.4-35 nM) against a panel of 21 tumor cell lines characterized as either paclitaxel-sensitive (ovarian: A2780/DDP, A2780/TAX, OVCAR-3, breast: MCF-7, SKBR3, prostate: LNCaP, PC3, colon: HCT116, LS174T, MIP, lung: A549, LX-1, squamous cell: A431, leukemia: CCRF-CEM, K562, mouse lung: M109, MLF) or paclitaxel-resistant (A2780/DDP-R, A2780/TAX-R, HCT116/VM46, HCT116/VP35) (42). In addition, lactam **29** was as effective as Epo B in its ability to arrest proliferating HCT116 tumor cells during mitosis as measured by flow cytometry.

Only 3 additional epothilone B analogues **30-32** (Fig. 5) encompassing structural changes in Region C were

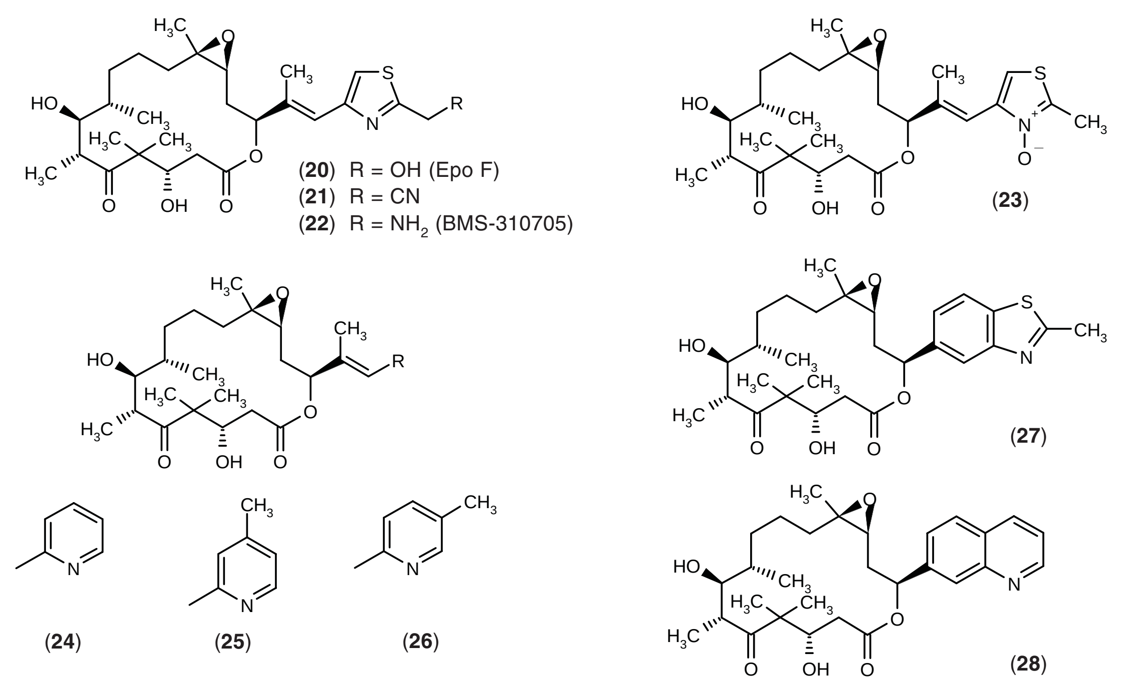


Fig. 4. Structural modifications of region B.

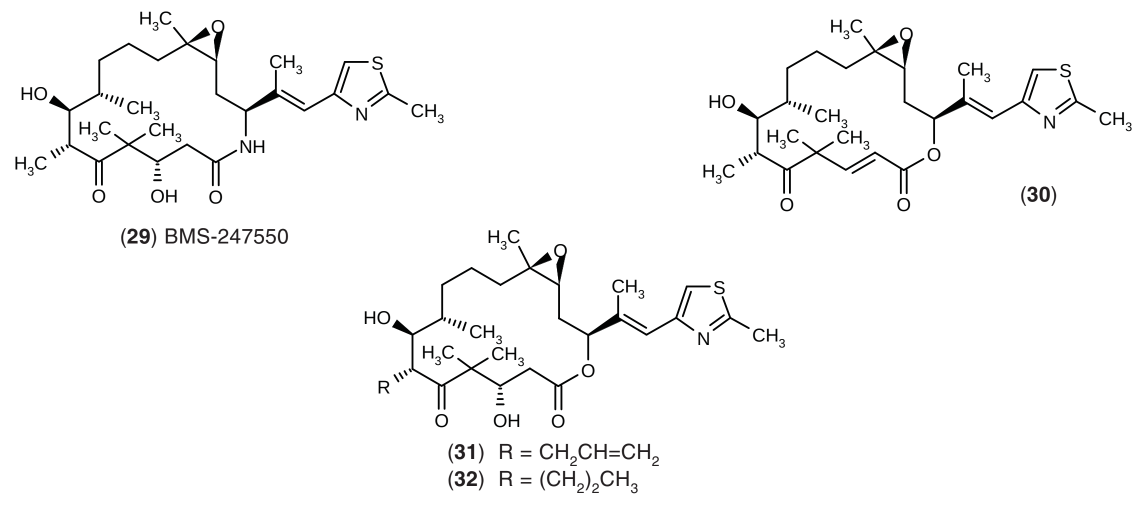
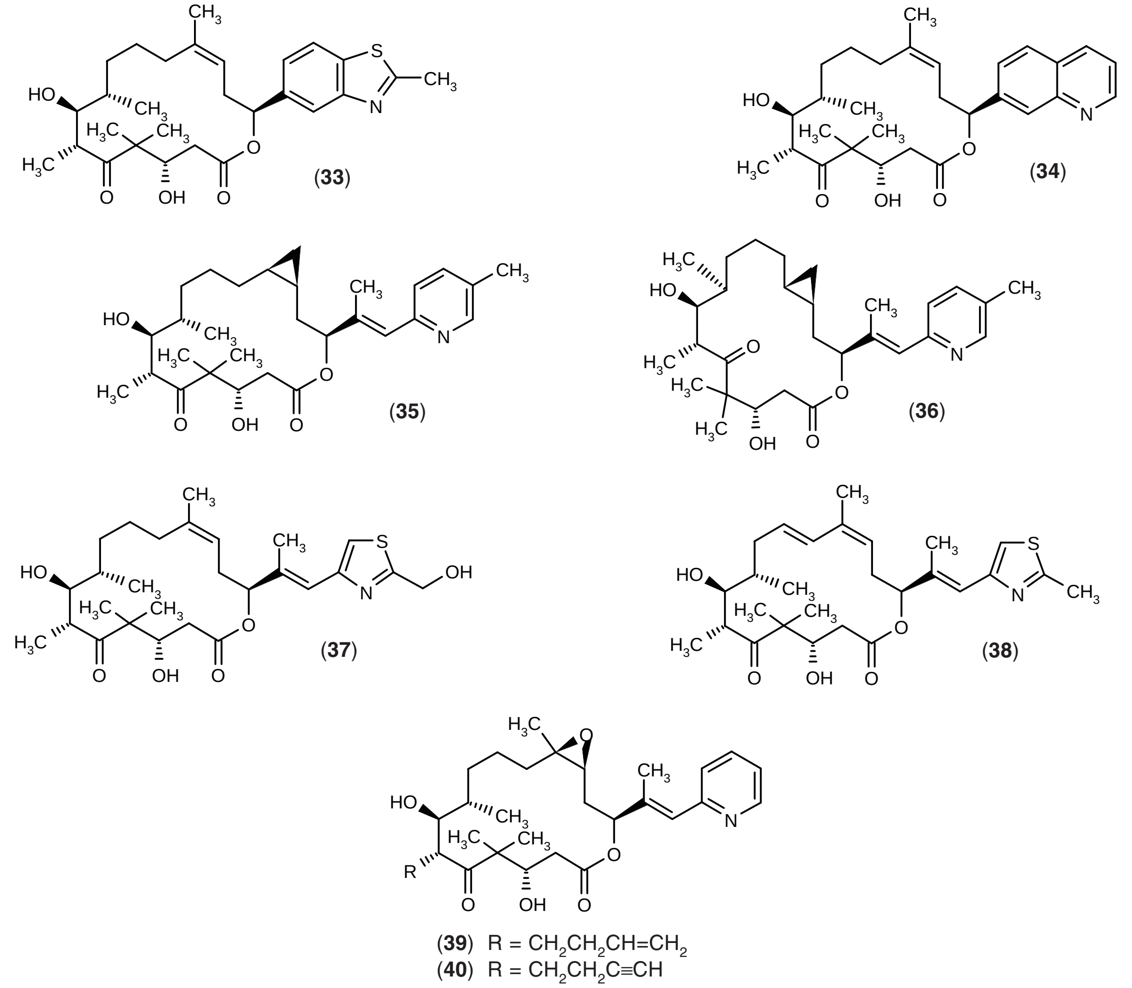


Fig. 5. Structural modifications of region C.

reported to have significant *in vitro* activity. *trans*-Enoate **30**, prepared by GBF scientists via semisynthesis involving the chemoselective dehydration of the 3-hydroxyl group, is only approximatley 10-fold less active than Epo B *in vitro* (43). The Schering AG group prepared C-6 modified epothilone B derivatives **31** and **32** utilizing a total synthesis strategy (33, 55). In cytotoxicity assays, analogues **31** and **32** are only 2- and 6-fold less potent than Epo B *versus* the MCF-7 cell line (IC_{50}s = 1.2 and 3.4 nM), respectively. Both compounds are equivalent to the natural product in the MDR NCI/ADR subline (IC_{50}s ~4 nM).

Combination analogues incorporating multiple structural modifications to the epothilone framework have been well documented (20-21, 51-55). These compounds have typically been obtained by total synthesis since, compared to other methods, this flexible approach offers broader access to structurally diverse analogues. The more interesting examples from a biology perspective are illustrated in Figure 6. Although not as cytotoxic as their



(**33**) (**34**) (**35**) (**36**) (**37**) (**38**)

(**39**) R = $CH_2CH_2CH{=}CH_2$
(**40**) R = $CH_2CH_2C{\equiv}CH$

Fig. 6. Combination analogues: structural modifications to more than one region.

epothilone B counterparts **27** and **28** (Fig. 4), the benzothiazole and quinoline containing analogues of Epo D (*i.e.*, **33** and **34**) are as effective as Epo D at inhibiting the growth of KB-3.1 and KB-8511 cells (IC_{50}s <0.6 nM) (32). The Scripps/Novartis team also recently described the biological activities of a series of 12,13-cyclopropyl epothilones with varying C-15 side chains (28). For instance, the corresponding pyridyl analogues **35** and **36** possess potent antiproliferative activities against the KB-3.1 and KB-8511 cells lines (IC_{50}s < 0.8 nM).

While pursuing variations to Epo D, the Memorial Sloan-Kettering group described the synthesis and *in vitro* profiles of a 21-hydroxylated version **37** (25-26), and a 10,11-unsaturated derivative **38** (34). Combination analogue **37** is in some instances a more potent cytotoxic agent than Epo D, and provides the additional advantage of improved water solubility (25). IC_{50} values for **37** are in the range of 1-12 nM against the CCRF-CEM, K562, HL-60, PC3, HT-29, MCF-7, MX-1, SK-OV-3 and UL-3-C drug-sensitive cell lines. Antiproliferative activity of **37** is compromised 17-30-fold against vinblastine-resistant leukemia cells, but essentially retained against other drug-resistant leukemia (teniposide, paclitaxel), mammary (adriamycin) and ovarian adenocarcinoma (paclitaxel) sublines. Epothilone analogue **38**, which differs from Epo D by the addition of a conjugated double bond in the polyketide backbone, was slightly less potent (2-6-fold) when compared to Epo D against the parental CCRF-CEM line (IC_{50} = 25 nM) and MDR sublines (34).

In further examples of active combination analogues, the Schering AG group has disclosed that bringing together a pyridyl-containing side chain at C-15 and unsaturated alkyl groups at C-6 affords **39** and **40** which are cytotoxic in the MCF-7 cell line (IC_{50}s = 3.1 and 1.3 nM) (33). While the propynyl substituted **39** maintained activity against the multidrug resistant NCI/ADR subline, the closely related butynyl analogue **40** was 7-fold less potent against this variant. Whether structural modifications are made in isolation or in combination with other changes, the research community has shared a great deal of success in discovering epothilone analogues with impressive *in vitro* biological activity. The demonstration of *in vivo* biological activity in animal models of cancer is a significant hurdle that must be overcome prior to initiation of human clinical studies.

In vivo antitumor activity

Surprisingly, despite the discovery of many epothilones with robust *in vitro* activity, as described above, relatively little *in vivo* pharmacology has been disclosed in the literature. Published or presented *in vivo* data has been limited to 2 natural macrolides, Epo B (**2**, epothilone B) and Epo D (**4**, epothilone D) and a handful of promising analogues: **37** (21-hydroxyepothilone D), **38** (10,11-dehydroepothilone D), **19** (26-fluoroepothilone B), **29** (epothilone B lactam) and **22** (21-aminoepothilone B). A variety of human xenograft and murine tumor models have been used to evaluate epothilones *in vivo*. Methods for conducting such experiments as well as reporting tumor responses to drug exposure varies considerably among laboratories. In general, antitumor efficacy data are represented as a ratio of tumor volume for treated *versus* control animals (T/C), the difference in time (days) for tumors in treated *versus* untreated mice to double in size or reach a target size (T-C), or tumor log cell kill (LCK) which is T-C divided by the time for a log increase in tumor volume (calculated as 3.32 times the tumor volume doubling time). While this guideline is sometimes overlooked, legitimate comparison of epothilone analogues to each other or to marketed anticancer drugs, such as paclitaxel, should take into consideration the optimal dose, vehicle and schedule for each individual drug.

Despite initial reports highlighting the *in vitro* antineoplastic activity of Epo B, preliminary studies at Memorial Sloan-Kettering (23) and Bristol-Myers Squibb (39) suggested that only modest *in vivo* antitumor activity was attainable with this agent. Eventually this lack of therapeutic effect was attributed to poor metabolic stability, unfavorable pharmacokinetic characteristics and a narrow therapeutic window in mice. Early studies at Memorial Sloan-Kettering (22) were conducted in SCID mice which were s.c. implanted with either drug-sensitive or MDR human leukemia cells (CCRF/CEM, CCRF-CEM/VBL). Periodic (6 doses) i.p. administration at 0.7 mg/kg and weekly (3 doses) i.v. or i.p. dosing at 1.5-3.0 mg/kg of Epo B, using water or DMSO as a vehicle, provided significant tumor shrinkage in these models (T/C range 0.14-0.42). In comparison studies with Epo D, modest (16-50%, T/C 0.84-0.5) tumor volume reduction was observed with Epo B against the human MX-1 mammary and SK-OV3 ovarian adenocarcinoma xenografts implanted in athymic nude mice when administered i.p. in 5 doses every other day (*i.e.*, q2dx5) at 0.3 mg/kg or 0.6 mg/kg (DMSO), and i.v. at 0.6 mg/kg (1:1 Cremophor®/EtOH) (23). Substantial decreases in average body weight (1-3 g) were noted following treatment with Epo B at both dose levels and deaths occurred (3 of 7 mice) at the 0.6 mg/kg dose. Furthermore, complete mortality was observed when Epo B was dosed i.p. in nontumor bearing nude mice at 0.6 mg/kg every day for a total of 4 doses (*i.e.*, qdx4).

Contrary to the reports suggesting that Epo B is not well tolerated in mice, Novartis has published general comments concerning the broad spectrum *in vivo* efficacy of this clinical agent (52). Stable disease or tumor regression was reported with Epo B in human lung, breast, colon and prostate tumor models, following single dose (4-6 mg/kg) or weekly (3-4 mg/kg) i.v. administration. Regressions were also noted in the MDR KB-8511 and HCT-15 tumor models. Interestingly, body weight losses (10-20%) and occasional deaths were observed during treatment, providing additional evidence for a relatively narrow therapeutic index, at least in mice.

The Memorial Sloan-Kettering group also evaluated 3 additional epothilones (Epo D, **37** and **38**) in drug-sensitive and MDR human tumor xenograft models (23-24, 26, 34). In early comparative studies in the MX-1, SK-OV-3 and MCF-7/Adr (MDR breast) adenocarcinoma models, Epo D dosed i.p. (DMSO) at 25-35 mg/kg on a q2dx5 schedule consistently gave superior antitumor effects with lower toxicities than Epo B (23). In the MX-1 experiment, a 15 mg/kg dose of Epo D resulted in 59% tumor growth inhibition (*i.e.*, T/C = 0.41), while the 25 mg/kg and 35 mg/kg dose levels produced a 96% growth inhibition and 1-3 cures (out of 6 mice) at about 20 days posttreatment. Substantial weight loss (> 2 g) was observed at 35 mg/kg, while all mice gained weight throughout dosing at the 25 mg/kg level. An average tumor growth inhibition of about 70% was reported for Epo D at 35 mg/kg doses in both the SK-OV-3 and MCF-7/Adr models. It was noted that 4 of 6 mice died of drug toxicity in the MX-1 and MCF-7/Adr experiments when Epo D was dosed at 15 mg/kg i.v. with Cremophor®/EtOH as the vehicle. A subsequent, comprehensive survey of doses, schedules, formulations and modes of administration for Epo D in MX-1 tumors in nude mice, showed that i.p. injection in DMSO (35 mg/kg, q2dx5) or 6-hour i.v. infusion in Cremophor®/EtOH (30 mg/kg, q2dx5 or 6) provided optimal efficacy with moderate toxicity (24). Utilizing the i.v. dosing schedule noted above for both Epo D and paclitaxel (20-24 mg/kg), Epo D was found to be superior to paclitaxel in the refractory MDR MCF-7/Adr (89 *vs.* 27% average tumor growth inhibition) and CCRF-CEM/TAX (1 cure *vs.* inactive) tumors (24). Again using the same i.v. route of administration, Epo D and paclitaxel both demonstrated remarkable efficacy in the MX-1 and HT-29 xenografts.

The 21-hydroxylated version of Epo D, **37**, demonstrated curative efficacy comparable to Epo D against human HCT116, MX-1, K562 and CCRF-CEM xenografts, when administered over a 6-hour i.v. infusion at 30 mg/kg (26). An added benefit of **37** is the reported 2.5-fold increase in water solubility (25). Despite the impressive *in vitro* profile of conjugated diene **38**, and in particular the antiproliferative effects against drug-resistant cell lines, this synthetic analogue of Epo D was found to be inactive *in vivo* (34). The lack of efficacy in murine tumor models was attributed to poor pharamcokinetic properties, based on the instability of drug in mouse plasma.

In vivo efficacy was demonstrated with i.v. administration of 26-fluoro Epo B, **19**, in 2 human prostate xenograft models: PC3 and MDA PCa 2b, which is a clinically derived, androgen-independent, metastatic (bone) prostate cancer model (30). Three i.v. injections of **19** given weekly (q7dx3) at 10 mg/kg produced tumor growth delays of 45 and 50 days in the PC3 and MDA PCa 2b models, respectively. In a separate MDA PCa 2b study, when compound **19** was dosed at 5 or 10 mg/kg (q4dx2) it was found to be superior to paclitaxel when tested at its maximum tolerated dose (MTD) of 40 mg/kg. Although no drug-related deaths occurred with compound **19**, substantial yet reversible body weight loss (30%) was observed during dosing at 10 mg/kg. Based on these studies in human prostate models, it appears that this close structural analogue of Epo B may have an acceptable therapeutic index, as equivalent doses of Epo B resulted in significant cases of morbidity and mortality.

The preclinical antitumor effects of lactam analogue **29** have been clearly demonstrated in both paclitaxel-sensitive and -resistant murine tumor models (42). In an ovarian carcinoma (A2780) model, mice were treated i.v. with 6.3 mg/kg of **29** every other day for a total of 5 doses (q2dx5) yielding significant antitumor effects as expressed by log cell kill (LCK value of 1 or greater indicating an active response). Lactam **29** compared favorably to paclitaxel in this model, with LCK values of 2 or greater for both agents. This epothilone analogue also showed similar antitumor effects against other paclitaxel-sensitive tumors (human colon carcinomas HCT116 and LS174T). Depending on dose and schedule, this agent was curative in > 50% of the animals bearing HCT116 tumors.

Importantly, **29** demonstrated robust activity against several paclitaxel-resistant tumor models. An MDR variant of HCT116 (HCT116/VM46) was equally sensitive to **29** (LCK = 2.4) as compared to the parent tumor line, whereas paclitaxel was poorly active in this study (LCK = 0.55). In addition to overcoming the MDR mechanism of resistance, **29** was equally effective against A2780Tax which is a tumor model with acquired paclitaxel-resistance due to tubulin mutations (grown from 1A9PTX22 cells, see Background discussion). Three additional paclitaxel-resistant models were developed via direct implantation of patient biopsies into immunocompromised mice: Pat-7 ovarian carcinoma, Pat-21 breast carcinoma, and Pat-26 pancreatic carcinoma. In each case, **29** elicited an active response, which was superior to optimally dosed paclitaxel.

In yet other studies, **29** was shown to have significant antitumor activity using a variety of schedules (q2dx5, q4dx3 and q8dx3), routes of administration (i.v. and p.o.) and tumor host animals (mice and rats) (42). In an uncommon comparison of preclinical agents from different laboratories, **29** was found to be inferior to Epo D against the MX-1 and K562 tumor models (26). As mentioned above, such comparisons of preclinical activity have questionable value unless both agents are compared at optimal dose/schedule and proper control experiments are included in the study.

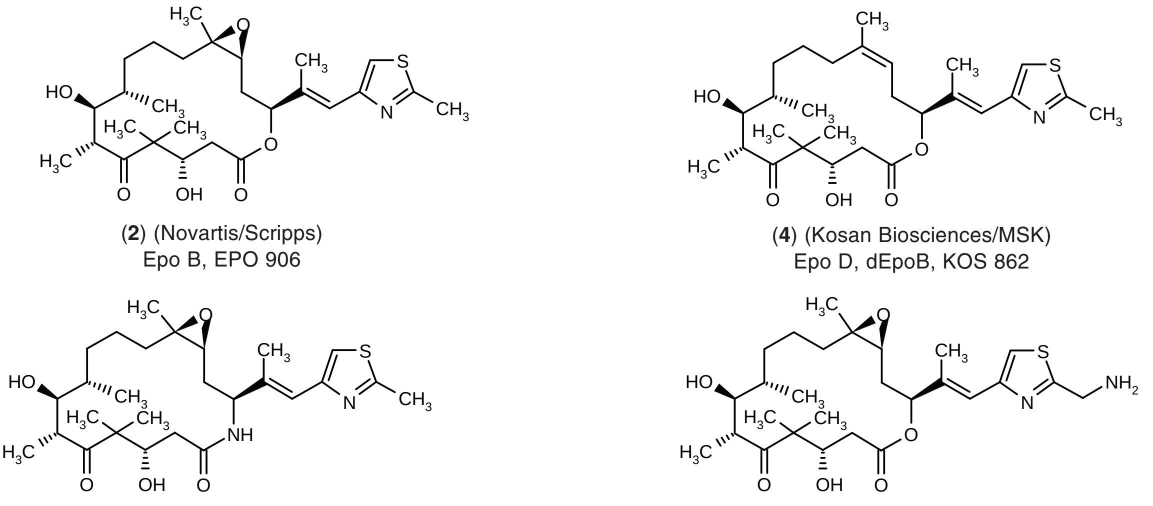
The Bristol-Myers Squibb and GBF collaboration recently reported preclinical *in vivo* activity for a 21-aminoepothilone, **22** (38-39). This derivative has good water solubility (1 mg/ml) and exhibited *in vivo* antitumor effects similar to optimally dosed lactam analogue **29**. For instance, 3 i.v. doses of 12 mpk of **29** administered every 4 days (q4dx3) elicited a potent antitumor effect (LCK = 2.4) in the Pat-7 xenograft model described above (39). Similar antitumor effects were observed in other xenograft models (Pat-21, A2780Tax). Importantly, this epothilone analogue demonstrated *in vivo* activity (LCK > 1) at doses well below the MTD, which hints at the possibility of achieving an acceptable therapeutic window in humans.

Clinical development

Reports in the literature and at scientific meetings indicate that, so far, 4 epothilones have been studied in a clinical setting (Fig. 7): Epo B (**2**, EPO-906), Epo D (**4**, KOS-862), epothilone B lactam (**29**, BMS-247550) and 21-aminoepothilone B (**22**, BMS-310705). Clinical supplies of these materials have been prepared by a variety of means. Fermentation of overproducing mutants of *S. cellulosum* to provide Epo B is quite feasible on a large scale to support drug development. As mentioned earlier, scientists at Novartis and Kosan Biosciences have independently identified the polyketide synthase (PKS) gene responsible for epothilone biosynthesis. Initial studies with heterologous expression of this gene in *S. coelicolor* and *M. xanthus* suggest that future production scale fermentations in other microorganisms will be efficient alternatives (13, 16-17).

As a minor fermentation product of *S. cellulosum*, Epo D has required other methods for production of multigram quantities. Memorial Sloan-Kettering's total synthesis approach has undergone extensive optimization, leading to a practical means for preparing clinical supplies of Epo D (56). Alternatively, Bristol-Myers Squibb has developed a titanium-mediated deoxygenation of Epo B affording a one-step semisynthesis of this agent (36). Kosan has taken the intriguing approach of modifying the biosynthesis to exclude the final epoxidation step, to give Epo D (16-17). Thus, the epothilone PKS was heterologously expressed in a variant of *M. xanthus* which, by design, lacked the P450 epoxidase responsible for the final step in the Epo B biosynthesis. It is likely that this latter method will serve as the preferred method for Kosan's ongoing clinical development of Epo D (57).

As noted above, the fermentation of *S. cellulosum* to provide Epo B is reasonably efficient and can even provide sufficient natural product to support the large scale semisynthesis of other agents. For example, both BMS-247550 and BMS-310705 were prepared by



(**2**) (Novartis/Scripps)
Epo B, EPO 906

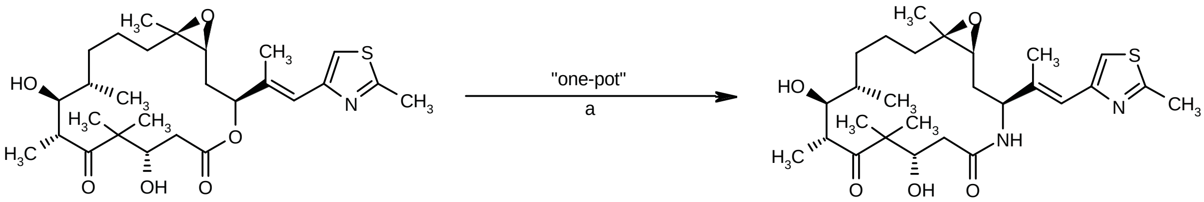
(**4**) (Kosan Biosciences/MSK)
Epo D, dEpoB, KOS 862

(**29**) (Bristol-Myers Squibb/GBF)
BMS-247550

(**22**) (Bristol-Myers Squibb/GBF)
BMS-310705

Fig. 7. Epothilones currently undergoing clinical trials.

Scheme 1: Semisynthesis of Epo B-lactam BMS-247550[a]



(**2**) Epo B

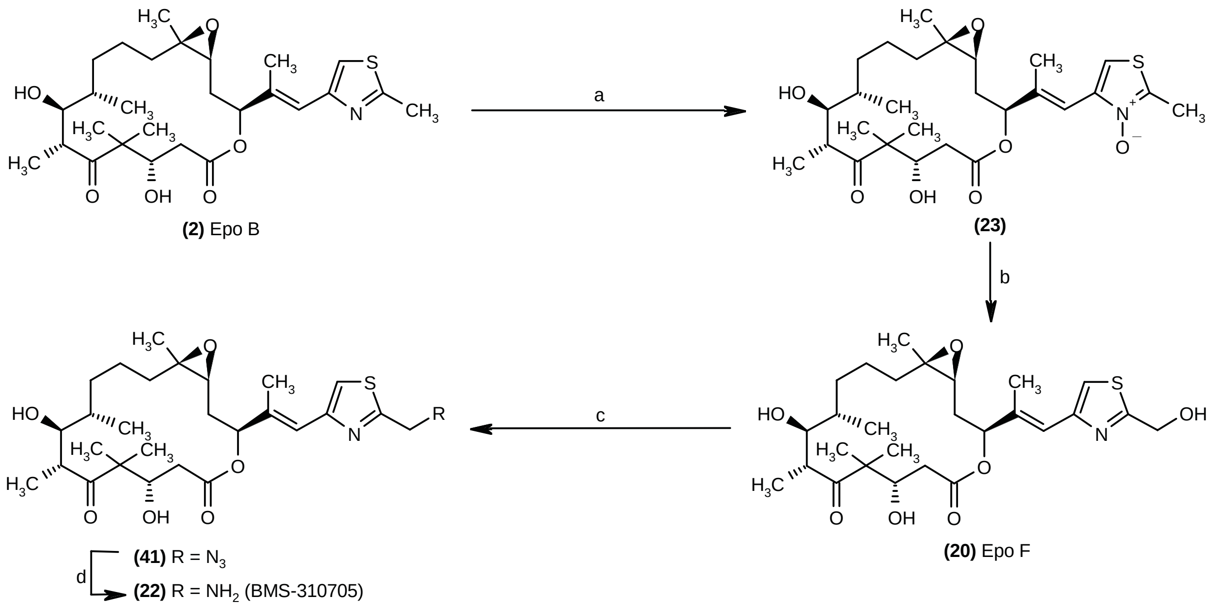
(**29**) BMS-247550

[a](a) Pd(PPh$_3$)$_4$, 10 mol % NaN$_3$, degassed THF-H$_2$O, 45 °C, 20 min, then PMe$_3$, 1-2 h, then MeCN-DMF (20:1), EDCI-HOBt, 25 °C, 4-12 h, 20-25%.

semisynthesis using *S. cellulosum*-derived Epo B as a starting material. The lactone to lactam conversion yielding BMS-247550 required 3 chemical steps which could be achieved in a single reaction vessel without the need for isolation of intermediate products, further adding to the efficiency of the semisynthesis (Scheme 1) (41, 58). GBF's semisynthesis of BMS-310705 from Epo F involved a 2-step process as shown in Scheme 2 (38). Epothilone F itself was obtained from Epo B by chemical means (40) or by biotransformation (19).

The clinical evaluation of epothilones began in early 1999 with Novartis' phase I trials of EPO-906 (Epo B). Most of the clinical data in this field are only available in the form of meeting abstracts and posters. However, a much needed review of the early phase I data was recently published (59). The primary objectives of phase I trials are to assess drug safety and define a recommended dose for advanced clinical trials. It is becoming clear that the nature and extent of toxicities of the epothilones are highly dependent on the dosing schedule and method of administration. Two EPO-906 trials have examined i.v. dosing at 7-day (36 patients) and 21-day (42 patients) intervals (60-61). In both of these trials, maximum tolerated doses (MTD) of 2.5 mg/m^2 and 8 mg/m^2, respectively, elicited gastrointestinal side effects (diarrhea) as the dose limiting toxicity (DLT). While other toxicities were minimal for patients on the 7-day schedule, the higher dose on a 21-day schedule caused fatigue, nausea and peripheral neuropathy as secondary side effects. Obtaining objective responses is difficult in a patient population having advanced disease and extensive histories of prior chemotherapy or radiation therapy. Still, 2 partial responses were noted in the 7-day interval trial and 1 partial response was noted in the 21-day interval trial. Phase

Scheme 2: Semisynthesis of BMS-310705[a]



(2) Epo B

(23)

(20) Epo F

(41) R = N_3

(22) R = NH_2 (BMS-310705)

[a](a) *m*-CPBA, CH_2Cl_2, 48%; (b) 1. $(CF_3CO)_2O$, 2,6-lutidine, CH_2Cl_2, 2. aq. NH_4OH, MeOH, 81% for two steps; (c) DPPA, DBU, THF, 94%; (d) PMe_3, THF-H_2O, 91%

II evaluation of EPO-906 is in progress, but there have been no additional reports on the clinical efficacy of this agent.

A recent report described the preliminary clinical evaluation of KOS-862 (Epo D), sponsored by Kosan Biosciences (62). The trial was at an early stage of dose escalation and pharmacokinetic analysis of a 21-day interval dosing schedule. At this stage of the ongoing trial, there were no reported DLTs or objective responses in the 21 patients included in the study. Other phase I trials with KOS-862 are reportedly underway to examine daily and weekly dosing schedules.

In this drug class, BMS-247550 is by far the most extensively studied agent owing in part to the robust collaboration between Bristol-Myers Squibb and the National Cancer Institute. Phase I trials sponsored by these groups involved similar dosing schedules as those used with EPO-906. Results from 3 trials exploring a 21-day interval between doses indicated that the MTD was 40-50 mg/m^2 (63-65). In general, prolonged neutropenia, peripheral neuropathy and fatigue were dose limiting. A case of hypersensitivity, perhaps due to formulation with Cremophor EL®, resulted in the addition of antihistamine pretreatment to the regimen. Among these studies several partial responses in cancers of the lung, breast, ovary and skin were noted. Interestingly, a pharmacodynamic analysis indicated that there is a positive correlation of microtubule bundle formation in peripheral mononuclear cells and plasma drug exposure in patients receiving i.v. doses of BMS-247550 (66).

With this dosing regimen of BMS-247550 (50 mg/m^2 infused over 1 h every 21 days), similar toxicities were observed in 4 phase II studies, which resulted in dose reduction or other treatment modification. In a phase II trial in soft tissue sarcoma (24 patients), overall survival benefit is pending but time to disease progression was short (67). A second study in metastatic gastric cancer (23 patients) exhibited 9% partial responses and 52% stable disease (68). In a third phase II study in non-small cell lung cancer (NSCLC; 29 patients) where patients had received prior platinum-based therapy, 14% of patients on BMS-247550 had a partial response and 45% of patients had stable disease (69). Lastly, in a trial in metastatic breast cancer (27 patients), partial responses were observed in 25 and 63% of taxane-refractory and taxane-naive patients, respectively (70). Including patients with stable disease, the response rates climbed to 75 and 89%, respectively. Based on the cumulative nature of the neurotoxicity and experience in other ongoing trials (*vide infra*), most of these studies were modified and results are pending on alternate approaches such as one using the same schedule but a lower dose (32 or 40 mg/m^2) infused over 3 h or a second regimen involving daily dosing for 5 days (6 mg/m^2) followed by a 2-week treatment break.

In 2 phase I studies (53 patients total) evaluating a 7-day interval between doses of BMS-247550, the MTD fell in the 20-30 mg/m^2 range depending on infusion time and patient histories of prior chemotherapy (71-72). Severe fatigue was a side effect common to both studies, whereas only one of the studies highlighted neutropenia as dose limiting. In one of the studies, the cumulative nature of peripheral neuropathy was addressed by introducing treatment breaks after 3 consecutive weekly doses (72). This latter study noted 3 partial responses (colon, ovary and head/neck) and also reported pharmacokinetic parameters including appreciable oral bioavailability of BMS-247550 (%F = 54 at a 25 mg/m^2 dose).

In yet a different schedule of administration, a phase I study (27 patients) investigating 5 daily doses followed by a 16-day rest period determined a MTD of 6 mg/m^2 (73). Compared to other regimens, this schedule appeared to attenuate many of the previously observed side effects. A majority of patients on the study had received prior taxane therapy, and still, objective responses were noted in breast and cervical cancer patients. Importantly, the findings from this single study have greatly influenced the direction of several ongoing phase II trials, as noted above.

Early results from 2 phase I studies evaluating combination therapy have been reported. In one study, patients with advanced metastatic prostate cancer received up to 40 mg/m^2 of BMS-247550 (i.v.; once every 21 days) in combination with estramustine phosphate (74). At a dose of 35 mg/m^2, side effects were minimal. Significant reduction in PSA measurements and improvement in other clinical disease markers prompted the initiation of phase II evaluation of BMS-247550 in this setting. A second combination study (24 patients) with carboplatin noted that unexpected severe hematologic toxicity resulted in readjusting the carboplatin dose (75). Two partial responses were noted in this latter study.

The newest epothilone to enter clinical evaluation is BMS-310705 (76). In one phase I trial (29 patients), the drug appeared to be tolerated at doses up to 50 mg/m^2 and exhibited 2 partial responses (lung, ovarian). Importantly, no hypersensitivity reactions were observed, perhaps due to the improved water solubility of BMS-310705 and the absence of Cremophor EL® in the drug formulation.

Summary and outlook

The dedicated efforts of many chemists, pharmacologists and oncologists have combined with the activities of countless regulators, hospital administrators and patients, resulting in a remarkable worldwide endeavor to bring epothilone-related drugs closer to commercialization and the therapeutic benefit of cancer patients. The clinical results to date suggest that 1 or more of these agents will likely find a place in cancer therapy. It should be no surprise that the clinical development of these agents has not been straightforward. Cytotoxic agents have historically required dutiful experimentation with dose, schedule, formulation and tumor type before settling on optimal modes of administration. It is not clear if these agents will supplant current tubulin-targeted therapies (*i.e.*, taxanes), since additional clinical experience is certainly required before making such an assessment. It is clear, however, that the potential to exploit tubulin polymerization as a chemotherapy target with a drug that can overcome resistance mechanisms has galvanized a significant portion of the oncology community.

Future hurdles for these agents include finding the right tumor types and dosages for most effective single agent therapy, laying out a clear path to rapid identification of optimal combination therapies and dealing with the clinical emergence of epothilone resistance. The first of these will likely be addressed by additional clinical investigation and the incorporation of pharmacogenomic methods to aid in identifying appropriate patient populations. As noted above, combination studies of epothilones and other drugs are underway but these efforts should be supported by additional *in vitro* studies to help define synergistic drug combinations with the greatest potential for clinical benefit. Lastly, in depth understanding of epothilone resistance at the molecular level will aid in the development of new drugs that are not crossresistant with clinically investigated epothilones (66, 77). As demonstrated in this account, a growing array of promising new epothilones offers significant potential for addressing these hurdles in the future.

Acknowledgements

The authors respectfully acknowledge Mr. Lyndon Cornelius, Dr. David Lebwohl, Dr. Francis F.Y. Lee, Dr. Thomas Griffin and Dr. John Hunt for critical reviews of this manuscript.

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