

Epothilones and Their Analogs – Potential New Weapons in the Fight Against Cancer

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Abstract: Epothilones are a new class of microtubule depolymerization inhibitors, which inhibit the growth of a broad range of human cancer cell lines *in vitro* with low nM or sub-nM IC₅₀s. Unlike many other cytotoxic anticancer agents epothilones are also active *in vitro* against multidrug-resistant cell lines and epothilone B exhibits potent *in vivo* antitumor activity in multidrug-resistant tumor models. We have prepared various types of synthetic and semi-synthetic analogs of epothilones and we have studied the effect of the corresponding structural modifications on *in vitro* tubulin polymerization and antiproliferative activity. Epoxide ring opening, replacement of the epoxide moiety by amide groups or a 1,2-disubstituted imidazole ring, or the incorporation of phenylene moieties in the C(9)–C(12) region all lead to a substantial loss in *in vitro* activity. In contrast, expansion of the 3-membered oxirane ring to a 5-membered 1,3-dioxolane system, either *cis*- or *trans*-fused to the 16-membered macrocycle, is reasonably well tolerated. Substitution of a 2-methyl benzothiazole moiety for the natural (2-(2-methyl-thiazol-4-yl)-1-methyl-)ethenyl side-chain results in analogs with more potent antiproliferative activity than natural epothilones.

Keywords: Antiproliferative activity · Antitumor activity · Epothilones · Pharmaceutical chemistry · Potent analogs · Structure-activity relationships

1. Background and Introduction

The effective treatment of cancer is one of the major problems in modern medicine and the search for novel anticancer drugs represents one of the most important, but also one of the most challenging endeavors in contemporary drug discovery. An important milestone in the history of modern cancer chemotherapy is marked by the introduction into clinical practice of Taxol® (paclitaxel) in 1992, a compound which has been hailed to represent 'the perhaps most important addition to the chemotherapeutic armamentarium against cancer over the past several decades' [1]. Unlike most other traditional cytotoxic anticancer drugs, which inter-

fere with some aspect of DNA replication or nucleotide metabolism [2], paclitaxel owes its growth inhibitory activity to the ability to interact with cellular microtubules and to prevent proper passage of cells through mitosis and ultimately cell division [1][3].

Microtubules are hollow filaments of *ca.* 240 Å diameter, which are composed of the 55 kD proteins α - and β -tubulin as the constituent subunits. Apart from many other critical functions, microtubules are of particular importance for the formation of the mitotic spindle (an array of microtubules with the shape of an American football), which provides the structural framework for the physical segregation of sister chromatids in the process of cell division. A critical requirement for the proper assembly of the mitotic spindle and the subsequent movement of the sister chromatids to the spindle poles is the ability of microtubules to lengthen and shorten in response to specific cellular signals. This phenomenon is commonly referred to as 'dynamic instability' and it is now believed that micro-

tubule-interacting drugs exert their effect by inhibition of microtubule dynamics [4]. However, notwithstanding this appealing unifying model, microtubule-interacting drugs can be divided into two distinct categories, namely those which *destabilize* microtubules and inhibit tubulin polymerization *in vitro*, such as the vinca alkaloids, and those which lead to *increased* microtubule stability, such as paclitaxel and docetaxel (Taxotere®) [5]. After its mechanism of action had been established in 1980 [6], paclitaxel (including its analogs or derivatives) was the only compound known to act as a microtubule stabilizer for more than ten years. It was only in 1995 that a second group of natural products, *epothilones A and B* (Fig. 1) was recognized by a group at Merck Research laboratories to share paclitaxel's ability to stabilize preformed microtubules under depolymerizing conditions and to promote the polymerization of tubulin heterodimers *in vitro* [7][8]. Microtubule binding was shown to be competitive with paclitaxel, which suggests that epothilones and paclitaxel

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bind to the same, or at least largely overlapping, site(s) on β -tubulin [7].

Until that time the discovery of epothilones by the groups of Reichenbach and Höfle several years earlier [12]

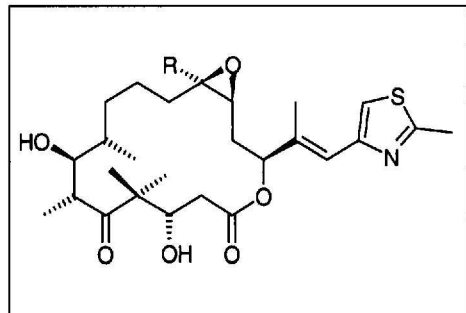


Fig. 1. Structures of epothilones A (R = H) and B (R = CH₃).

had gone almost unnoticed, but the elucidation of their paclitaxel-like mode of action followed by the disclosure of their absolute stereochemistry in mid-1996 [13] has triggered widespread interest in these molecules throughout the scientific community and has made them the subject of drug discovery programs in several major pharmaceutical companies. In addition to the mechanistic similarities between epothilones and paclitaxel, these activities were further fuelled by the fact that epothilones, in contrast to paclitaxel, were found to be only poor substrates for the P-gp efflux pump (a 170 kd transmembrane protein) and therefore were also active *in vitro* against P-gp over-expressing, paclitaxel-resistant human cancer cell lines [7][14]. This suggests that epothilone-derived anticancer drugs might eventually be useful for the treatment of drug-resistant tumors.

The widespread interest in the chemistry and biology of epothilones is reflected in the fact that they have been the target of numerous total syntheses [15]. In addition, a host of synthetic [15][16] and semi-synthetic [17] analogs of epothilones have been reported in the literature, mostly originating from the laboratories of Danishefsky [16] and Nicolaou [15]. This has led to a rather comprehensive understanding of the structure-activity relationship (SAR) of this class of natural products in a remarkably short (given the complexity of the molecules) period of time. Major drug companies which, based on the patent literature, have initiated epothilone-related drug discovery programs include Schering AG, Bristol-Myers-Squibb (BMS), and Novartis. In addition, a significant drug discovery effort in the epothilone area is underway at the Sloan-Kettering Cancer Center in New York [16].

In this report we want to review some selected aspects of our epothilone-based drug discovery program at Novartis. This will include the biochemical and biological characteristics of natural epothilones as well as the synthesis and *in vitro* SAR of specific synthetic and semi-synthetic analogs. To put our own work into context, we shall also briefly review the most relevant general features of the epothilone SAR, as they have emerged over the last three years from the work of different laboratories. However, it is clearly beyond the scope of this article to provide a comprehensive overview on every aspect of the biology and SAR of epothilones that has been studied in the literature to date [15][16].

2. Epothilones A and B – *In vitro* and *in vivo* Biological Activity

As indicated above, epothilones, like paclitaxel, are microtubule-stabilizing agents, *i.e.* they can prevent the Ca²⁺ or cold-induced depolymerization of pre-formed microtubule polymers *in vitro* [14]. The microtubule-stabilizing properties of these compounds are also manifest in their ability to *promote* the polymeri-

zation of tubulin heterodimers into microtubule polymers in the absence of guanosine triphosphate (GTP), which is usually required in the polymerization reaction [5]. Induction of tubulin polymerization, rather than microtubule stabilization itself, is commonly used as a biochemical readout to assess the interaction of microtubule stabilizers with tubulin in a quantitative fashion. This may either involve the determination of absolute EC₅₀-values (*i.e.* the concentration of compound required to produce half-maximal polymerization of tubulin under a specific set of experimental conditions); alternatively, the degree of tubulin polymerization may be determined at a single, *fixed* compound concentration and compared to the effect achieved by a specific reference compound (*e.g.* GTP, paclitaxel, or epothilone B at defined concentrations). Although the percentage values produced by the latter method do not represent an exact quantitative measure for the ability of a compound to interact with tubulin, they are still useful for an orientating initial categorization in the course of SAR studies with an extended set of analog structures (*vide infra*).

As illustrated by the tubulin polymerization data summarized in Table 1, pac-

Table 1. Induction of tubulin polymerization by epothilones and paclitaxel.

	% Tubulin polymerization ^{a)}	EC ₅₀ [μ M] ^{b)}
Epothilone A	69	1.12
Epothilone B	90	0.67
Paclitaxel	49	1.88

^{a)} Induction of polymerization of porcine brain microtubule protein by 2 μ M of test compound relative to the effect of 25 μ M of epothilone B, which gave maximal polymerization (85% of protein input). ^{b)} Concentration required to achieve half-maximal tubulin polymerization. Tubulin polymerization was determined using a modified version of the centrifugation assay described in [18].

Table 2. IC₅₀-values [nM] for net growth inhibition of human carcinoma cell lines by epothilone A and B in comparison to paclitaxel^{a)}

Cell Line	Epothilone A	Epothilone B	Paclitaxel
A549 (lung)	2.67 \pm 0.71 (15)	0.23 \pm 0.05 (15)	3.19 \pm 1.52 (16)
HCT-116 (colon)	2.51 \pm 0.43 (17)	0.32 \pm 0.06 (17)	2.79 \pm 1.64 (19)
PC-3M (prostate)	4.27 \pm 0.52 (16)	0.52 \pm 0.10 (16)	4.77 \pm 3.01 (17)
MDA-MB-231 (breast)	1.31 \pm 0.51 (15)	0.13 \pm 0.04 (15)	1.43 \pm 0.87 (16)

^{a)} Cells were exposed to drugs for 3–5 d, allowing for at least two population doublings. Cell numbers were estimated by quantification of protein content of fixed cells by methylene blue staining [19]. Data are presented as mean \pm SD, with the number of individual experiments indicated in parentheses.

Table 3. IC₅₀-values [nM] for growth inhibition of drug-resistant human cancer cell lines by epothilones in comparison to paclitaxel. Values in parentheses indicate relative resistance, i.e. IC₅₀ (drug-resistant line)/IC₅₀ (parental line)^{a)}.

Cell Line	Epothilone A	Epothilone B	Paclitaxel
KB-31 (epidermoid)	2.10	0.19	2.31
KB-8511 ^{b)}	1.90 (0.9)	0.19 (1.0)	533 (230)
1A9 (ovarian)	2.00	0.06	2.00
1A9PTX22 ^{c)}	3.00 (1.5)	0.10 (1.7)	43 (22)
MCF-7 (breast)	1.49	0.18	1.80
MCF-7/ADR ^{d)}	27.5 (18)	2.92 (16)	9105 (5060)

^{a)} For experimental details cf. legend of Table 2. ^{b)} P-gp overexpression/MDR [20a].
^{c)} Ala364→Thr mutation in β-tubulin [20b]. ^{d)} Multiple resistance mechanisms/MDR.

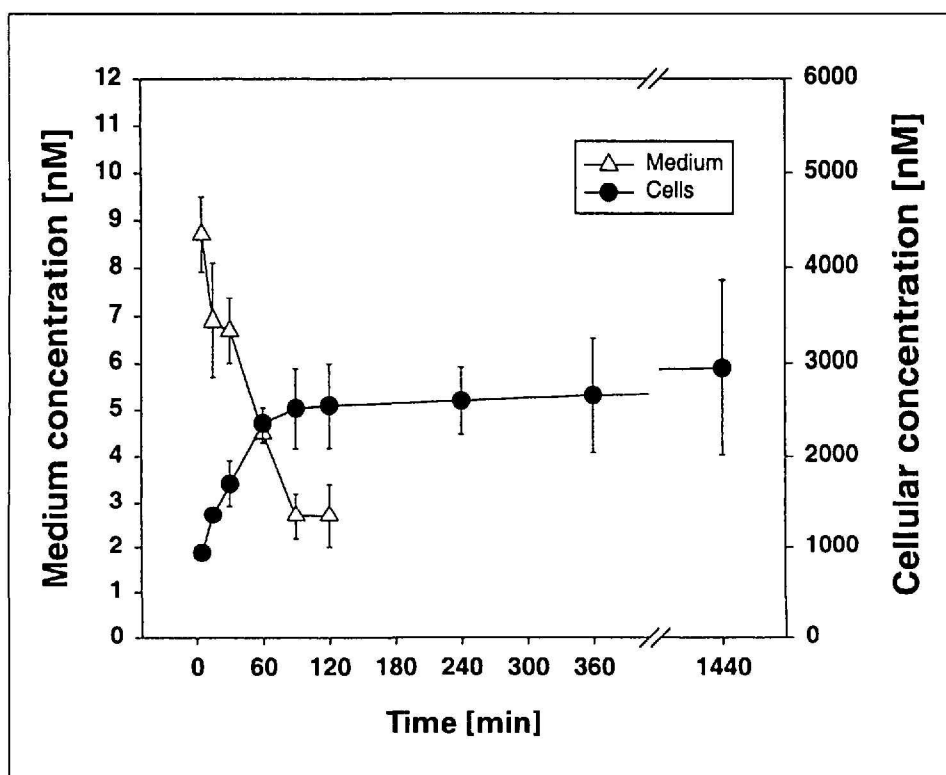


Fig. 2. Time-dependent accumulation of epothilone B in HeLa cells. HeLa cells were seeded into 10 cm dishes (1×10^6 cells/dish) and allowed to proliferate to approximately 70% confluence. Epothilone B was pre-diluted in DMSO and added to medium at a final concentration of 10 nM (vehicle concentration 0.1%). Following incubation for the indicated time periods, cells were washed with phosphate-buffered saline and harvested. Cell pellets were extracted with acetonitrile and the cell-associated amount of drug was quantified by μ -HPLC-MS. Cellular drug concentrations were calculated based on an estimated single cell volume of 2.8 pL. Data are presented as mean \pm SEM of 3 independent experiments. Medium values for time points beyond 120 min were below the limit of quantification (1.7 nM).

litaxel, epothilone A, and epothilone B all promote *in vitro* tubulin polymerization with low μ M EC₅₀s. Both epothilones are more potent inducers of tubulin polymerization than paclitaxel, with epothilone B being the most active of the three compounds (EC₅₀ = 0.67 μ M vs. 1.12 μ M for epothilone A and 1.88 μ M for paclitaxel).

At the cellular level, interference with microtubule functionality during mitotic spindle formation causes cell cycle arrest at mitosis following exposure of cells to nM concentrations of epothilones. This is eventually followed by cell demise through programmed cell death (apoptosis). Accordingly, epothilones inhibit the growth of a broad range of human cancer

cell lines *in vitro* with nM or even sub-nM IC₅₀s. This is illustrated in Table 2 for a selected set of four human cancer cell lines, each representing one of the major types of solid human tumors.

Epothilone B is a more potent inhibitor of human cancer cell growth than paclitaxel, which is in line with its more pronounced effects on microtubule stability *in vitro*. The antiproliferative activity of epothilone A is comparable to that of paclitaxel. We and others [7] have demonstrated that EC₅₀s for epothilone-induced mitotic arrest correlate closely with IC₅₀s for cytotoxicity (cell death), but the molecular mechanisms connecting these events have not been elucidated.

One of the most significant differences between epothilones and paclitaxel, but also other standard cytotoxic anticancer agents, consists in the fact that the former can also inhibit the growth of multidrug-resistant cancer cells [7][14]. This is illustrated in Table 3 for a series of drug-sensitive/drug-resistant cell line pairs (with the resistant line being derived from the sensitive one through selection with a standard cytotoxic agent), which are characterized by different types of resistance mechanisms.

As pointed out in the introductory section, the activity of epothilones against multidrug-resistant cells *in vitro* holds the promise that epothilone-derived anticancer agents might eventually be useful in the treatment of multidrug-resistant tumors, which represents one of the most serious problems in cancer chemotherapy.

Regarding the correlation between tubulin polymerization effects and antiproliferative activity, there appears to be a discrepancy between the fact that the induction of tubulin polymerization by epothilones *in vitro* is only achievable with μ M EC₅₀s, whereas cellular activity is observed at nM concentration levels. We have been able to resolve this apparent discrepancy by demonstrating that epothilones (like paclitaxel [21]) accumulate intracellularly several hundred-fold over external medium concentrations, such that nM medium concentrations in cell culture experiments effectively translate into μ M concentration levels inside cells (Fig. 2). Intracellular epothilone concentrations thus become comparable to the concentrations required to affect tubulin polymerization and microtubule stability *in vitro*.

Based on the above *in vitro* data we have conducted extensive studies to characterize the *in vivo* antitumor activity of epothilone B. As a general conclusion,

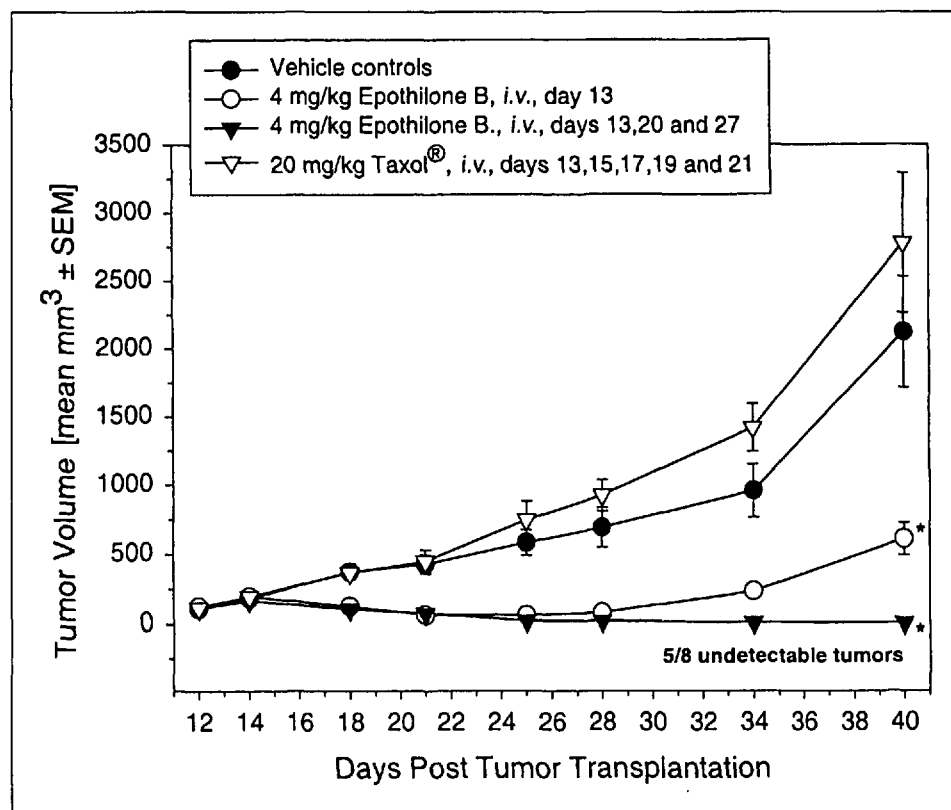


Fig. 3. Antitumor effects of epothilone B and Taxol[®] against subcutaneously transplanted human KB-8511 epidermoid carcinoma in female BALB/c nude mice. Tumor fragments of approximately 25 mg were transplanted into the left flank of each female BALB/c *nu/nu* mouse ($n = 8$ per group). Intravenous treatments were started on day 13 after tumor transplantation. Taxol[®] was given at 20 mg/kg every second day for 10 days (d 13, 15, 17, 19, 21). Epothilone B was administered once at 4 mg/kg on day 13, or once weekly at 4 mg/kg (d 13, 20, 27). The experiment was terminated on day 40, when the animals were sacrificed (except for mice whose tumors had regressed to an undetectable size). Statistical analyses of differences in tumor volumes and body weights used Dunnett's test to compare treatment groups to controls. Data with p -values of < 0.05 for tumor volumes and body weights at the end of the experiment vs. start of treatment are indicated (*). Starting weight of mice was between 25 and 30 g.

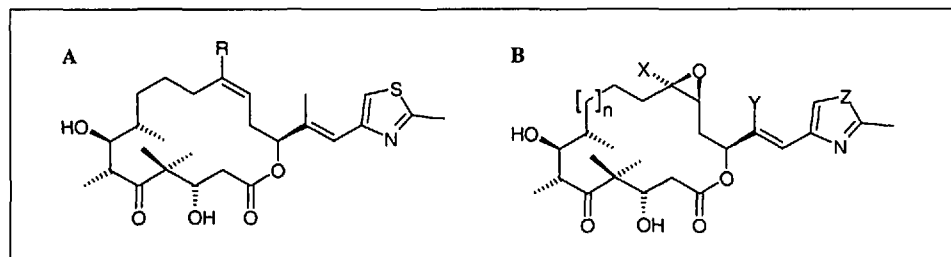


Fig. 4. Structural templates for the discussion of various aspects of the epothilone SAR (for explanations *cf.* text)

epothilone B has proven to be a potent inhibitor of tumor growth in a broad range of nude mouse xenograft models (*i.e.* nude mice bearing solid human tumors), albeit with a relatively narrow therapeutic window. As an example the data summarized in Fig. 3 illustrate the effect of the compound on the growth of P-gp overexpressing Taxol[®]-resistant human epidermoid carcinoma KB-8511.

While Taxol[®] is completely ineffective in this model, *i.v.* treatment with epothilone B, either given as a single

dose of 4 mg/kg or on a weekly schedule of 4 mg/kg (3 administrations), results in profound inhibition of tumor growth and tumor regressions. For the weekly regimen 5 out of 8 mice were found to be tumor-free even 60 d after the end of the treatment period.

Based on its potent *in vivo* antitumor activity in Taxol[®]-resistant models, Phase I clinical trials with epothilone B have been initiated by Novartis in 1999. However, it should be kept in mind that epothilone B treatment in experimental

animal models at efficacious doses is also associated with significant body loss. (The maximum body weight loss observed in the above KB-8511 experiment was 12% for the weekly dosing regimen (d 34) and 7% after single dose administration (d 14)). The availability of analogs with an improved therapeutic index would thus be highly desirable and this premise forms the basis for our analog and derivatization program, part of which will be discussed in the following section.

3. Synthesis and SAR of Epothilone Analogs

3.1. General Aspects

Driven by the intriguing biological profile of epothilones, substantial efforts have been made over the last three years to establish a comprehensive understanding of the SAR for this new class of microtubule inhibitors [15–17]. Some of the most relevant SAR features that have emerged from this work, originating mostly from the laboratories of Nicolaou and Danishefsky, are summarized in the following (Fig. 4):

- The presence of a C(12)-C(13) epoxide moiety is not absolutely required for efficient microtubule stabilization and potent antiproliferative activity, which decreases only about 10-fold upon reduction of the epoxide moiety to a *cis* olefin (Fig. 4A, $R = H, CH_3$) [22]. Likewise, the BMS group has recently demonstrated that the replacement of the oxirane ring by a cyclopropane, thiiran, or variously N-substituted aziridine moieties is well tolerated [23].
- Modification of the 26-methyl group in epothilone B through incorporation of relatively small and apolar substituents (Fig. 4B, $X = CH_2F, CH_2Cl, C_2H_5, n-C_3H_7, CH=CH_2, Y = CH_3, Z = S$) produces analogs which are only slightly less potent than epothilone B itself [22]. Polar substituents (*e.g.* $X = CH_2OH$) may lead to a significant decrease in activity [24].
- Ring contraction or expansion via the removal of existing or the incorporation of additional CH_2 -groups in the C(9)-C(11) region (Fig. 4B, $n = 0, 2, 3, X = H, Y = CH_3, Z = S$) both cause a substantial loss in biological potency [25].
- Removal of the C(8) methyl group [26a], simultaneous inversion of stereochemistry at C(6) and C(7) as well as inversion of stereochemistry at C(3) are all detrimental for biological activity [22a].

v) The replacement of the thiazole ring either by an oxazole (Z = O) [22a] or various pyridine moieties [27] is well tolerated. Likewise, the presence of the allylic methyl group at C(16) is not required for potent biological activity (Y = H) [28]. Substitution of a simple phenyl moiety for the 2-methyl thiazole ring in epothilone B causes a decrease in antiproliferative activity of *ca.* one order of magnitude [28].

The lactam analog **1** of epothilone B has recently been reported by the BMS group to possess antiproliferative activity which is comparable to that of epothilone B (Fig. 5) [23b].

In a collaboration with Prof. Schinzer at the University of Magdeburg we have independently prepared the lactam analog **2** of deoxyepothilone A [29], which we found to be *ca.* 20-fold less potent than deoxyepothilone A on the KB-31 cell line (Fig. 5). Likewise, the epothilone B lactam **1** in our cellular system proved to be *ca.* 10-fold less active than epothilone B itself (Fig. 5). Danishefsky has recently reported the total synthesis of the lactam analog **3** of deoxyepothilone B (Fig. 5) [30], which he found to be '4.8-fold less active than deoxyepothilone B'. Compound **1** is currently undergoing clinical trials sponsored by BMS [23b].

3.2. Modifications at C(12) and/or C(13)

One of the initial directions in our analog program involved the introduction of structural changes in the epoxide region (C(12) and C(13)), either through direct chemical modification of the natural products or through total synthesis of specific analogs which could not be accessed from the natural products as starting materials. This strategy was driven by synthetic as well as structural considerations: On one hand, the epoxide moiety

was perceived to represent the most reactive functionality in the molecules, offering the opportunity to prepare a variety of different derivatives through simple nucleophilic ring opening reactions; on the other hand, previous work had demonstrated that the epoxide oxygen was not absolutely essential for potent antiproliferative activity, thus raising the question whether other structural elements (apart from a *cis* double bond; *vide supra*) might be tolerated at the C(12)/C(13) site.

As illustrated in Scheme 1, acid-catalyzed hydrolytic epoxide opening in epothilone A leads to a inseparable mixture of *trans* diols **4a/b**, which were then further elaborated into the corresponding acetonides **5a** and **5b** [31]. It should be noted that acid-catalyzed epoxide ring opening in epothilones requires very carefully controlled reaction conditions; otherwise various types of rearrangement reactions take precedence over simple epoxide hydrolysis (*cf.* also [17c]).

The epothilone A-derived C(12)/C(13) *cis* diols were obtained *via* deoxyepothilone A, which was prepared through reduction of epothilone A with 3-methyl-2-selenoxo-benzothiazole [32] (Scheme 1). Standard *cis*-dihydroxylation with OsO₄/NMO gave *cis* diols **6a** and **6b**, which were again converted into the respective acetonides **7a** and **7b** [31]. Reaction of epothilone A with nucleophile sources other than water (HCl, MgBr₂·xEt₂O, NaI/TMS-I, LiN₃, Mg(OMe)₂) leads to preferential, but not completely selective ring opening at C(12). Very similar chemistry has been independently reported by the group of Höfle [17c].

None of the various diols **4a/b**, **6a/b** or amino alcohol **9** showed any appreciable biological activity, with IC₅₀s for growth inhibition of KB-31 or KB-8511 cells being above 1 μM in all cases. In contrast, azido alcohol **8** is at least 10-fold more potent (IC₅₀s on KB-31 and

KB-8511 cells of 61 nM and 64 nM, respectively), which may indicate that the loss in activity for **4a/b**, **6a/b** and **9** cannot be simply ascribed to increased conformational flexibility [33]. However, the interpretation of changes in cellular activity is not straightforward, as they may be caused by a combination of changes in target affinity, cellular penetration, and metabolic stability. We have not determined EC₅₀-values for induction of tubulin polymerization for any of the above compounds, but none of them (including **8**) showed measurable induction of tubulin polymerization at 2 μM compound concentration (*vs.* 69% for epothilone A) [34].

As illustrated in Table 4, a dramatic increase in antiproliferative activity is observed upon conversion of **4a** and **6a** into the corresponding acetonides **5a** and **7a**, which are 30 to 100-fold more potent than their respective diastereoisomers **5b** and **7b** [31] and only 10 to 15-fold less potent than epothilone A itself. This indicates that the size of the ring fused to the C(12)–C(13) single bond can be significantly increased without a dramatic loss in biological potency. Moreover, the data for **5b** also illustrate that, given the proper absolute stereochemistry at C(12) and C(13), activity is retained even upon moving from a *cis*- to a *trans*-fused system. This is in agreement with previous results obtained for *trans*-epothilones A [22][35].

A different approach to C(12)/C(13) modifications in epothilones is highlighted by the synthetic analog structures **10–12** shown in Fig. 6 which incorporate an N-alkyl amide group (**10**, **11**) or a 1,2-disubstituted imidazole moiety (**12**) in place of the C(12)/C(13) epoxide [36].

Assuming a preference of the N-alkyl amide bond for a *cis* conformation (as a consequence of conformational constraints imposed by the macrocycle), these latter analogs were conceived as potential mimetics of deoxyepothilone B (*cf.* Fig. 5) [37]. Unfortunately, all of these compounds are at least 1000-fold less potent antiproliferative agents than deoxyepothilone B (Fig. 5), indicating that the simple stabilization of a *cis*-conformation about the C(12)–C(13) bond does not suffice to produce potent biological activity [37]. Both steric as well as polarity arguments might be raised to rationalize the lack in activity of compounds **10–12**, but these arguments are difficult to reconcile with the data obtained for analogs **5a** and **7a** (which are also characterized by a substantial increase in steric bulk in the C(12)/C(13)

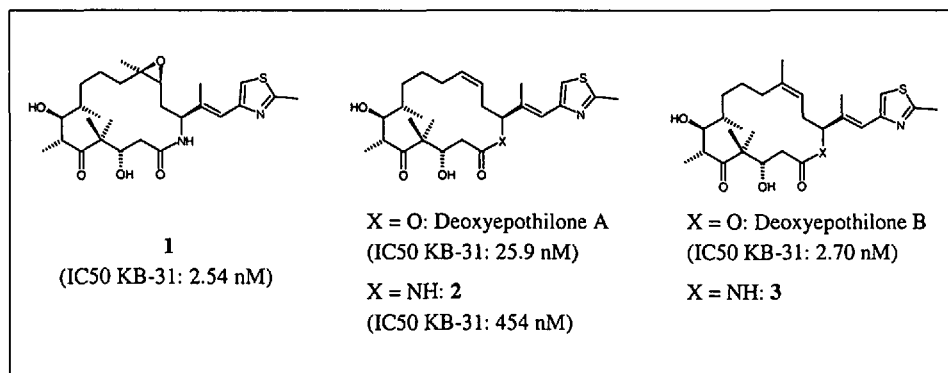
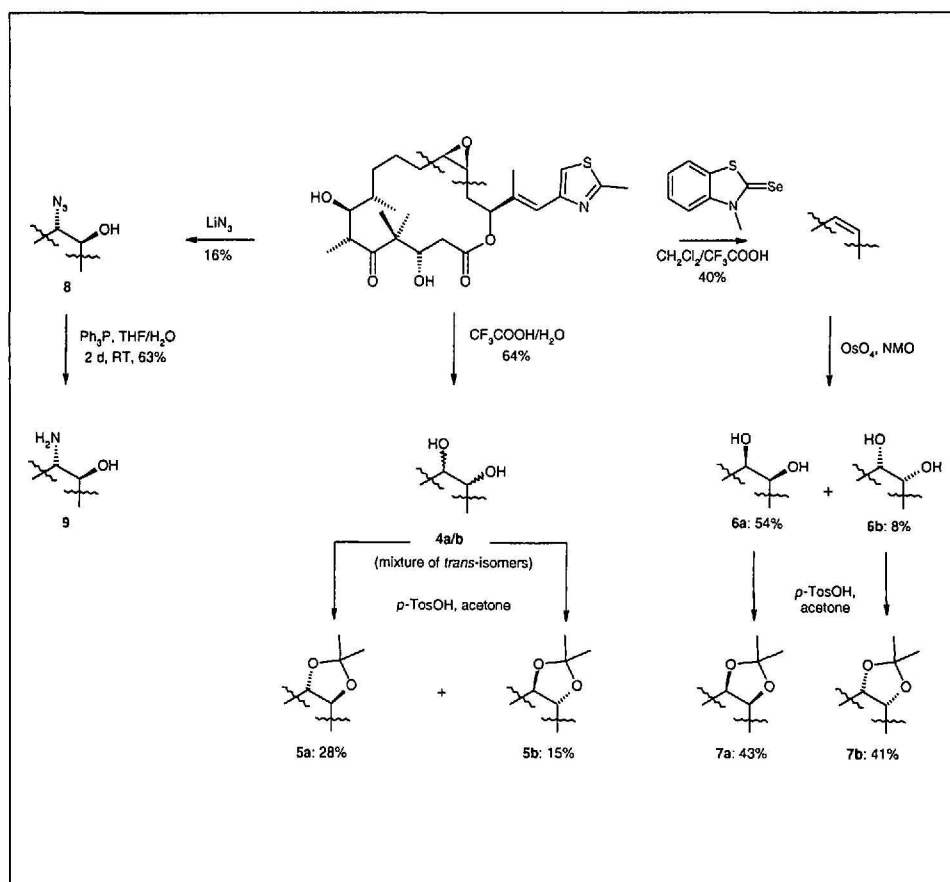


Fig. 5. Lactam-based analogs of epothilones. Values in parentheses refer to growth-inhibitory activity against the human epidermoid cancer cell line KB-31.



Scheme 1. Synthesis of C(12)/C(13)-modified analogs of epothilone A.

Table 4. Induction of tubulin polymerization and growth inhibition of human carcinoma cell lines by C(12)/C(13)-modified epothilone analogs **5** and **7**^a.

Compound	%Tubulin Polymerization	IC ₅₀ KB-31 [nM]	IC ₅₀ KB-8511 [nM]
5a	26	23	10
5b	< 10	> 1000	> 1000
7a	17	30	17
7b	< 10	> 1000	> 1000

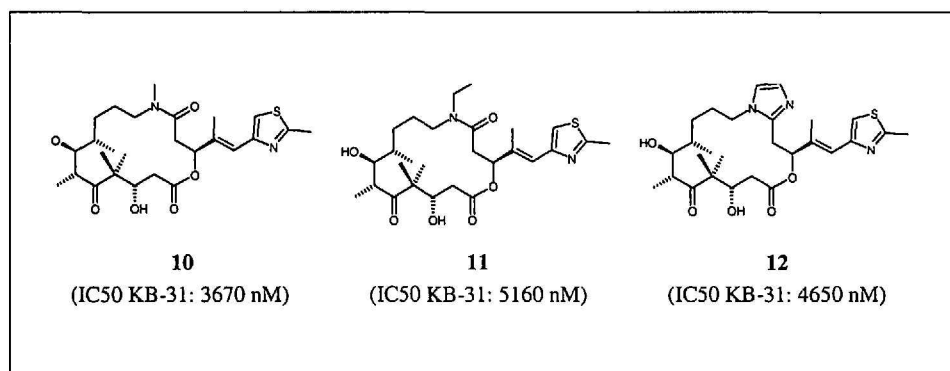
^a) For experimental conditions cf. Tables 1 and 2.

Fig. 6. Amide- and imidazole-based analogs of epothilones. Values in parentheses refer to growth-inhibitory activity against the human epidermoid cancer cell line KB-31.

region) and recent results from the Danishefsky laboratory, demonstrating that an analog incorporating a *lipophilic* 1,2-phenylene moiety in place of the more polar amide groups or the imidazole ring in analogs **10–12**, likewise is several hundred-fold less active than deoxyepothilone B [26b].

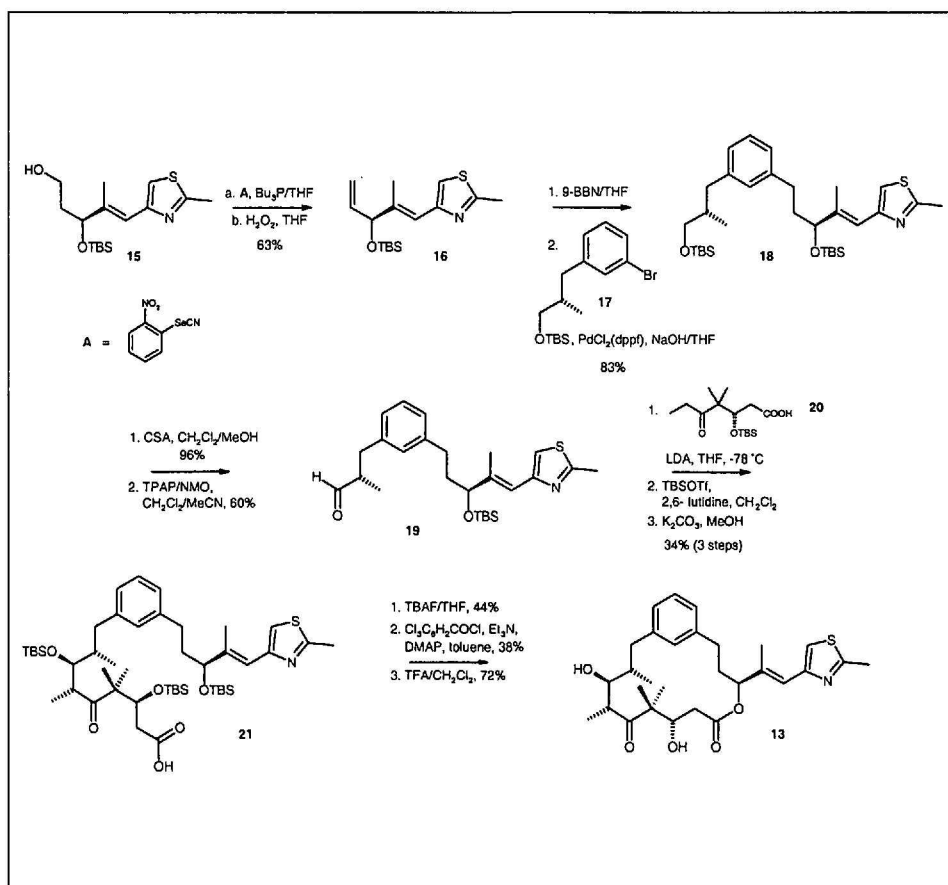
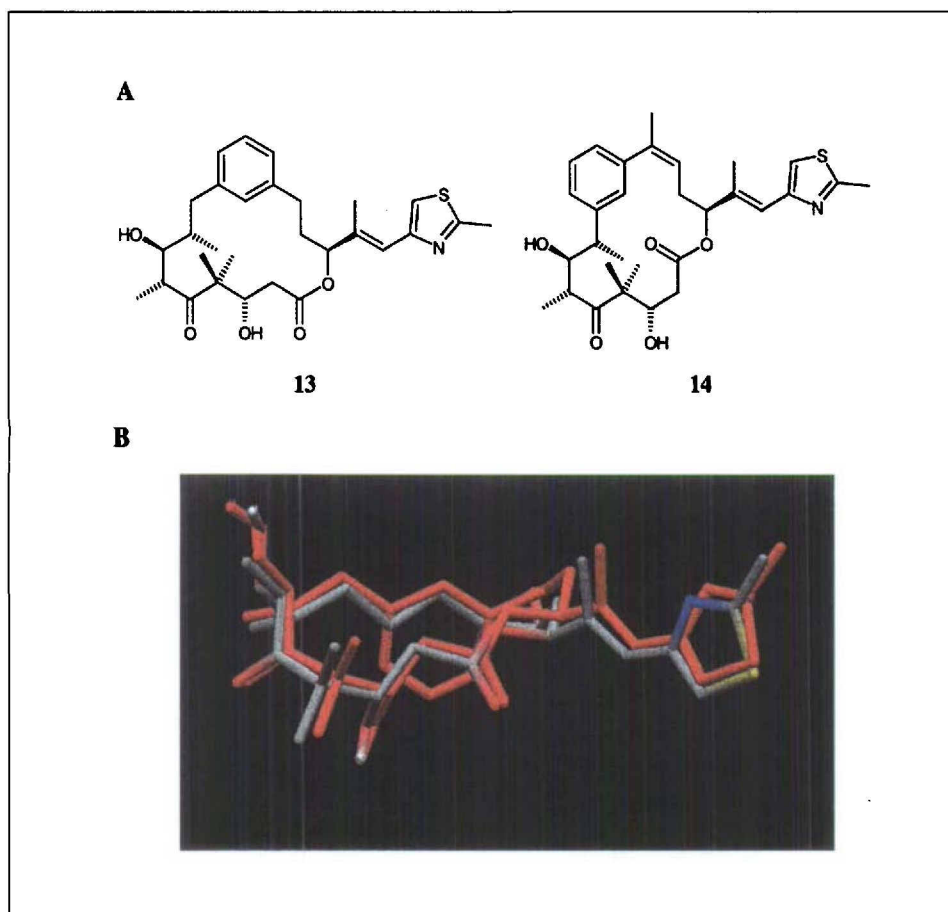
3.3. Modifications in the C(9)–C(12) Region

An alternative approach that we have pursued for modifications situated in the Northern hemisphere of epothilones is based on an epothilone pharmacophore model that was derived in our laboratory from a comparative analysis of the X-ray crystal structure of epothilone B [13] with those of paclitaxel and discodermolide [38][39]. According to this model, the bioactive conformation of epothilone B is very closely related to its X-ray crystal structure, with the three bonds between C(8)/C(9), C(9)/C(10), and C(10)/C(11) all adopting an *anti*-periplanar conformation. The model suggests that the incorporation of *meta*-substituted phenyl rings in the C(9) to C(12) segment, such as in compounds **13** or **14** (Fig. 7A), should lead to a stabilization of the purported bioactive conformation in this potentially rather flexible region of natural epothilones. This is illustrated in Fig. 7B, which shows an overlay between a low energy conformation of **13** (in red) and the presumed bioactive conformation of epothilone B (in gray).

The key steps in the synthesis of analog **13** are outlined in Scheme 2 [40].

The critical junction between fragments C(7)–C(12) and C(13)–C(15) was achieved through alkyl Suzuki coupling between aryl bromide **17** and olefin **16** in excellent 83% yield [41]. Transformation of this coupling product to the aldehyde **19** and subsequent aldol reaction with the dianion of the O-protected β -hydroxy acid **20** (comprising the C(1)–C(6) fragment of epothilones) [42] yielded the desired *anti*-Felkin *syn* aldol product with 5:1 selectivity [43]. The synthesis was completed by partial deprotection of **21** with TBAF, followed by Yamaguchi macrolactonization [44], which proceeded in moderate yield, and removal of the TBS protecting groups with $\text{CF}_3\text{COOH}/\text{CH}_2\text{Cl}_2$.

Unfortunately, epothilone analogs **13** and **14** proved to be devoid of any appreciable biological activity (data not shown). This may either indicate that our pharmacophore model does not appropriately reflect the bioactive conformation of epothilones; alternatively, the addi-

Scheme 2. Synthesis of the conformationally constrained epothilone analog **13**.Fig. 7. A: Structures of conformationally constrained epothilone analogs **13** and **14**. B: Overlay of a low energy conformation of **13** (in red) with the hypothetical bioactive conformation of epothilone B (in gray).

tional steric bulk associated with the phenylene moiety could conceivably more than outweigh any possible activity gain arising even from proper conformational stabilization.

3.4. Side-chain Modifications

Previous work on side-chain modified epothilone analogs has focused on modifications of the thiazole moiety at the 2- and 4-position [17a, b][22a][45], the replacement of the thiazole ring by other heterocyclic structures [22][27] or a simple phenyl group [22], and the importance of the allylic methyl group attached to C(16) (*cf.* [28]). As a part of these studies and in a collaborative effort with Prof. Nicolaou's group at the Scripps Research Institute in La Jolla, CA, we have recently shown that the replacement of the 2-methyl thiazole moiety in epothilone B by various types of substituted pyridine rings can lead to analogs with enhanced antiproliferative activity [27].

However, in addition to analogs incorporating an olefinic double bond as a linker between the macrolactone ring and different types of heterocyclic structures, we have also studied a new type of modification, which involves rigidification of the entire side-chain through incorporation of the C(16)–C(18) olefinic double bond into a fused hetero-aromatic ring system. The prototypic example for this type of structures are the benzothiazole-derived analogs **22** and **23** shown in Fig. 8. Our first generation synthesis of **22** is summarized in Scheme 3 [47].

Key steps for the construction of this deoxyepothilone B analog include i) the Pd⁰-catalyzed coupling between vinyl iodide **24** [46] and the zincate derived from alkyl iodide **25** [47], ii) the aldol reaction between aldehyde **27** and the dianion of O-protected β-hydroxy acid **20** [42], and iii) the Yamaguchi-type macrolactonization of seco-acid **29** [44] and subsequent removal of the O-protecting groups. Apart from the low yield in the macrolactonization step (which could be improved at a later stage, *vide infra*) this strategy was primarily hampered by the low stereoselectivity of the aldol reaction between aldehyde **27** and β-hydroxy acid **20** and the difficulty in separating the resulting mixture of isomers. As a consequence we have elaborated a second generation approach for the synthesis of **22/23** and related analogs, which in essence involves construction of the critical C(6)–C(7) linkage *prior* to the formation of the C(11)–C(12) bond, thus obviating the need for the separation of aldol isomers for each new analog prepared (Scheme 4) [46].

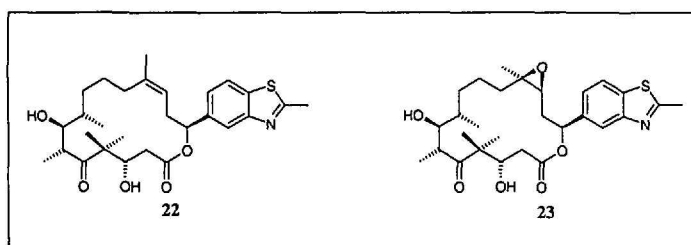
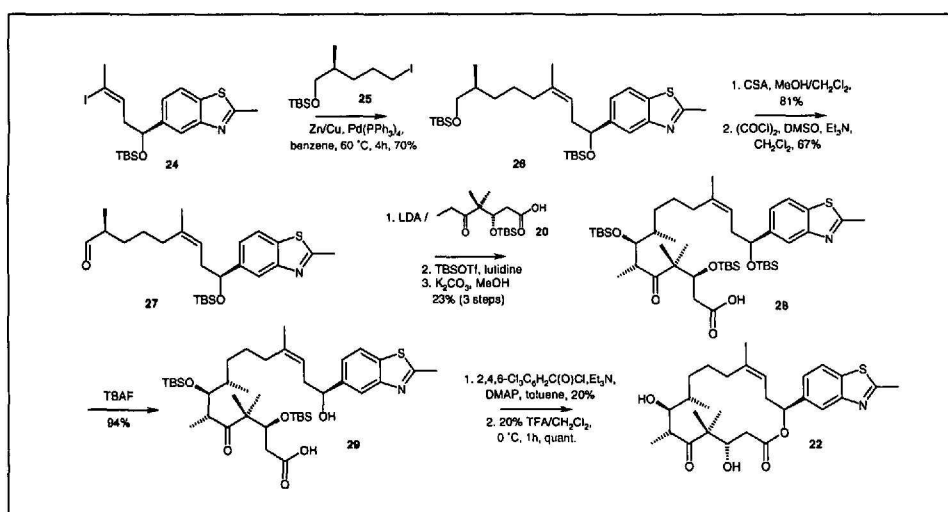
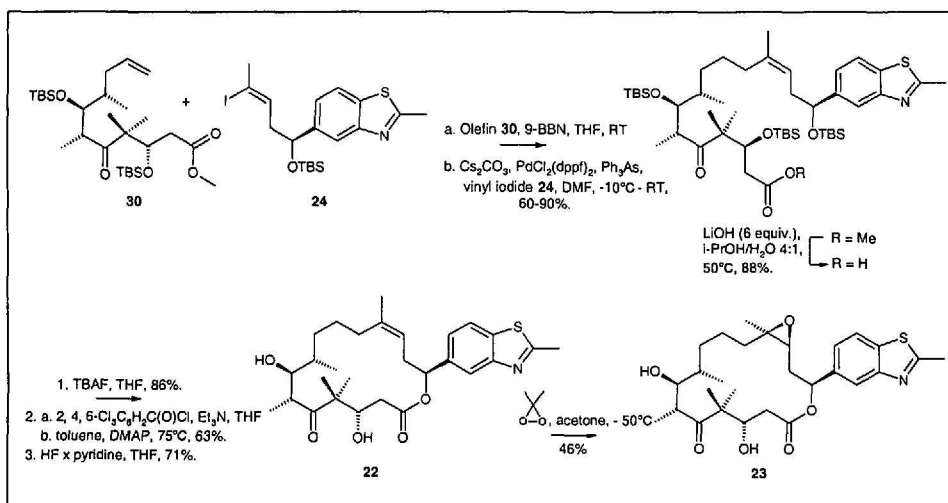


Fig. 8. Structures of side-chain modified epothilone analogs **22** and **23**.



Scheme 3. Synthesis of deoxyepothilone B analog **22**: First generation approach.



Scheme 4. Synthesis of epothilone analogs **22** and **23**: Second generation approach.

Table 5. Induction of tubulin polymerization and growth inhibition of human carcinoma cell lines by side-chain modified epothilone analogs **22** and **23**^{a)}.

Compound	%Tubulin Polymerization	Growth inhibition (IC50-values [nM])			
		A549 (lung)	HCT-116 (colon)	PC-3M (prostate)	MDA-MB-231 (breast)
22	76	1.00	1.22	1.55	0.50
23	83	0.07	0.17	0.28	0.04
Epothilone B	90	0.23	0.32	0.52	0.13
Deoxyepothilone B	83	4.62	4.48	7.40	2.08
Epothilone A	69	2.67	2.51	4.27	1.31

^{a)} For experimental conditions cf. Tables 1 and 2.

As illustrated in Scheme 4, an additional feature of this second generation approach consists in the use of a (borane-based) Suzuki-type coupling between **24** and olefin **30** to establish the C(11)–C(12) bond [46], which proved to be a more reliable reaction than the zincate-based coupling shown in Scheme 3. It should also be noticed that the yield in the macrolactonization step could be significantly improved when the reaction was performed at higher dilution (1 mM) and at 75 °C rather than at RT [44]. Epoxidation of **22** with dimethyldioxirane [48] proceeded with *ca.* 6:1 selectivity and provided epoxide **23** in 46% yield after chromatographic purification. The same strategy was employed for the synthesis of a series of analogs of **22/23** incorporating benzimidazole and quinoline type side-chains [49].

As illustrated in Table 5 for a selected set of human cancer cell lines, epothilone analogs **22** and **23** are generally more potent inhibitors of human cancer cell proliferation than the respective parent compounds deoxyepothilone B and epothilone B. The activity increase is slightly more pronounced for **22**, which is a 3 to 5-fold more potent antiproliferative agent than deoxyepothilone B across all cell lines investigated in our laboratory; in addition, the compound is clearly more potent than epothilone A. Similar observations have been made for related analogs incorporating quinoline or benzimidazole-based side-chains [49]. This is an intriguing finding, as only few other modifications have been described in the literature so far which lead to analogs with enhanced *in vitro* activity over natural epothilones [27]. As indicated by the tubulin polymerization data presented in Table 5, the observed increase in anti-proliferative activity does not seem to be a consequence of more effective interactions with tubulin, but may rather be related to parameters such as cell penetration or intracellular accumulation, although we have not explicitly investigated these aspects. Given the fact that deoxyepothilone B has been suggested in the literature to be a more attractive anti-tumor agent than epothilone B (due to reduced toxicity) [50], the improved anti-proliferative activity of **22** over deoxyepothilone B could make this compound a potentially interesting candidate for further development.

4. Conclusions

Epothilones are a new class of microtubule inhibitors with activity against multidrug-resistant cancer cells *in vitro* and (in the case of epothilone B) have also been demonstrated to possess potent *in vivo* antitumor activity in Taxol®-resistant tumor models. Among the naturally occurring epothilones, epothilone B is currently undergoing Phase I clinical trials. Apart from the promising *in vitro* and *in vivo* profile of naturally occurring epothilones (including deoxyepothilone B), the epothilone structural framework undoubtedly represents a highly attractive template for anticancer drug discovery. This is reflected in the large number of synthetic and semi-synthetic analogs of epothilones which have been described in the literature over the last three to five years. Although very few modifications have been demonstrated to result in truly enhanced antiproliferative activity, a number of analogs have been identified in different laboratories, whose *in vitro* activity is within one order of magnitude of that of the (respective) natural products (e.g. **5a**, **7a**). Among these, the epothilone B lactam analog **1** has advanced to Phase I clinical trials (BMS). It should be noticed, however, that all of the more potent analogs reported so far do not incorporate any gross structural deviations from the natural product leads. As illustrated by compounds **10–14** (and also other examples studied in our laboratories) more rigorous modifications of the macrocyclic framework lead to a dramatic loss in biological activity. In the course of our work on epothilone side-chain modifications we have now discovered a new class of analogs (exemplified by **22** and **23**) with generally improved antiproliferative activity over epothilone B/deoxyepothilone B. These compounds could represent interesting candidates for anticancer drug development.

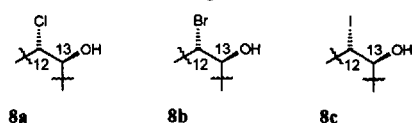
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Activity decreases in the order **8c** (IC50s of 6.5 nM and 5.4 nM on KB-31 and KB-8511, respectively) > **8b** (IC50s of 11.2 nM and 5.2 nM) > **8a** (IC50s of 96.7 nM and 85.5 nM). In all three cases IC50s were determined for 2:1 – 4:1 mixtures of the 12- and 13-halo isomers, respectively, as no isomer separation could be achieved for any of these regioisomeric mixtures. For **8b** and **8c** interpretation of the biological data may additionally be complicated by the fact that these compounds under the assay conditions could conceivably revert, at least partially, to epothilone *A*. In preliminary experiments in aqueous phosphate buffer, pH 7/dioxane 1/1 epothilone *A* was regenerated from **8c** (4:1 mixture of regioisomers, *vide supra*) with a half-life of *ca.* 12 d. On the other hand, **8a** has been reported to be completely resistant to epoxide formation even under strongly basic conditions [17c].

- [34] At first glance the lack of measurable induction of tubulin polymerization by azido alcohol **8** may be taken to indicate that the antiproliferative activity of this compound is not primarily caused by interference with microtubule functionality. However, it should be kept in mind that the cellular activity of **8** is *ca.* 30-fold lower than that of epothilone *A*. Assuming a strictly linear correlation between induction of tubulin polymerization and antiproliferative activity *in vitro* (which is a gross oversimplification), an EC50 for induction of tubulin polymerization of *ca.* 30 μ M would be predicted for **8** (cf. Table 1 for EC50 of epothilone *A*). Based on data for other compounds (e.g. **5a**) EC50s in this range do not translate into tubulin polymerization values significantly above 10% at 2 μ M compound concentration (if

- at all). Taking into consideration that cellular activity will depend not only on target affinity *in vitro*, the lack of tubulin polymerization induction *in vitro* under our specific assay conditions thus does *not* imply that interaction with cellular microtubules is not at the origin of the antiproliferative activity of **8**.
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