Comparative Profiling of the Novel Epothilone, Sagopilone, in Xenografts Derived from Primary Non–Small Cell Lung Cancer

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Abstract

Purpose: Characterization of new anticancer drugs in a few xenograft models derived from established human cancer cell lines frequently results in the discrepancy between preclinical and clinical results. To take the heterogeneity of tumors into consideration more thoroughly, we describe here a preclinical approach that may allow a more rational clinical development of new anticancer drugs.

Experimental Design: We tested Sagopilone, an optimized fully synthetic epothilone, in 22 well-characterized patient-derived non–small cell lung cancer models and correlated results with mutational and genome-wide gene expression analysis.

Results: Response analysis according to clinical trial criteria revealed that Sagopilone induced overall responses in 64% of the xenograft models (14 of 22), with 3 models showing stable disease and 11 models showing partial response. A comparison with response rates for established drugs showed the strong efficacy of Sagopilone in non–small cell lung cancer. In gene expression analyses, Sagopilone induced tubulin isoforms in all tumor samples, but genes related to mitotic arrest only in responder models. Moreover, tumors with high expression of genes involved in cell adhesion/angiogenesis as well as of wild-type TP53 were more likely to be resistant to Sagopilone therapy. As suggested by these findings, Sagopilone was combined with Bevacizumab and Sorafenib, drugs targeting vascular endothelial growth factor signaling, in Sagopilone-resistant models and, indeed, antitumor activity could be restored.

Conclusion: Analyses provided here show how preclinical studies can provide hypotheses for the identification of patients who more likely will benefit from new drugs as well as a rationale for combination therapies to be tested in clinical trials.

Lung cancer remains the leading cause of cancer-related mortality in Europe and the United States, and non–small cell lung cancer (NSCLC) accounts for approximately 70% to 80% of all lung cancer cases (1). The outlook at diagnosis is poor, with 70% of NSCLC patients having inoperable or metastatic disease leading to survival rates of 4 to 6 months if left nontreated (2, 3). Combination chemotherapy is considered to be the standard of care for patients with advanced NSCLC (4–6).

Although mouse xenograft models derived from established human cancer cell lines have undoubtedly furthered the understanding of the antitumor activity of novel anticancer agents, these models have several disadvantages. Depending on the number of cell passages, xenografts can behave very differently to the primary tumor (7), and combined with other deficiencies in preclinical approaches (reviewed in ref. 8), this can reduce the relevance of established xenograft models for predicting the probability of success of anticancer drugs in clinical studies in some tumor localizations. Analysis of antitumor activity in patient-derived xenograft models has provided a more accurate selection process for the identification of agents that have activity in clinical trials, suggesting that some of these models may provide a useful hint for activity in the clinic (9). Furthermore, correlations between the growth of xenograft models derived directly from patient tumors and the clinical prognosis of donor patients have been reported (10, 11). In the future, the establishment and use of patient-derived human tumor xenografts may therefore play a key role in the search for more efficacious cancer treatments (12–16).

Recent advances in the genome-wide analysis of gene expression using oligonucleotide microarrays have allowed the determination of molecular characteristics...
Translational Relevance

Here, we describe the use of a panel of 22 patient-derived non–small cell lung cancer xenograft models to support the clinical development of the new anticancer drug Sagopilone. In this heterogeneous panel, response to Sagopilone treatment was determined in an integrative preclinical phase II design. Genome-wide gene expression and mutational analysis were used to identify the predictive markers for response and explore the mechanism of Sagopilones antitumor activity in vivo. Tumors with wild-type TP53 as well as the high expression of genes involved in cell adhesion/angiogenesis were more likely to be resistant to Sagopilone therapy. Therefore, drugs targeting tumor angiogenesis were combined with Sagopilone in resistant non–small cell lung cancer xenografts, and indeed, combination therapy resulted in restored antitumor activity. The analyses show how preclinical studies can guide the identification of patients that will more likely benefit from the drug and provide a rationale for combinations to be tested in clinical trials.

present in xenograft models that mirror tumor behavior and relate to disease progression and survival (17). As current regimens for treatment of NSCLC rely on combination therapy, some of which may have no benefit for particular patients, the ability to identify and assess antitumor activity in well-characterized xenografts in correlation with particular genetic or molecular characteristics may aid the development of new therapeutic regimens.

Microtubules play vital roles in mitosis, comprise a major component of the cytoskeleton, and influence cell shape; they are therefore a validated target for many established chemotherapeutic agents, including Vinca alkaloids, taxanes, and epothilones (18–21). Sagopilone (ZK-EPO) is the first fully synthetic epothilone with improved efficacy and tolerability compared with other epothilones and taxanes (22, 23). It is rapidly and efficiently taken up by cells, evading P-glycoprotein efflux pumps (22, 23), and it is marked in vitro and in vivo activity in multiple cell lines and human tumor models (22, 23). Sagopilone has shown significant clinical activity in phase II studies including in ovarian and prostate cancer, glioma, and melanoma (reviewed in ref. 24).

In the preclinical study presented here, we evaluated the activity, efficacy, and tolerability of Sagopilone in a series of well-characterized NSCLC xenografts derived from primary patient tumors (15). With 64% overall responses in a response analysis according to clinical trial criteria, a high level of efficacy of Sagopilone in NSCLC compared with established drugs was revealed. Genome-wide gene expression profiling combined with pathway analysis showed an increased expression of several α and β tubulin genes after treatment with Sagopilone in all models, whereas genes related to mitotic arrest were induced preferably in Sagopilone responder models. Additionally, increased basal expression of genes involved in cell adhesion/angiogenesis were observed in cell adhesion/angiogenesis in xenograft models that do not respond to Sagopilone. In these models, combination of Sagopilone with drugs targeting vascular endothelial growth factor (VEGF) signaling led to an enhanced antitumor activity compared with either agent alone. Moreover, mutational analyses revealed increased response rates in models with mutations in the TP53 gene or low TP53 mRNA levels. The study results presented here give further insight into the cellular mechanisms related to Sagopilone response as well as first ideas on potential biomarkers and rational combination therapy. Sagopilone has been tested in a phase II clinical trial for NSCLC, and marker genes derived from the study presented here can be measured and correlated with clinical outcome (25).

Materials and Methods

In vivo activity of sagopilone in xenograft models from primary patient tumors. From previously described primary NSCLC tumor specimens (15), a total of 22 models, obtained directly after surgery from patients with squamous cell carcinoma (n = 10), adenocarcinoma (n = 6), pleiomorphic carcinoma (n = 4), large cell carcinoma (n = 1), and dedifferentiated carcinoma (n = 1) were used. All animal experiments were conducted in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the welfare of animals and the German animal protection law, in addition to approval by local authorities. Tumors with low passage numbers were used and treatment groups consisted of five to six animals each. Treatment with Sagopilone or chemotherapeutic agents was started at palpable tumor size (50-100 mm³), the injection volume was 0.2 mL/20-g body weight. Tumor size was measured twice weekly in two dimensions using calipers, and tumor volume was calculated according to V = [length × (width)]²/2. Antitumor activity was defined on the basis of the Response Evaluation Criteria in Solid Tumors criteria (26), with tumor shrinkage (TS) including partial response, ≥30% decrease in tumor volume on day 21 after start of treatment, and complete response, complete disappearance of tumors. Tumor volume on day 21 after start of treatment of ≥30% and <+20% compared with the start of treatment was classified as stable disease (SD), and tumor growth of ≥20% was classified as progressive disease (PD). Models showing TS or SD were considered as showing overall response and were therefore classified as responders.

In the therapy experiments, Sagopilone (Bayer Schering Pharma AG) was dosed at 8 mg/kg i.v. on day 1 and day 10 after the start of treatment, representing the maximum tolerated dose for multiple treatments (22, 23). Evaluation of the reference drugs at doses and schedules that represent the maximum tolerated or efficient doses has been
**Table 1.** Probe sets regulated 24 h after treatment of xenografts with 10 mg/kg Sagopilone compared with vehicle-treated samples

A. Number of probe sets regulated in individual models (Welch test, 5-fold change, $P < 0.001$ as described in Materials and Methods)

<table>
<thead>
<tr>
<th>NSCLC model no.</th>
<th>Response to Sagopilone</th>
<th>No. of vehicle-treated xenografts</th>
<th>No. of Sagopilone-treated xenografts</th>
<th>No. of regulated probe sets</th>
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<tr>
<td>Lu7126</td>
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<td>5</td>
<td>5</td>
<td>209</td>
</tr>
<tr>
<td>Lu7187</td>
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<td>333</td>
</tr>
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B. Probe sets regulated 24 h after treatment of xenografts with Sagopilone in Sagopilone responder and nonresponder models; table represents overlap from Welch test ($P < 0.01$)

<table>
<thead>
<tr>
<th>Probe-set*</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Entrez gene ID</th>
<th>$P$: responder xenografts†</th>
<th>Ratio of means: $P$: responder xenografts†</th>
<th>$P$: nonresponder xenografts‡</th>
<th>Ratio of means: $P$: nonresponder xenografts‡</th>
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<tr>
<td>216323_x_at</td>
<td>H2-ALPHA</td>
<td>α-Tubulin isotype H2-α</td>
<td>113457</td>
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<tr>
<td>212242_at</td>
<td>TUBA1</td>
<td>Tubulin, α 1</td>
<td>7277</td>
<td>7.02E−09</td>
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<td>3.26E−04</td>
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<td>211714_x_at</td>
<td>TUBB</td>
<td>Tubulin, β polypeptide</td>
<td>203068</td>
<td>6.56E−07</td>
<td>1.37</td>
<td>6.40E−06</td>
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</tr>
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<td>Tubulin, β 3</td>
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<td>1.77E−04</td>
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<td>200977_x_at</td>
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<td>Tubulin, β 2</td>
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<td>1.41</td>
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<td>Tubulin, β polypeptide</td>
<td>203068</td>
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<td>TUBB3</td>
<td>Tubulin, β 3</td>
<td>10381</td>
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<td>TUBB2</td>
<td>Tubulin, β 2</td>
<td>10383</td>
<td>8.14E−06</td>
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<tr>
<td>212639_x_at</td>
<td>K-ALPHA-1</td>
<td>Tubulin, α, ubiquitous</td>
<td>10376</td>
<td>8.46E−06</td>
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<tr>
<td>211750_x_at</td>
<td>TUBA6</td>
<td>Tubulin α 6, tubulin α 6</td>
<td>84790</td>
<td>7.89E−05</td>
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<td>213646_x_at</td>
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<td>10376</td>
<td>3.91E−04</td>
<td>1.12</td>
<td>6.21E−03</td>
<td>1.12</td>
</tr>
</tbody>
</table>

(Continued on the following page)
described earlier (15). Twenty-four hours before the end of the experiment, the control group (5-10 animals) was randomly split into two groups with equal numbers of animals. One half of the animals (three to five animals) was treated with the vehicle PBS and the other half (three to five animals) were treated with Sagopilone at 10 mg/kg i.v., corresponding to the maximum tolerated dose for single dosing (22, 23). At the end of the experiment, the animals were sacrificed and tumor tissues were snap frozen in liquid nitrogen.

Additionally, the in vivo activity of Sagopilone was examined either alone or in combination with Sorafenib and Bevacizumab in Lu7187, Lu7612, and in two human adenocarcinoma–derived cell lines (A459 and LXF). Sagopilone antitumor activity was examined at 8 mg/kg (one single bolus i.v. injection) with two or three treatment cycles every 14 d. Sorafenib-tosylat, 100 mg/kg (Bayer Healthcare AG), was given once daily per os and Bevacizumab (Roche Pharma) was applied at 0.5 mg/kg daily i.p. starting on the day of the first Sagopilone dose. Daily dosing schedules of antiangiogenic therapy were chosen to ensure continuous VEGF inhibition. Tumor volume and body weight were measured twice weekly.

**Gene expression profiling.** RNA from snap-frozen tumor tissue samples was extracted, DNase I digested, measured, and quality controlled as described earlier (15). For array hybridization, the One-Cycle eukaryotic target labeling kit from Affymetrix was used according to the manufacturer's instructions. Briefly, 2 μg of high-quality total RNA were reverse transcribed using T7 tagged oligo-dT primer in the first-strand cDNA synthesis reaction. After the RNase H–mediated second-strand cDNA synthesis, the double-stranded cDNA was purified using the Agencourt RNAclean kit with magnetic beads (Beckman Coulter) according to the manufacturer's instructions. Purified cDNA served as the template for the subsequent in vitro transcription reaction that generates biotin-labeled complementary RNA. The biotinylated complementary RNA was cleaned using the Agencourt RNAclean kit and fragmented with the fragmentation buffer provided in the One-Cycle eukaryotic target labeling kit from Affymetrix (Affymetrix, Inc.), followed by hybridization to the GeneChip HGU133Plus2.0 (Affymetrix), which contains 54,675 probe sets. The GeneChips were washed and stained with streptavidin-phycocerythrin on a GeneChip Fluidics Station 450 (Affymetrix). After washing on a GeneChip Fluidics Station 450, the arrays were scanned on an Affymetrix GeneChip 3000 scanner with autoloader and barcode reader (Affymetrix). A total of 167 HGU133Plus2.0 arrays were hybridized (Table 1A).

**Table 1.** Probe sets regulated 24 h after treatment of xenografts with 10 mg/kg Sagopilone compared with vehicle-treated samples (Cont’d)

<table>
<thead>
<tr>
<th>Probe-set†</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Entrez gene ID</th>
<th>P: responder xenografts†</th>
<th>Ratio of means: responder xenografts†</th>
<th>P: nonresponder xenografts‡</th>
<th>Ratio of means: nonresponder xenografts‡</th>
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<td>TUBB-</td>
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<td>8.16E−03</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Abbreviation: N/a, not applicable.

†Each lane represents one probe set on the Affymetrix Arrays; cross-reactivity might occur within tubulin β family or within tubulin α family but not across families.

‡Welch test in all Sagopilone responder models between vehicle- and Sagopilone-treated samples.

§Welch test in Sagopilone nonresponder models between vehicle- and Sagopilone-treated samples; P <1.00E-3 and fold changes above 1.3 are in bold.
models are available. The quality check of the gene expression data was done as described earlier (15). The probe intensities on each array were summarized with the MAS5.0 summarization algorithm. The analysis of the probe set–specific signal intensities was done with the Expressionist Pro 4.0 Analyst (GeneData) software. The data set was median normalized.

Statistical analyses. The identification of differentially expressed genes in Sagopilone-treated versus vehicle-treated xenografts (n = 2-5 for each model and condition) was determined using t tests with Welch correction. The analysis was carried out for each model individually as well as all Sagopilone-treated responder xenografts.

Moreover, differentially expressed genes between Sagopilone responder models (TS+SD: 14 models, 54 samples, 2-5 independent samples for each model) and nonresponder models (PD, 8 models, 37 samples, 2-5 independent samples for each model) at baseline, which means only looking at vehicle-treated samples were determined using a t test with Welch correction.

P value and fold change cutoffs that were chosen for the reported genes are described with the corresponding analyses in the Results section. Probe sets differentially expressed as the chosen P value and fold change cutoff were subjected to pathway analysis using the Metacore software (GeneGo).

Quantitative real-time reverse transcription-PCR. Aliquots of the total RNA from representative tumors of each model were reverse transcribed using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) containing 3 μg RNA, according to the manufacturer's instructions. Real-time PCR was carried out using predesigned Taqman assays and the Taqman Fast Universal Master Mix on an Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems Applied Deutschland GmbH) as described earlier (15). Four genes were selected for quantitative real-time PCR analysis: 

- Carbonic anhydrase IX (CA9, assay ID Hs00154208_m1),
- carbonic anhydrase XII (CA12, assay ID Hs01080906_m1),
- integrin α 6 (ITGA6, assay ID Hs01040998_m1), and 
- ephrin receptor A4 (EPHA4, assay ID Hs00177874_m1). Results were normalized to the average expression levels of human glyceraldehyde-3-phosphate dehydrogenase (4326317E) and human Cyclophilin A (4326316F). The relative expression of each gene was determined on the basis of the comparative threshold cycle method (ΔΔCT method) using the median gene expression level in the responder models as reference.

Results

Antitumor activity of Sagopilone and NSCLC standard chemotherapies in patient-derived tumor xenografts. In vivo antitumor activity of Sagopilone was compared in patient-derived tumor xenografts with five standard chemotherapeutic agents for the treatment of NSCLC, i.e., Etoposide, Carboplatin, Gemcitabine, Paclitaxel, and Vinorelbine (15). Responses in two of these models over time are presented in Fig. 1. Complete tumor eradication and a superior level of activity were observed after Sagopilone treatment compared with all other agents in the Lu7298 model (Fig. 1A). Sagopilone slightly delayed tumor progression in the Lu7198 model (Fig. 1B); however, after initial tumor growth inhibition, tumor progression was observed on day 21 after the start of treatment. Therefore, when assessed according to clinical Response Evaluation Criteria in Solid Tumors criteria (26), this xenograft model is considered to be nonresponsive to all anticancer drugs tested including Sagopilone.

Overall, Sagopilone showed antitumor responses in 64% of the xenograft models (14 of 22), with 3 models showing SD and 11 models showing TS (Supplementary Table S1; Fig. 2). The number of cases that responded to Sagopilone were higher than those observed for Paclitaxel and Gemcitabine (each 43%, 9 of 21), and Carboplatin (23%, 5 of 22). Sagopilone also induced growth inhibition in 11, 7, and 6 models in which tumor progression was reported after Carboplatin, Gemcitabine, and Paclitaxel treatment, respectively (Supplementary Table S1; Fig. 2).

Although a Gemcitabine dose of 120 mg/kg i.p. every 3rd
day was determined as maximal tolerated dose in previous experiments and also was derived from literature data (27). the mice we used in the experiments presented here showed less tolerability toward this agent, and in four xenograft models (Lu7198, Lu7336, Lu7612, and Lu7668) 50% or more toxic deaths occurred during the course of the experiment. Vinorelbine was omitted from this analysis as it showed overall lower response rates (15).

Identification of differentially expressed genes between vehicle- and Sagopilone-treated xenograft samples. To elucidate the short-term molecular effect of Sagopilone, xenograft tumor samples obtained 24 hours after treatment

Fig. 2. Waterfall plots showing the change in median tumor volume of all 22 patient-derived NSCLC xenograft models 21d after the initiation of Sagopilone (A), Carboplatin (B), Paclitaxel (C), and Gemcitabine (D) treatment. Analysis by clinical criteria; median change in tumor volume of >+20% is considered tumor progression (black columns); change in tumor volume of >−30% to <+20% is considered SD (gray columns); change in tumor volume of <−30% is considered TS (white columns).
with Sagopilone (10 mg/kg) were compared with vehicle (PBS) by global gene expression analyses. The respective ratio of means and $P$ value for each probe set were determined in a $t$ test with Welch correction (Welch test). First, differentially expressed genes for each model (three to five independent samples per condition) were analyzed in the 18 different xenograft models, in which vehicle-treated and Sagopilone-treated samples were available. In Table 1A, the number of regulated probe sets (Welch test, ellipse with either $P < 0.001$ or 5-fold change) for each individual model is shown, ranging between 143 (for Lu7612) and 638 (for Lu7336) with a very small overlap of genes regulated by Sagopilone treatment between the xenograft models (Supplementary Fig. S1).

Additionally, Welch tests on all vehicle-treated xenografts versus all Sagopilone-treated xenografts were done for responder and nonresponder models. These analyses revealed 140 probe sets regulated at a $P$ value below 0.01 in the responders (Supplementary Table S2; Table 1B) compared with 59 probe sets regulated by Sagopilone treatment in the nonresponder ($P$ value below 0.01; Supplementary Table S3; Table 1B). The overlap between these two gene lists consisted of 15 probe sets (Table 1B; Fig. 3A and B) that are upregulated after Sagopilone treatment. This list that hence includes genes that are regulated by Sagopilone treatment irrespective of the tumor response consists exclusively of tubulin $\alpha$ and $\beta$ isoforms. Thus, tubulin $\alpha$ and $\beta$ isoforms are upregulated with high statistical significance in most of the xenograft models 24 hours after Sagopilone treatment (Table 1B; Fig. 3B).

In addition to the 15 probe sets that showed upregulation in the Sagopilone responder and nonresponder models, 43 probe sets were upregulated and 82 probe sets were downregulated only in the responder models (Supplementary Table S2), whereas 13 probe sets were upregulated and 31 probe sets were downregulated only in the nonresponder models (Supplementary Table S3). Scrutinizing the pathways and processes represented in these two gene lists using the Metacore software, in genes that are part of the Pathway Map “Cell cycle: Spindle assembly and chromosome separation,” as for example, AURKB, CCNB1, NEK2, KIF11, CDC20, genes related to the mitotic arrest phenotype are highly significantly upregulated.
(P < 10⁻³; Supplementary Table S4A and Fig. S2) in the responder models—this correlated well with the phenotype observed after treatment of cancer cells with Sagopilone in vitro (22, 23). In contrast, performing the Metacore pathway analysis on the genes that are regulated after Sagopilone treatment in the nonresponder models, none of the pathway maps contained genes that were significantly overrepresented in the gene list, suggesting that no major cellular phenotype was induced by Sagopilone in the nonresponder models (Supplementary Table S4B).

Identification of differentially expressed genes between Sagopilone responder and nonresponder models at basal level (nontreated samples). Next, it was of interest to identify genes that differentiate Sagopilone responder from nonresponder models at baseline status, i.e., in nontreated tissue samples, as such material would be in practice frequently available from surgery. These genes or the pathways they are involved in might serve as potential predictors for Sagopilone sensitivity. Therefore, a Welch test was done comparing global gene expression patterns between all vehicle-treated responder models (TS+SD; 14 models, 54 samples) and all vehicle-treated nonresponder models (PD, 8 models, 37 samples). This analysis revealed 687 probe sets upregulated in nonresponder models, corresponding to 401 unique genes and 330 probe sets downregulated in nonresponder models, corresponding to 241 unique genes using a P value cutoff of <10⁻⁵ and a minimum fold change of 1.5 (Supplementary Table S5). According to pathway analysis using the Metacore software, the main pathways upregulated in the nonresponder models contain genes related to cell adhesion and ephrin signaling (Supplementary Fig. S3), as for example, CDC42, EPHA4, EPHB1, FAP1, GRB10, HRAS, and PAK1. Genes of this pathway have also been shown to play important roles in VEGF-induced angiogenesis (28, 29). A closer look at the gene list revealed more angiogenesis-related genes as well as hypoxia/HIF1α target genes among the genes upregulated in the nonresponder models, as for example, the carbonic anhydrases IX and XII (CA9 and CA12), the BH3 only protein BNIP3L, the glucose transporter SLC2A1 (also known as GLUT1), the integrin ITGA6, matrix metallopeptidase 11 (MMP11), and Jumonji domain containing 2B (JMJD2B) (Supplementary Table S5, refs. 30–34). Examples of gene expression levels of these genes are shown in Fig. 4A. The corresponding results from the global gene expression analysis were validated by real-time PCR gene expression analysis for the tumor-relevant candidates EPHA4, ITGA6, CA9, and CA12 (Fig. 4C). The ratios between responder and nonresponder models detected with the quantitative real-time PCR were similar to the results from the Affymetrix array analysis. Taking together these results suggest that pathways related to cell adhesion/angiogenesis and hypoxia play a role in the resistance of NSCLC xenograft models to Sagopilone and therefore suggest a combination of Sagopilone with antiangiogenic drugs in Sagopilone nonresponder models.

Response to Sagopilone and mutation status of cancer genes. No correlation between KRAS (18% mutations with altered protein sequences) and epidermal growth factor receptor (EGFR; 18% mutations, no altered protein sequence; ref. 15) mutations as well as EGFR mRNA levels and Sagopilone response was observed (data not shown; Supplementary Table S6). Interestingly, a significant correlation between TP53 mutational status and Sagopilone response was observed (P < 0.05, in a Wilcoxon test; Supplementary Table S6), with TP53 mutations reducing transcriptional activity of the protein5 (15), present in 71.4% (10 of 14) xenografts that responded to Sagopilone, in contrast to only 25% (2 of 8) of Sagopilone nonresponders. Notating the data on gene expression levels of the TP53 transcript with the mutational status and Sagopilone response showed that in addition to the high rate of TP53 mutations in Sagopilone responder models in two of the four xenograft models with wild-type TP53 that responded to Sagopilone, the expression levels of the TP53 mRNA were very low (Fig. 4B), supporting the hypothesis that an inactive tumor suppressor protein TP53 is present in the Sagopilone responder models (12 of 14; 85.7%) in contrast to Sagopilone nonresponder models (2 of 8, 25%).

In vivo activity of Sagopilone alone and in combination with Sorafenib or Bevacizumab in xenograft models. As gene expression analysis between Sagopilone responders and nonresponders revealed the upregulation of genes involved in angiogenesis/hypoxia in the nonresponder models, in vivo activity of Sagopilone in combination with Bevacizumab, a VEGF-binding antibody, was examined in xenografts with activated angiogenesis/hypoxia pathways (Fig. 5A). We selected one of the patient-derived NSCLC xenograft model with a strong activation of hypoxia pathways (Lu7612) and with the A549 and LXF, two established lung cancer cell line models that are Sagopilone nonresponders when grown as xenografts. For A549, a high expression of genes related to hypoxia has been shown (Fig. 4C). Treatment with Sagopilone (8 mg/kg) combined with Bevacizumab (0.5 mg/kg, daily administration) reported the largest reduction in tumor growth compared with either agent alone in the A549 and LXF models. The Lu7612, which displayed strong activation of hypoxic pathways in the gene expression analysis, was already strongly responsive to the Bevacizumab treatment alone. Therefore, the combination of Bevacizumab with Sagopilone provided only a small additional increase in activity that was, however, not significant (Fig. 5A).

The antitumor activity of Sagopilone was further examined in four xenograft models with an activated cell adhesion/angiogenesis/hypoxia pathway in combination with Sorafenib, a multitarget kinase inhibitor of VEGF receptor and RAF-kinase, known to play a role in cell adhesion, angiogenesis, as well as the hypoxic response (Fig. 5B). Synergistic antitumor activity was observed after 8 mg/kg Sagopilone and 100 mg/kg Sorafenib, compared with either agent alone in three models. The Lu7612 model again was strongly responsive to Sorafenib treatment alone and

5 http://www-p53.iarc.fr/
therefore only slightly enhanced activity of the combination was detectable. The activity observed after Sagopilone treatment alone or in combination with the two other agents assessed was consistently significant compared with control.

**Discussion**

Human tumors accumulate genetic and molecular abnormalities (16), leading to a broad heterogeneity, and xenograft panels that reflect these heterogeneities might have increased value for predicting the response of new therapeutic agents in the clinic (7, 35).

Consequently, it is important to use clinically relevant tumor models derived directly from patients, besides established human cancer cell line models that may have undergone clonal selection through multiple passages, in preclinical research to more accurately reflect the clinical situation and potentially also to predict the response to a novel therapeutic agent. These models may also help to define patient groups that are more likely to benefit from treatment and, in addition, to identify a rationale for combination therapies.

Here, we determined the efficacy of Sagopilone in 22 patient-derived xenograft models (15) and reported that...
Fig. 4 Continued. C, mean-normalized gene expression of genes from B by quantitative real-time PCR analysis; the Sagopilone response is indicated (black columns, PD; gray columns, SD; white columns, TS); columns, mean of two to five independent tumors per model in B and C, and SDs of triplicate measurements of one representative sample in D; bars, SEM; Norm Sign Int, normalized signal intensity.
Sagopilone suppressed/inhibited tumor growth in a greater number of tumors compared with Carboplatin, Paclitaxel, and Gemcitabine. Etoposide and Vinorelbine have been shown before to have lower response rates in these models (15). The response of these models to standard therapies are similar to the response rates that are observed in the clinic, and also the mutation rates of key oncogenes and tumor suppressor genes such as KRAS, TP53, and EGFR (15) exhibit the mutation rates for NSCLC that are contained in public databases such as the COSMIC database. Sagopilone has previously shown antitumor activity in several

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6 http://www.sanger.ac.uk/genetics/CGP/cosmic/
tumor models including breast, cervical, ovarian, small-cell lung cancer, melanoma, and prostate cancer (22, 23). Although epothilones and taxanes bind to the same site on tubulin (36, 37), Sagopilone provokes a more rapid and potent tubulin polymerization than these agents and escapes resistance mechanisms mediated by drug transporters. These characteristics of Sagopilone may explain why it could potentially show more potent antitumor efficacy than other tubulin-targeting agents (22, 23).

Following an integrative research approach, we analyzed changes in global gene expression induced by single treatment with Sagopilone for 24 hours. Comparing the changes in gene expression after the Sagopilone treatment of individual models, we found very little overlap between the models. This indicates on the one hand a high degree of heterogeneity in the xenograft models, which reflects the heterogeneity in primary NSCLC specimens with regard to molecular characteristics. Analogous findings have been reported recently in a similar study done with the HDAC inhibitor MS-275 in primary colon cancer xenografts (38). Interestingly, a highly significant upregulation of tubulin α and tubulin β isoform mRNAs was observed in xenograft samples treated with Sagopilone and this regulation was independent of the response of the xenograft models to Sagopilone. This highlights that tubulins are the primary molecular target for Sagopilone and further suggests that the upregulation of tubulin isoforms is a positive feedback mechanism of tumor cells following exposure with the microtubule-stabilizing agent, potentially to increase the pool of “free” tubulins. This observation has not been described in literature before, rather, in contrast, a recent report on cell lines selected for Patupilone resistance by long-term culture in the presence of Patupilone showed decreased levels of tubulin β 3 (TUBB3) isoforms (39). These analyses were done after culturing cell lines for several weeks, which may result in different effects than those observed after short-term treatment with an epothilone in vivo. In general, increased levels of TUBB3 have been associated with resistance to Paclitaxel in several tumors including NSCLC, ovarian, prostate, and pancreatic tumors (40–45). In our studies, however, we did not detect differences in TUBB3 mRNA expression between Sagopilone responders and nonresponders neither at basal levels nor after treatment with Sagopilone. These results suggest that the use of tubulin expression may not represent a predictive marker of Sagopilone response in NSCLC.

Comparing genes regulated by Sagopilone treatment between responder and nonresponder models, it was obvious that the genes most consistently induced in the responder models after Sagopilone treatment are involved in regulation of the cell cycle, especially in spindle assembly and chromosome separation. This panel of genes is typically induced during mitosis, consistent with a mitotic arrest phenotype observed 24 hours after a single treatment with Sagopilone in responsive models. This is in line with earlier observations of mitotic arrest followed by apoptosis or mitotic catastrophe being the main mechanism of cell killing by Sagopilone in vitro (22). In contrast, no clear phenotype could be associated with the changes in gene expression in nonresponder models after treatment with Sagopilone.

As the response of NSCLC tumors to chemotherapy differs between patients, with objective response rates varying between 20% and 40% (46), identifying predictive markers of response or resistance to therapeutic agents represents an important therapeutic goal. Here, we found that genes associated with cell adhesion, the hypoxia pathway, and neoangiogenesis were expressed at higher levels in xenografts that did not respond to Sagopilone. Hypoxia triggers pathways that drive angiogenesis and tumor progression, and the presence of genes associated with these pathways has previously been associated with a negative prognosis and resistance to therapy (47, 48). Upregulation of CA9 and CA12 gene expression, in particular, has been detected in a large number of common malignancies and is implicated in tumor development (31). The data presented here show that the combination of Sagopilone with an inhibitor of angiogenesis such as Bevacizumab or Sorafenib results in an enhanced antitumor effect in tumor models with an activated HIF1α/hypoxia pathway. This finding should be followed up in additional preclinical models as well as in clinical studies.

Additionally, the expression of mutant, activated EGFR and KRAS and the reduced expression or mutation of TP53 in NSCLC tumor cells has been found to have prognostic significance in the development and progression of NSCLC. Alterations in EGFR and KRAS are implicated in cellular proliferation, invasion, and metastasis, whereas loss of TP53 function interferes with apoptosis (49–51). No correlations were found between overexpression or mutations of EGFR and K-RAS genes and Sagopilone activity, suggesting that Sagopilone may be active in patients with NSCLC tumors with these changes. Our data show that activity of Sagopilone correlated with antitumor activity in models with low expression or expression of mutant TP53. There are literature reports of a poorer prognosis in patients expressing mutant TP53 with standard treatments. This correlation has not been observed in studies with microtubule-targeting agents (52, 53). These data suggest that Sagopilone may be active where these other chemotherapies are not, in particular, in platinum-resistant NSCLC patients, in which a substantial proportion of patients express mutant TP53 (54). Because Sagopilone has increased microtubule-binding properties compared with Paclitaxel or Patupilone (22), and is active in TP53 mutant models, it holds promise of being an effective treatment for NSCLC patients with tumors expressing mutant TP53. Tumors harboring TP53 mutations with the loss of DNA checkpoint control may be more readily driven into apoptosis and mitotic catastrophe after treatment of Sagopilone, which induces a strong G2-M arrest (22).

In conclusion, results have been generated from a large set of patient-derived xenograft models through genomewide gene expression analyses and mutation analysis of...
selected genes to identify potential markers of response and refractoriness to Sagopilone in NSCLC. Clinical investigation of the marker genes (e.g., CA9, CA12, EPHA4, and ITGA6) together with TP53 gene expression and mutation analysis will be undertaken to assess the predictive value for patients with tumors who will respond to Sagopilone therapy. Sagopilone showed a high level of activity in clinically relevant NSCLC models and these data agree with previous preclinical (22, 23) investigations.

Sagopilone has been tested as monotherapy in a phase II clinical trial in platinum-resistant NSCLC (25, 55, 56). Although the number of partial response has only been 5%, Sagopilone has induced SD in 43% to 45%. An analysis of TP53 mutational status and gene expression in these tumors is planned to assess the relationship of these parameters with response.

Discourse of Potential Conflicts of Interest

Employment and ownership interest of Bayer Schering Pharma AG (S. Hammer, A. Sommer, U. Klar, and J. Hoffmann), employment (I. Fichtner, M. Becker, J. Rolf, and J. Hoffmann), and ownership (I. Fichtner and J. Hoffmann) interest of EPO GmbH.

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