Natural Products

DOI: 10.1002/ange.200602785

Total Synthesis and Antitumor Activity of ZK-EPO: The First Fully Synthetic Epothilone in Clinical Development**

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The clinical success of paclitaxel (PT) in the treatment of ovarian and breast cancer strongly contributed to the assessment that tubulin is one of the best clinically validated targets in tumor therapy. However, one disadvantage with PT and other taxane analogues is their recognition by cellular efflux mechanisms, such as the p-glycoprotein (p-gp), which contribute to a loss of activity in cells overexpressing the multidrug-resistance (MDR) phenotype.

In 1995 Bollag et al.[1] reported that the natural product class of epothilones mimics the biological activity of PT with respect to its action on the tubulin system. Thus, the cell cycle is arrested in the G2/M phase and the cells are driven into mitotic catastrophe and/or apoptosis. In contrast to PT, epothilones possess the potential to overcome MDR in vitro and, most importantly, in vivo.[2–5] These findings stimulated intensive research activities in chemistry, pharmacology, and medicine.

Epothilones were first discovered by Reichenbach, Höfle, and co-workers who characterized the compounds they had isolated from the myxobacterial strain *Sorangium cellulosum*.[6,7] They also described their cytotoxic potential in a patent application filed in 1991.[8]

The first total syntheses of epothilone A were reported in 1996/1997 by the research groups of Danishefsky, Nicolaou, and Schinzer.[9–11] Since we had no access to the more potent compound epothilone B, from the start of our project we focused on total synthesis to evaluate the potential of this compound class. During our own investigations, the total syntheses of epothilones B and D were reported.[12–15] Epo-

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[**] We thank Siegfried Bäsler, Stefan Barm, Angela Bieseke, Klaus Burmeister, Klaus Cornelius, Gisbert Depke, Ralf Günther, Wolfgang Halfbrodt, Frank Halwass, Karola Henschel, Hiltraud Hillmann, Klaus-Dieter Joachim, Frank Kuczynski, Hue-Quan Lao, Günter Michl, Gerhard Neumann, Christian Pingel, Ingrid Pissarczyk, Peter Pretsch, Bodo Röhr, Oliver Schenk, Michael Schirner, Jürgen Skupsch, Marion Slopianka, Daniela Wrobel, and Henk Zimmermann for their excellent scientific and technical assistance.

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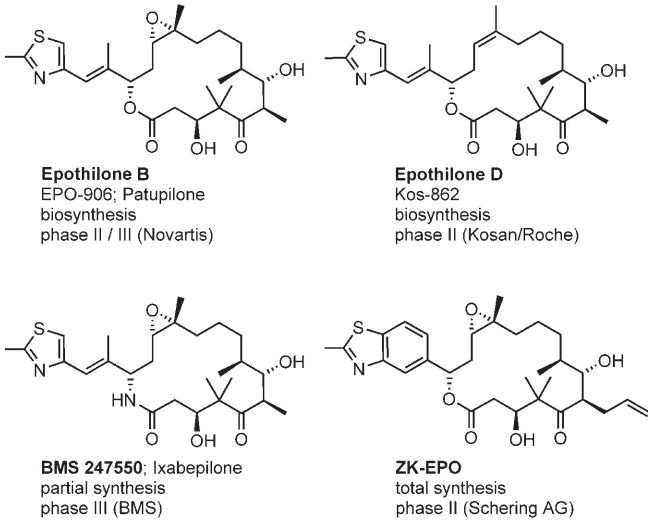
thilone B proved to be highly active in proliferation assays, but also highly toxic in animal models. On the basis of these results we started a lead optimization program, with the primary goal being to maintain the high activity of the natural compound while improving the therapeutic window.

A highly convergent total synthesis enabled us to introduce structural modifications at nearly every position of the macrolactone. We found that epothilone D and most of its analogues had a significantly reduced activity compared with epothilone B. Even though they are not good substrates for p-gp, a delayed cellular uptake and an easy cellular release was observed, which strengthened our focus on compounds of the epothilone B type.[16]

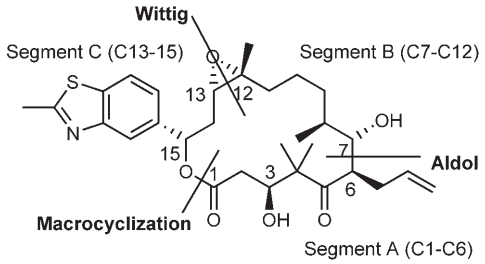
To increase the chemical as well as the enzymatic stability we replaced the lactone by a lactam moiety, and were surprised to discover that the compounds were then well recognized by MDR mechanisms thus resulting in a taxane-like profile. Several other structural modifications have since been identified that also change the compound profile in this unwanted direction. During extensive studies on structure/property—as well as structure/activity—relationships we learned that it is possible to further improve the activity of the natural compounds in multi-drug resistant cell lines and to reduce the toxicity in vivo without reducing the antitumor efficacy. The side chain at C15[17] and the residue at C6 were identified as key positions for tuning tolerability and efficacy.[18,19] From about 350 active[20] epothilone analogues synthesized in our laboratories, we have chosen ZK-EPO (**20**) on the basis of its outstanding preclinical properties for clinical development. This compound combines high activity and efficacy, a fast and efficient cellular uptake, no recognition by efflux mechanisms, and an improved therapeutic window.

Scheme 1 shows the four epothilones currently undergoing advanced clinical development.[21,22] The natural compounds epothilones B and D are produced by biosynthesis and the lactam BMS 247550 by partial synthesis starting from epothilone B. We now disclose the chemical structure of ZK-EPO (**20**) as well as the synthetic route developed in our research.



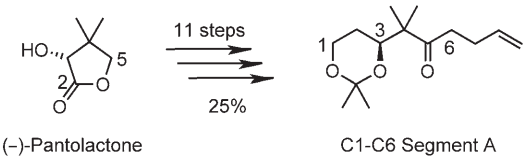
Scheme 1. Epothilones in advanced clinical trials, their names, production method, and development status.

We applied a highly convergent strategy based on our lead-optimization program that offered good flexibility for introducing structural modifications at nearly every position of the 16-membered-ring skeleton. This strategy is reflected in our synthetic route to ZK-EPO (Scheme 2) where we applied a Wittig reaction to connect C12 and C13, an aldol reaction[10,11] to establish stereocenters C6 and C7, and finally a Yamaguchi cyclization for macrolactonization.[12]



Scheme 2. Strategic bond formation in the construction of ZK-EPO.

Segment A is constructed from a chiral pool synthesis starting with (−)-pantolactone (Scheme 3).[23] Even though 11 steps were required, this synthesis allows the preparation of C6-modified building blocks in large quantities.

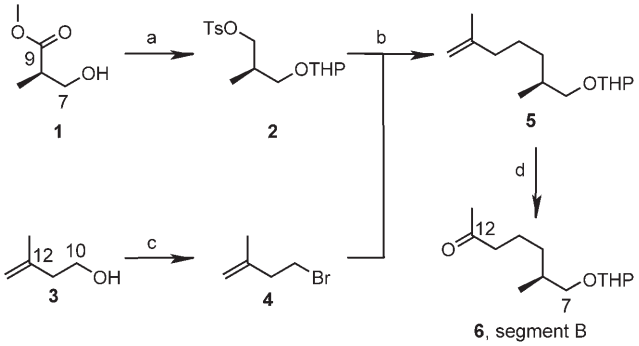


Scheme 3. Synthesis of the C1–C6 segment (segment A) from (−)-pantolactone.

Besides the possibility for structural modifications, segment B represents a central strategic synthon. By using an appropriate protecting-group strategy the segments can be connected either by the sequence C + B→CB + A→CBA or by the sequence A + B→AB + C→ABC. This flexibility was achieved during the synthesis of potential drug compounds through an 11-step synthesis that allowed us to incorporate the most valuable building blocks A or C last.[19]

After selecting ZK-EPO as the candidate for development, and favoring the connection sequence C + B→CB + A→CBA, the synthesis of segment B can be shortened considerably (Scheme 4).

The readily available chiral starting material **1** (Roche ester) is first protected as a tetrahydropyranyl ether. After reduction of the ester moiety, the resulting primary alcohol is derivatized to tosylate **2**. Coupling of **2** with 2-methyl-1-butenyl-4-magnesium bromide (**4**) in the presence of a catalytic amount of Li_2CuCl_4[24,25] leads to **5**. Bishydroxylation of the double bond followed by oxidative degradation provides methyl ketone **6** in an overall yield of 47% starting from **1**.



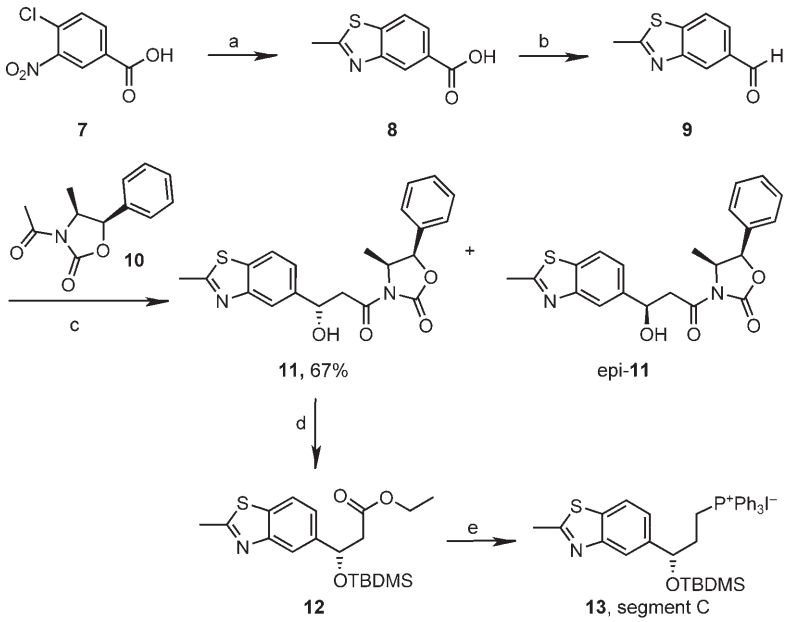
Scheme 4. Stringent synthetic route to the C7–C12 segment (segment B). Reagents and conditions: a) 1. DHP, pTsOH, CH_2Cl_2; 2. LAH, diethyl ether, RT, 87%; 3. TsCl, pyridine, RT, 97%; b) Mg/Li_2CuCl_4 (cat.), THF, −70°C to RT, 75%; c) NBS, PPh_3, CH_2Cl_2, RT, 51%; d) OsO_4, $NaIO_4$, H_2O, THF, RT, 74%. THP = tetrahydropyranyl, DHP = dihydropyran, pTsOH = toluene-4-sulfonic acid, LAH = lithium aluminum hydride, Ts = toluene-4-sulfonyl, NBS = *N*-bromosuccinimide.

Benzothiazole **8** is obtained from benzoic acid **7** in a one-pot reaction (Scheme 5). Aldehyde **9** is prepared by a reduction–oxidation sequence and then submitted to an Evans aldol addition of 3-acetyl-(4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone (**10**) to establish the stereogenic center at C15. The excess of chiral auxiliary can be considerably reduced by the presence of zinc chloride to give a 8:2 mixture of diastereoisomers from which pure **11** is isolated directly by crystallization.[26] The relative stereochemistry was determined by X-ray analysis of **11**, thereby confirming the correct absolute configuration at C15.

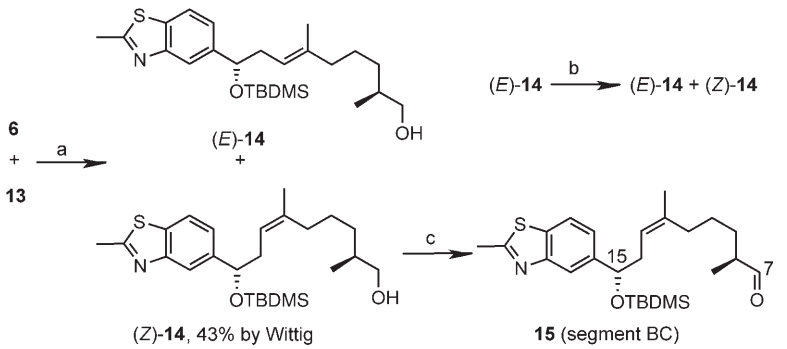
The most efficient way to recover the chiral auxiliary was transesterification to **12** and subsequent reduction to the corresponding alcohol, which is then transformed to crystalline phosphonium salt **13** (segment C). The overall yield starting from **7** is 16%.

Segments B (**6**) and C (**13**) are connected by a Wittig reaction to afford a nearly 1:1 mixture of *E*/*Z* isomers.[12] After removal of the tetrahydropyranyl ether the isomers can be separated by chromatography to yield stereochemically pure (*Z*)-**14** and (*E*)-**14** (Scheme 6). The configuration of the double bond was assigned unambiguously by NOE experiments. (*E*)-**14** can be isomerized by irradiation with light with a wavelength greater than 280 nm to give a 6:4 mixture of (*E*)-**14** and (*Z*)-**14** in a total yield of over 90%. Finally, alcohol (*Z*)-**14** is oxidized to aldehyde **15** (segment BC). The total yield is 36% (photochemical recycling not included).

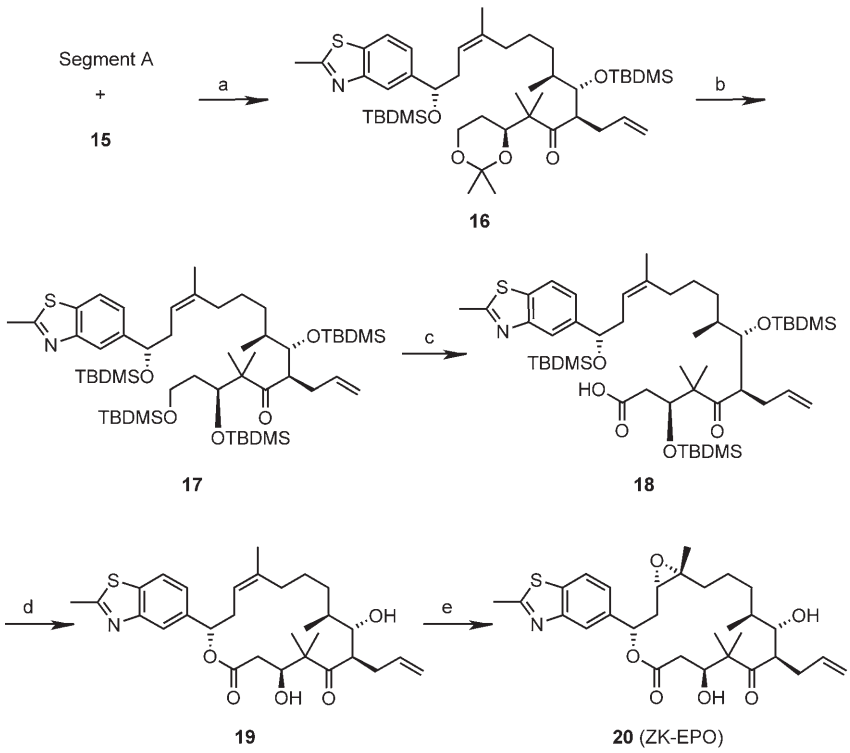
Aldehyde **15** was treated with segment A in an aldol reaction to yield **16** with good selectivity, along with minor amounts of its 6,7 diastereoisomer that is removed by chromatography (Scheme 7). The addition of zinc chloride significantly improves the diastereoselectivity, which normally decreases with increasing size of residues at C6. The remaining transformations to the target molecule are well established in the total synthesis of epothilones.[27–29] Removal of the ketal group in **16** followed by persilylation generates tetrasilyl ether **17**. The primary silyl ether is removed under mild acidic conditions followed by a two-step oxidation sequence which establishes carboxylic acid **18**. Next, the benzylic alcohol is released and the crude hydroxy acid subjected to Yamaguchi cyclization conditions to generate the 16-membered macrolactone. The remaining protecting groups are removed to yield epothilone D analogue **19**. The addition of hexafluorosilicic acid significantly



Scheme 5. Synthesis of the C13–C15 segment (segment C). Reagents and conditions: a) Na_2S, Ac_2O, HOAc, reflux, 71%; b) 1. LAH, THF, RT to reflux, 71%; 2. SO_3·pyridine, Et_3N, CH_2Cl_2/DMSO, RT, 95%; c) *n*BuLi, $ZnCl_2$, THF, −70°C; d) 1. TBDMSCl, imidazole, DMF, RT, 91%; 2. $Ti(OEt)_4$, EtOH, reflux, 91%; e) 1. DIBAH, toluene, CH_2Cl_2, −40°C, crude; 2. I_2, PPh_3, CH_2Cl_2, RT, 92%; 3. PPh_3, toluene, 100°C, 87%. TBDMS = *tert*-butyldimethylsilyl, DIBAH = diisobutylaluminum hydride.



Scheme 6. Synthesis of C7–C15 segment (segment BC). Reagents and conditions: a) 1. NaHMDS, THF, 0°C to RT, 83%; 2. pTsOH (cat.), EtOH, RT, 86%; b) *hν*, toluene/acetone, RT, 92%; c) Swern oxidation, crude. HMDS = 1,1,1,3,3,3-hexamethyldisilazane.



Scheme 7. Synthesis of the C1–C15 segment (segment ABC) and completion of the synthesis of ZK-EPO (**20**). Reagents and conditions: a) LDA, $ZnCl_2$, THF, −70 °C; 64 %; b) 1. pTsOH (cat.), EtOH, RT, 97 %; 2. TBDMSOTf, 2,6-lutidine, CH_2Cl_2, −70 °C to 0 °C, 96 %; c) 1. CSA, CH_2Cl_2, MeOH, RT, 80 %; 2. Swern oxidation, crude; 3. $NaOCl_2$, NaH_2PO_4, 2-methyl-2-butene, THF, H_2O, *t*BuOH, 0 °C to 15 °C, 85 %; d) 1. TBAF, THF, RT, crude; 2. Yamaguchi cyclization, 60 %; 3. HF·pyridine, hexafluorosilicic acid, THF, RT, 87 %; e) DMDO, acetone, CH_2Cl_2, −78 °C, 71 % + 10 % β-epoxide. LDA = lithium diisopropylamide, Tf = trifluoromethanesulfonyl, CSA = camphorsulfonic acid, TBAF = tetrabutylammonium fluoride, DMDO = 3,3-dimethyldioxirane.

facilitates the cleavage of both silyl ethers in terms of reaction time, yield, and insensitivity to the quality of different batches of HF·pyridine. Epoxidation of the double bond affords the α-epoxide ZK-EPO (**20**) with high stereoselectivity and in 15 % overall yield along with minor amounts of the biologically less active β-epoxide.

The longest linear sequence (C→CB→ZK-EPO) involves 22 steps and occurs in an overal yield of 0.9 %. With this synthetic route we succeeded in producing 36 g of compound, which was sufficient to perform nearly all the preclinical investigations.

The relative and absolute configuration of ZK-EPO was confirmed by X-ray analysis (Figure 1, left). It is interesting to note that the observed conformation differs considerably from that of epothilone B (Figure 1, right):[30] in ZK-EPO, both hydroxy groups as well as the epoxide are oriented towards the center of the macrocyle. A similar conformation was postulated for 14(*S*)-methyl-epothilone B, as deduced from its corresponding 14(*R*)-methyl-epothilone D analogue—the latter showing significantly reduced antiproliferative activity relative to its 14(*S*) epimer which adopts a conformation similar to epothilone B.[31] These investigations suggest that the conformation of ZK-EPO in the crystal lattice might not be the conformation adopted at the common taxane/epothilone tubulin binding site.

A panel of 20 different human tumor cell lines was exposed to different concentrations of ZK-EPO and compared with PT (Figure 2, top), doxorubicin (adriamycin ADM), cisplatin (CDDP), and camptothecin (CPT, Figure 2, bottom). The degree of growth inhibition was measured three days after initiation of drug treatment. The activity of each compound is given as a concentration that inhibits cell proliferation by 50 % (IC_{50} values). Cell lines marked in red show significant resistances to standard cytotoxic drugs. ZK-EPO proved to be highly active in all of these proliferating human tumor cell lines, irrespective of their MDR phenotype, and showed a mean IC_{50} value below 1 nM.

To determine whether ZK-EPO also influences nonproliferating nontumor cells, ZK-EPO was tested on proliferating or confluent (nonproliferating) human immortal keratinocyte HaCat cells and the results compared with those with PT.[32] The growth of proliferating HaCat cells was potently inhibited by PT and ZK-EPO while only minor effects were observed on confluent cells. This observation indicates a very low toxic potential on normal, nondividing cells.

Even modest structural modifications may have considerable effects on the recognition through MDR-related pathways; this is illustrated by comparing the antiproliferative activity in the sensitive MDR(−) human breast cancer cell line MCF-7 (PT-sensitive) with the multidrug-resistant MDR(+) cancer cell line NCI/ADR (PT-insensitive; Figure 3). Epothilone B is highly active towards the

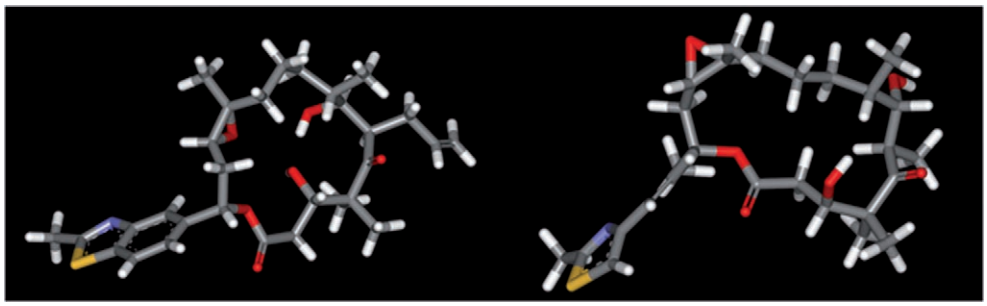


Figure 1. The absolute and relative configuration of ZK-EPO (**20**; left) and comparison with the conformation of epothilone B (right).

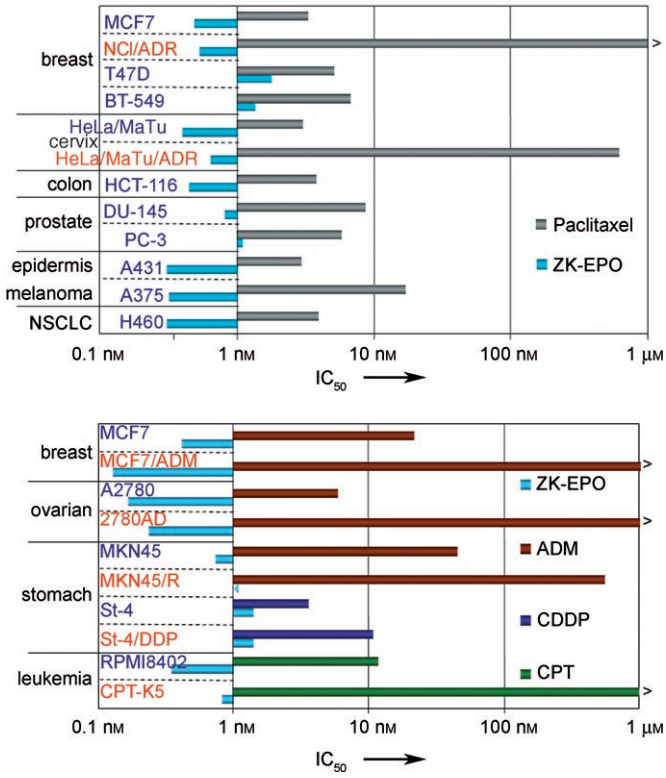


Figure 2. ZK-EPO efficiently inhibits cell proliferation of different human tumor types in vitro.

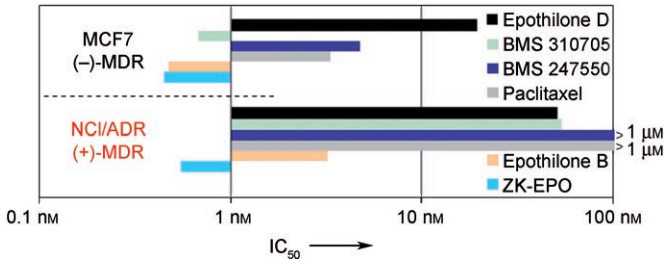


Figure 3. ZK-EPO maintains its high antiproliferative activity even in tumor cell lines overexpressing the MDR phenotype.

MDR(−) cell line and also displays considerable activity towards the MDR(+) cell line, although the activity is reduced about tenfold. If the lactone is replaced by a lactam moiety (for example, in BMS247550[33,34]), the activity at the MDR(−) cell line is significantly reduced and is nearly lost with the MDR(+) cell line, thus resulting in a PT-like profile. Replacement of the methyl group on the thiazole ring in the side chain of epothilone B by an aminomethylene group (for example, as in BMS310705[35]) generates a compound that shows nearly the same activity towards the MDR(−) cell line as epothilone B, but the activity towards the MDR(+) cell line is reduced about 100-fold. The natural product epothilone D possesses comparable activity in both cell lines, but with a significantly reduced overall activity.[36] ZK-EPO, however, maintains its high activity also towards the MDR(+) cell line. This finding is an indication that, in contrast to other epothilones or PT, ZK-EPO is not exported by the p-gp.

In the next experiment we tested whether ZK-EPO or epothilone B are still recognized by efflux mechanisms. The action of verapamil on drug accumulation in cells is often used to show that the efflux is mediated by the MDR1 p-gp. Thus, 10 μM verapamil was added together with paclitaxel, epothilone B, or ZK-EPO to NCI/ADR cells and the cellular growth estimated after three days of exposure. As expected, verapamil renders NCI/ADR cells responsive to growth inhibition by 100 nM PT. Furthermore, verapamil decreases the IC_{50} value for growth inhibition by epothilone B from 2.4 to 0.3 nM, but does not further enhance the potent antiproliferative effects of ZK-EPO on p-gp that overexpress NCI/ADR cells.[32] These data further indicate that ZK-EPO is not recognized by cellular efflux pumps such as p-gp.

The following examples demonstrate that the activity seen in vitro also translate into in vivo models. Human cervix cancer cells (HeLa/MaTu) from large cervix tumors were xenotransplanted into nude mice. The growth of these HeLa/MaTu cervix tumors could be efficiently inhibited by treatment with PT (12 $mg\,kg^{-1}$ once daily for 5 consecutive days; data not shown). A single treatment with ZK-EPO resulted in an almost complete inhibition of tumor growth whereas the untreated control showed rapid growth (Figure 4, top). Treatment with ZK-EPO resulted in a dose-dependent inhibition of the tumor growth. Response was seen at all doses after one initial treatment. Further tumor growth was completely inhibited at a dose of 8 $mg\,kg^{-1}$. At the highest dose level (8 $mg\,kg^{-1}$, application at days 6 and 25) the compound was well tolerated, as demonstrated by a modest reduction in body weight (about 5%) and a time to recover of less than seven days (Figure 4, bottom).

In the multidrug-resistant HeLa/MaTu/ADR cervix cancer model PT did not show an antitumor effect, while treatment with ZK-EPO resulted in an almost complete inhibition of tumor growth, whereas the untreated control showed rapid growth (Figure 5, left). A single application of 6 $mg\,kg^{-1}$ of ZK-EPO resulted in a complete regression in 50% of these highly resistant tumors. A second dose was not given in this experiment to determine the time to progression. Severe toxic side effects were not observed with these treatment schedules.

The models discussed are selected from tumor indications suitable for chemotherapy and include breast, ovarian, small cell lung, non-small cell lung, prostate, and colorectal cancers. ZK-EPO shows high efficacy in all these tumor types, including both MDR-negative and MDR-positive tumors.[37]

In addition, ZK-EPO displays high efficacy in tumor types normally not sensitive to chemotherapy. Among these tumor types are pancreatic, cholangio, renal cell, and hepatocellular cancers, melanoma, and brain tumors. An example of a melanoma cancer model demonstrating the high efficacy of ZK-EPO is depicted in Figure 5 (right).

Our preclinical data indicate that ZK-EPO exhibits a more potent antitumor activity than taxanes (for example, paclitaxel) and second-generation epothilones such as ixabepilone (BMS247550). ZK-EPO eludes the cellular efflux pumps normally responsible for MDR and has activity against both taxane-resistant and taxane-sensitive tumor models. ZK-EPO uptake may be unaffected by MDR-pump activity, thus

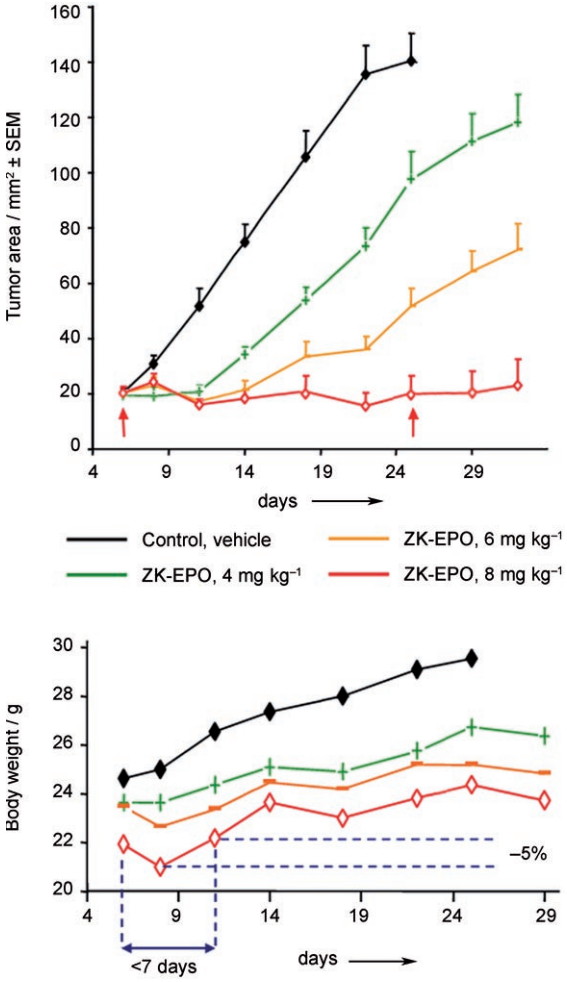


Figure 4. Therapeutic effect of ZK-EPO in nude mice bearing human cervix xenografts HeLa/MATU after intravenous injection. Changes in tumor area over time are shown (top). Evaluation of body weight as a parameter for drug-related toxicity during treatment with ZK-EPO. During the treatment period the body weight is measured twice weekly. The change in body weight over time is shown (bottom). The red arrows indicate when ZK-EPO was administered.

suggesting efficient maintenance within tumor cells. Therefore, ZK-EPO may be clinically effective as a first-line therapy that delays or prevents MDR-based resistance.

Following successful phase I clinical trials,[38] the potential of ZK-EPO in the treatment of different human tumors is now beeing evaluated in an extensive phase II clinical trials program.

Received: July 13, 2006
Published online: September 27, 2006

Keywords: clinical development · epothilones · natural products · total synthesis

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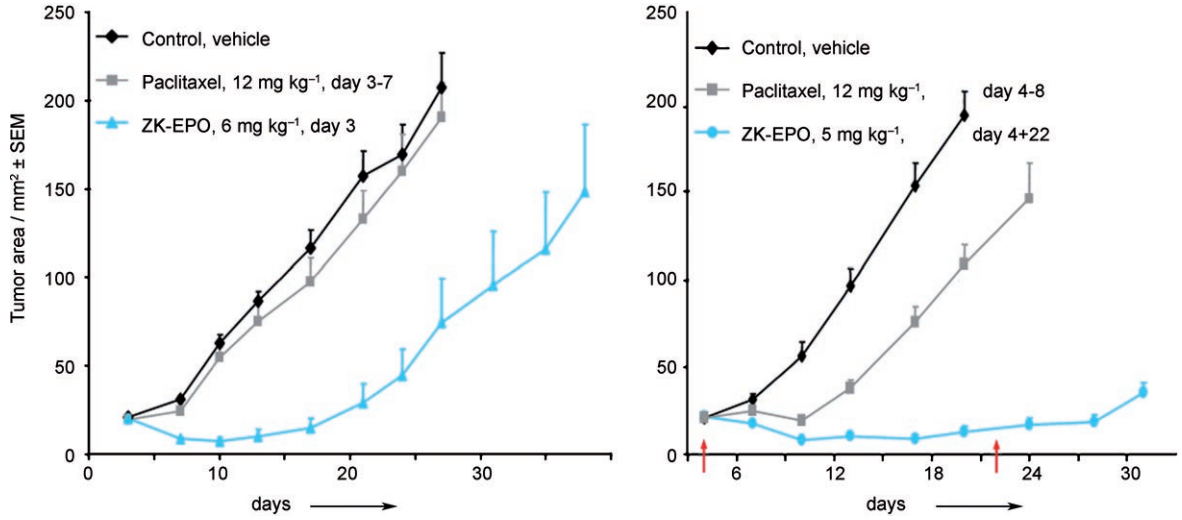

Figure 5. Tumor-inhibiting effect of ZK-EPO and PT in the human multidrug-resistant HeLa/MaTu/ADR cervix tumor (left) and A-375 melanoma (right) xenograft models in nude mice after intravenous injection. The red arrows indicate when ZK-EPO was administered.

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[18] Since the IUPAC nomenclature of epothilone D is completely different from epothilone B we prefer to use the numbering depicted in Scheme 2 throughout the following discussions.
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