Improved Cellular Pharmacokinetics and Pharmacodynamics Underlie the Wide Anticancer Activity of Sagopilone

Jens Hoffmann, Illo Vitale, Bernd Buchmann, Lorenzo Galluzzi, Wolfgang Schwede, Laura Senovilla, Werner Skuballa, Sonia Vivet, Rosemarie B. Lichtner, José M. Vicencio, Theoharis Panaretakis, Gerhard Siemeister, Hermann Lage, Lisa Nanty, Stefanie Hammer, Kevin Mittelstaedt, Sebastian Winsel, Julia Eschenbrenner, Maria Castedo, Carine Demarche, Ulrich Klar, and Guido Kroemer

1 Bayer Schering Pharma AG, TRG Oncology; 1Charité, Institute of Pathology, Humboldt University; and 1Institute for Chemistry and Biochemistry, Freie Universität, Berlin, Germany; 4Institut National de la Sante et de la Recherche Medicale, U848; 5Institut Gustave Roussy; and 6Université Paris Sud 11, Villejuif, France

Abstract

Sagopilone (ZK-EPO) is the first fully synthetic epothilone undergoing clinical trials for the treatment of human tumors. Here, we investigate the cellular pathways by which sagopilone blocks tumor cell proliferation and compare the intracellular pharmacokinetics and the in vivo pharmacodynamics of sagopilone with other microtubule-stabilizing (or tubulin-polymerizing) agents. Cellular uptake and fractionation/localization studies revealed that sagopilone enters cells more efficiently, associates more tightly with the cytoskeleton, and polymerizes tubulin more potently than paclitaxel. Moreover, in contrast to paclitaxel and other epothilones [such as the natural product epothilone B (patupilone) or its partially polymerizing] agents, sagopilone is not a substrate of the P-glycoprotein efflux pumps. Microtubule stabilization by sagopilone caused mitotic arrest, followed by transient multinucleation and activation of the mitochondrial apoptotic pathway. Profiling of the proapoptotic signal transduction pathway induced by sagopilone with a panel of small interfering RNAs revealed that sagopilone acts similarly to paclitaxel. In HCT 116 colon carcinoma cells, sagopilone-induced apoptosis was partly antagonized by the knockdown of proapoptotic members of the Bcl-2 family, including Bax, Bak, and Puma, whereas knockdown of Bcl-2, Bcl-XL, or Chk1 sensitized cells to sagopilone-induced cell death. Related to its improved subcellular pharmacokinetics, however, sagopilone is more cytotoxic than other epothilones in a large panel of human cancer cell lines in vitro and in vivo. In particular, sagopilone is highly effective in reducing the growth of paclitaxel-resistant cancer cells. These results underline the processes behind the therapeutic efficacy of sagopilone, which is now evaluated in a broad phase II program. [Cancer Res 2008;68(13):5301–8]

Introduction

Microtubules constitute dynamic tubulin structures that can be targeted for the treatment of cancer due to their critical role in mitosis and other cellular processes (reviewed in refs. 1–3). Taxanes, which include paclitaxel, are tubulin-targeting agents that have been widely used in the treatment of breast cancer, non–small-cell lung cancer (NSCLC), melanoma, ovarian cancer, and prostate cancer (4). The strong cytotoxic effect of paclitaxel results from mitotic arrest followed by apoptotic cell death (5). However, the therapeutic use of taxanes is limited by an intrinsic or acquired resistance phenomena and poor solubility, which requires Cremophor EL formulations (6, 7). Among the multiple reasons accounting for taxane resistance, two mechanisms seem particularly important, namely overexpression of P-glycoprotein (P-gp) efflux pumps (8) and β-tubulin mutations (9, 10).

Epothilones constitute a new alternative class of tubulin-binding compounds (11, 12). Some epothilones exhibit enhanced cytotoxicity against P-gp–overexpressing tumor cells, compared with paclitaxel (13–15), and certain epothilone derivatives are poorly recognized by P-gp pumps (16, 17). This suggests that some epothilones may overcome multidrug resistance (MDR) mechanisms, which would augment their therapeutic potential. Natural epothilones, however, are not optimal candidates for anticancer therapy for a number of reasons, including a limited therapeutic index and poor tolerability (18–20). Because of this, novel epothilone derivatives have been synthesized and investigated in preclinical models (17–20).

Sagopilone (ZK-EPO) is the first fully synthetic epothilone undergoing clinical trials (21). Previous results from our group indicated that sagopilone is not exported by P-gp efflux pumps and that it exerts cytotoxic effects against a panel of tumor models (21). Here, we investigate the molecular mechanisms underlying the cytolytic activity of sagopilone that involves mitotic blockade followed by activation of the mitochondrial apoptotic pathway. In particular, we identify proteins of the Bcl-2 family as major regulators of sagopilone-induced cell death. Moreover, we show that sagopilone is far more effective than other microtubule stabilizers in inducing the polymerization of tubulin in vitro and that it exhibits an improved intracellular pharmacokinetics, in part due to the fact that it is not a substrate of P-gp efflux pumps. Finally, we extend our previous observations and confirm the anticancer activity of sagopilone against a wide variety of human cancers, including those refractory to conventional therapy, in vitro and in vivo.
Materials and Methods

Chemicals. cis-Diammineplatinum(II) dichloride (cisplatin), DNase I, docetaxel, doxorubicin hydrochloride (Adriamycin hydrochloride), paclitaxel, and verapamil hydrochloride were purchased from Sigma-Aldrich. [3H]paclitaxel (10.1 Ci/mmol) was obtained from Moravek Biochemicals, Inc., whereas the Chk1 inhibitor UCN-01 was from the National Cancer Institute. Epothilone B (patupilone) and D, ixabepilone, and sagopilone were produced in the Bayer Schering Pharma AG Laboratories through total syntheses (21). The pan caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethylketone (Z-VAD-fmk) was purchased from Bachem AG. Stock solutions were prepared and stored as previously detailed (18).

Cellular uptake of radiolabeled compounds. Tumor cells (3 x 10^5) were seeded in 24-well plates in 0.5 mL of growth medium per well. After 2 d, the medium was replaced with a fresh one containing either 3.5 or 70 nmol/L radiolabeled sagopilone or paclitaxel. At the indicated time points, cells were washed twice with growth medium, lysed in 0.3 mL PBS containing 0.2% (w/v) SDS (Sigma-Aldrich) and 0.25 units/mL DNase I, followed by radioactivity quantification in LS3801 liquid scintillation counter (Beckman Coulter, Inc.). Cell number was estimated DNase I, followed by radioactivity quantification in LS3801 liquid scintillation counter (Beckman Coulter, Inc.). Cell number was estimated.

Subcellular distribution of radiolabeled sagopilone and paclitaxel. A549 cells (2 x 10^6) were cultured in 10-cm-diameter Petri dishes for 2 d, then the medium was replaced with 5 mL of a fresh one containing 5 µCi (corresponding to 35 nmol/L) radiolabeled sagopilone or paclitaxel. After 2 h, cellular fractionation was performed by means of the ProteoExtract kit (Merck KGaA) according to the manufacturer’s instructions, followed by immunoblot analysis and quantification of radiolabeled sagopilone and paclitaxel. A549 cell fractions prepared in the same manner were also subjected to immunoblot analysis with antibodies specific for α-tubulin (Sigma-Aldrich), glyceraldehyde phosphate dehydrogenase (GAPDH), and histone H3 (both from Millipore).

Results

Subcellular distribution of sagopilone and its effects on tubulin dynamics. Radioactive sagopilone was efficiently taken up by A549 NSCLC cells, resulting in a maximal intracellular concentration of 6.6 pmol/10^6 cells, which was reached as early as 2 h after addition of the compound. In comparison, paclitaxel accumulated to a maximal concentration of 5 pmol/10^6 cells only after 24 hours of incubation (Fig. 1A). Subcellular fractionation assays showed that, after 2 hours of treatment, sagopilone almost exclusively localizes to the cytoskeletal compartment of cells, whereas paclitaxel mainly associates with membranes and nuclei (Fig. 1B). Stabilization of microtubules by drugs is known to reduce the solubility of tubulin, resulting in a redistribution of the protein from the cytosolic (soluble) to the cytoskeletal (insoluble) fraction. This relocation was more pronounced after a short treatment (2 hours) with sagopilone than after an equivalent incubation with paclitaxel (Fig. 1C) and correlated with the higher intracellular concentration attained by sagopilone compared with paclitaxel at this time point. The improved effect of sagopilone on tubulin polymerization, as well as its strong association with microtubules, was consistently observed in several cell lines of distinct origin, including NSCLC A549 (Fig. 1), epidermoid cancer A-431, cervical carcinoma HeLa/MaTu/Adr, and ovarian carcinoma NC1/Adr cells (not shown). The association of sagopilone with polymerized microtubules in the cytoskeletal fraction suggests that sagopilone displays a particularly potent effect on tubulin polymerization. This was confirmed in vitro using a fluorescence-based assay. Sagopilone induced an accelerated tubulin polymerization in vitro, compared with paclitaxel and the natural product epothilone B (patupilone; Fig. 1D). Thus, initial polymerization rates (mean ± SE, n = 8) of patupilone and paclitaxel amounted to 70 ± 7% and 30 ± 3%, respectively, of those observed for sagopilone. Altogether, these data support the hypothesis that sagopilone exhibits favorable subcellular pharmacokinetic and pharmacodynamic properties.

Sagopilone activates mitochondrial apoptosis after cell cycle arrest. In contrast to the normal bipolar mitosis observed in untreated cells (Supplementary Fig. S1A), sagopilone induced mitotic figures characterized by monopolar (Supplementary Fig. S1B), tripolar (Supplementary Fig. S1C), and higher-order multipolar spindles (Supplementary Fig. S1D). Kinetic analysis revealed that sagopilone first reduced the number of cells reaching anaphase and telophase (witnessing mitotic arrest at metaphase) and then multinucleation (Supplementary Fig. S1E). In addition, sagopilone activated the spindle assembly checkpoint at concentrations as low as 100 pmol/L (i.e., lower than the IC50; see below), as indicated by the recruitment of Bub1 to mitotic spindles (Supplementary Fig. S1F and G). Following the arrest in the G2-M phase of the cell cycle (maximal at 24 hours), an increasing percentage of cells underwent apoptosis and exhibited a sub-G1 DNA content (Supplementary Fig. S1H). As confirmed by transmission electron microscopy, at 48 hours, a substantial fraction of cells exhibited multinucleation or had broken down into apoptotic bodies (Fig. 2). Videomicroscopic observation of cells expressing a histone H2B-GFP fusion protein (that allows for the visualization of chromatin) further showed that sagopilone induces a mitotic blockade followed by multinucleation and/or apoptosis (Supplementary Figs. S2 and S3 and Videos S1-S5).

Sagopilone-induced cell death was accompanied by the release of cytochrome c from mitochondria (resulting in a diffuse cytochrome c-specific immunofluorescence staining pattern), followed
by caspase-3 proteolytic activation (detected with an antibody that recognizes an epitope specifically accessible on active caspase-3; Fig. 2B; ref. 26). In addition, cells treated with sagopilone lost their mitochondrial transmembrane potential \( (\Delta F_m) \) before they died (Fig. 2C). Sagopilone-induced \( \Delta F_m \) dissipation was inhibited by overexpression of the viral mitochondrial inhibitor of apoptosis (vMIA, a Bax antagonist) from human cytomegalovirus (Fig. 2C; refs. 27, 28). Next, we aimed at identifying the apical caspases activated in response to sagopilone, by means of a biotinylated caspase inhibitor (biotin-VAD.fmk), which "traps" the first activated caspases by covalently binding to their heavy subunit (22). When biotin-VAD.fmk and sagopilone were coinjected into cells and 48 hours later biotinylated proteins were purified from cell extracts, caspase-9 (but not caspase-8) could be detected (Fig. 2D). These results suggest that caspases are activated as a consequence of mitochondrial cytochrome c release, through a canonical pathway that involves caspase-9 and caspase-3 as the initiator and executioner caspases, respectively (29).

Sagopilone induces cell death through an apoptotic pathway that implicates the Bcl-2 family of proteins. With a panel of previously validated siRNAs that target several apoptosis regulatory proteins, we compared the apoptotic pathways triggered by cisplatin, paclitaxel, and sagopilone in HCT 116 colorectal cancer cells. Paclitaxel- and sagopilone-induced cell death exhibited a similar pattern of modulation by distinct siRNAs, which was rather dissimilar from that observed with cisplatin (Fig. 3A). Thus, although all these agents induce apoptosis via the mitochondrial pathway, they act through distinct upstream regulators. Among the most efficient inhibitors of paclitaxel-induced apoptosis were siRNAs that knock down proapoptotic proteins from the Bcl-2 family (in particular Bax and Bak, alone or in combination, Bim and Puma) as well as caspase-2 and caspase-3. The knockdown of these proteins also inhibited apoptosis triggered by sagopilone, although to a lesser extent, whereas Bim and Puma were not involved in cisplatin-induced cell death. Conversely, constituents of the permeability transition pore complex like the adenine nucleotide translocase (ANT) isoform 2 and the voltage-dependent anion channel isoforms 1 and 2 were implicated as proapoptotic effectors in cisplatin-induced apoptosis, but had no effect on the lethal pathways initiated by paclitaxel and sagopilone. Moreover, we found ANT3 depletion sensitized cells to the apoptotic effects of paclitaxel and sagopilone but not of cisplatin. Of note, knockdown of Bcl-X\(_{L}\) and/or Bcl-2 sensitized cells to paclitaxel and sagopilone (Fig. 3A), supporting the general conclusion that mitochondrial membrane permeabilization (which is inhibited by Bcl-X\(_{L}\) and Bcl-2) is a major rate-limiting step in sagopilone-induced apoptosis. Accordingly, the genetic knockout of the proapoptotic Bcl-2/Bcl-X\(_{L}\) antagonist bax partially reduced cell death induced by sagopilone in mouse embryo fibroblasts (Fig. 3B) and human HCT 116 colon cancer cells (Fig. 3C). On the other hand, the ablation of p53 (Fig. 3B and C) or the transgenic overexpression of the caspase-inhibitory protein p35 (Fig. 3D) had
Figure 2. Sagopilone promotes mitotic catastrophe and mitochondrial apoptosis. A, human colon carcinoma HCT 116 cells were treated with 30 nmol/L sagopilone and processed for transmission electron microscopy observation. After 18 h of incubation, sagopilone-treated cells manifested alterations in the arrangement of chromosomes, which appeared dispersed in the cytoplasm outside the metaphase plate (I). Alternatively, or in addition, sagopilone induced the generation of multinucleated cells (II). After 36 h, the typical hallmarks of apoptosis were observed. White bars, picture scale. B, HCT 116 cells seeded on l-polylysine–coated coverslips were left untreated (Co) or treated with 10 nmol/L paclitaxel, patupilone, or sagopilone (Sag) for the indicated time, in the absence or presence of 50 μmol/L Z-VAD-fmk, followed by immunostaining for the detection of cytochrome c (Cyt c, green) and active caspase-3 (Casp-3a, red). Hoechst 33342 (emitting in blue) was used for nuclear counterstaining. Representative images obtained in control conditions and upon sagopilone treatment are shown (scale bars, 10 μm). White and black columns, mean percentage of cells (n = 3) characterized by Cyt c release (diffuse Cyt c) and caspase-3 activation (Casp-3a+), respectively; bars, SE. *, statistically significant differences (Student’s t test, P < 0.05), compared with untreated control cells. c, statistically relevant reductions in the percentage of Casp-3a+ cells by Z-VAD-fmk, compared with cells treated with the same concentration of microtubule poisons alone. C, cervical carcinoma HeLa cells stably transfected with pcDNA3.1 control vector (Neo) or with a plasmid encoding the viral mitochondrial inhibitor of apoptosis (vMIA) from human cytomegalovirus were left untreated or cultured with the indicated dose of sagopilone for 48 h, followed by cytofluorimetric quantification of viability (with propidium iodide, PI) and mitochondrial transmembrane potential [ΔΨm, with DiOC6(3)]. White and black columns, mean percentage of cells (n = 3) exhibiting ΔΨm loss alone (ΔΨm-) and in combination with plasma membrane rupture (PI+), respectively; bars, SE. *, statistically significant protection (Student’s t test, P < 0.05) observed in HeLa vMIA cells, compared with HeLa Neo cells treated with the same concentration of sagopilone. D, human colon carcinoma HCT 116 cells were either cultured in the simultaneous presence of sagopilone and biotin-VAD.fmk (b-VAD, which traps apical caspases) for 48 h (Pretreat b-VAD), or with sagopilone only for 42 h followed by the addition of biotin-VAD-fmk during the last 6 h of treatment (Posttreat b-VAD). Thereafter, biotinylated proteins were purified from cellular extracts and subjected to immunoblotting with antibodies that recognize caspase-3 and caspase-9.
only moderate effects on sagopilone-induced cell death. The siRNA-mediated down-regulation of Chk1 (but not of Chk2) sensitized cells to sagopilone-induced apoptosis (Fig. 3A), a finding that could be recapitulated by the pharmacologic Chk1 inhibitor UCN-01 (Supplementary Fig. S4A–D). In an additional series of experiments, we determined the effect of the genetic background on the clonogenic survival of human HCT116 colon carcinoma cells treated with spindle poisons. Although the absence of bax increased the relative resistance of these cells to taxanes and epothilones, including sagopilone, by a factor of 1.6 to 1.9 (P < 0.01, Student’s t test), the knockout of p53, p21, chk2, or 14-3-3s had no effects, underscoring the importance of Bax for the induction of apoptosis by microtubule-targeting agents (Supplementary Table SII). In HCT116 cells, p35 expression also conferred a moderate relative resistance, by a factor of 1.5 to 1.8.

A549-B480 is a clone of NSCLC A549 cells that has been selected by continuous culture in the presence of patupilone (30). These cells require the presence of 100 to 300 nmol/L patupilone for survival because of a series of mutations that lead to amino acid substitutions in class I β-tubulin (Gln292Glu and Val60Phe, in the first and second allele, respectively) and Ka1-tubulin (Leu195Met; ref. 30). Upon removal of patupilone, A549-B480 cells manifested a sequence of alterations (metaphase arrest, abnormal metaphases with monopolar and multipolar spindles, multinucleation, caspase-3 activation, and apoptotic chromatin condensation; Fig. 4A and B) that is strikingly similar to those observed in normal cells treated with microtubule-stabilizing drugs (Fig. 2). This cell death was partially suppressed by pharmacologic inhibition of caspases with Z-VAD-fmk and by siRNA-mediated depletion of Bax and/or Bak, and was enhanced following the depletion of Bcl-2 and/or Bcl-XL (Fig. 4C). The addition of sagopilone rescued A549-B480 cells from

Figure 3. Sagopilone-induced mitochondrial apoptosis is modulated by a specific set of cell death regulators. A, human colon carcinoma HCT 116 cells previously transfected with the indicated siRNAs for 24 h were left untreated or treated with 30 nmol/L sagopilone, 30 nmol/L paclitaxel, and 20 μmol/L cisplatin (CDDP), followed by viability assessment with a colorimetric assay. For each siRNA, columns report the assay-independent variable ΔWST-1 (defined in Supplementary Materials and Methods), which represents the difference between the residual viability observed upon treatment in transfected cells and the residual viability obtained in cells transfected with a control, scrambled siRNA (SCR). Columns, mean; bars, SE. Gray and black columns, statistically significant (Student’s t test, P < 0.05) negative and positive values of ΔWST-1, which in that order represent chemosensitization and cytoprotection toward chemotherapeutic agents. White columns, nonsignificant (n.s.) values (P > 0.05). Wild-type (wt), p35+/−, bax+/−, and bax+/-bax+/- mouse embryonic fibroblasts (B); p35+/−, bax+/−, p21+−, and 14-3-3s+− human colon carcinoma HCT 116 cells (C); and HCT 116 cells stably transfected with an empty vector (pCMV) or with a pCMV-based plasmid encoding the baculoviral inhibitor of caspases p35 (D) were treated with the indicated concentrations of sagopilone for 48 h. Thereafter, cells were stained with DiOC6(3) and propidium iodide for the cytofluorimetric detection of apoptosis-associated mitochondrial transmembrane potential (ΔΨm) dissipation and plasma membrane rupture. White and black columns, mean percentage of cells (n = 3) characterized by ΔΨm loss alone (ΔΨm−) and in combination with plasma membrane breakdown (PI+), respectively; bars, SE. *, statistically significant (Student’s t test, P < 0.05) differences in the response to sagopilone of cells characterized by the indicated genetic backgrounds, compared with control cells treated with the same concentration of drug.
patupilone withdrawal-induced apoptosis (Fig. 4D), which points to a large overlap between the mechanisms underlying the effects of patupilone and sagopilone. Consistent with this interpretation, 1,000 nmol/L sagopilone alone as well as 100 to 1,000 nmol/L sagopilone in association with 300 nmol/L patupilone were able to overcome the resistance of A549-B480 cells (which are characterized by an IC50 for patupilone of 480 nmol/L; ref. 30) to microtubule stabilizers, and provoked extensive cell death (Fig. 4D). Notably, the dose of sagopilone (10 nmol/L) required to substitute for patupilone (>100 nmol/L) was relatively low, further underscoring its elevated pharmacologic potential (Fig. 4D).

The pharmacokinetic profile of sagopilone contributes to its enhanced antitumor effect. Although sagopilone activates lethal pathways similar to those triggered by other epothilones and taxanes (Figs. 2–4), it incorporates more efficiently into cells (Fig. 1) and hence acts at a lower IC50 than these compounds, when tested on HCT 116 colon cancer cells (Supplementary Table SII). Similarly, sagopilone showed an enhanced antitumor activity compared with other epothilones in Adriamycin-resistant NCI/Adr cells, which are well known for the overexpression of P-gp efflux systems that confer a MDR phenotype (21). Inhibition of P-gp efflux pumps by verapamil (31) enhanced the sensitivity of NCI/Adr cells to patupilone; yet, it had no effect on the response of these cells to sagopilone (Supplementary Fig. S6A). The uptake of radiolabeled sagopilone and paclitaxel was evaluated in several cell lines, including MDR1-overexpressing HeLa/MA/Adr/Adr cells (Supplementary Table SIII). Generally, the uptake of 3.5 nmol/L sagopilone (range 5.9–11.8 pmol/106 cells) was higher than that of paclitaxel (range 0.2–3.6 pmol/106 cells), and this difference was particularly pronounced in HeLa/MA/Adr/Adr cells (5.9 pmol/106 cells for sagopilone versus 0.2 pmol/106 cells for paclitaxel). Similar results were observed when these agents were administered at a concentration of 70 nmol/L. Notably, verapamil increased the intracellular concentration of paclitaxel in Adriamycin-resistant HeLa/MA/Adr/Adr cells more than 6-fold; yet, it did not influence the intracellular concentration of radiolabeled sagopilone (Supplementary Table SIII), further demonstrating that sagopilone is not a substrate of P-gp efflux pumps. The rapid cellular uptake of sagopilone is consistent with its full antiproliferative effects on tumor cells even after a short incubation period (2 hours versus >72 hours for ixabepilone; Supplementary Fig. S5; refs. 32–35).

Broad anticancer activity of sagopilone in vitro and in vivo.

To evaluate the therapeutic range of sagopilone, its antiproliferative activity was compared with that of other microtubule-targeting agents on a panel of tumor cell lines. Sagopilone was more active than paclitaxel and other epothilones (such as ixabepilone or epothilone D) in all cell models evaluated (Supplementary Fig. S6b), including those that are intrinsically resistant to paclitaxel (e.g., Caco2 and HCT-15 colon cancer and Panc-1 pancreatic cancer cells) and resistant to standard anticancer agents (e.g., HeLa/MA/Adr/Adr and HeLa-RDB cervical cancer, HT-29-RDB colon cancer, and EPG75-257-RDB gastric cancer cells; Supplementary Fig. S6B). As determined on a panel of 49 tumor cell lines, the mean IC50 value of sagopilone was 0.64 nmol/L (range 0.3–1.8 nmol/L), which is much lower than that previously determined for ixabepilone (1.4–34.5 nmol/L) in 21 tumor models (36).

The range of activity shown by sagopilone in vitro was mirrored in vivo across different human cancer xenograft models, including those resistant to common antineoplastic agents like paclitaxel. Sagopilone was efficient in the treatment of a variety of human cancers growing in immunodeficient mice, as determined by

**Figure 4.** Sagopilone prevents apoptosis induced by patupilone withdrawal in A549-B480 cells. A and B, NSCLC. A549-B480 cells were cultured on L-polylysine–coated coverslips in the presence of 300 nmol/L patupilone, which was then removed for the indicated recovery time. Thereafter, samples were processed for immunofluorescence microscopy aimed at detecting β-tubulin (green fluorescence) and active caspase-3 (Casp-3a, red fluorescence). Hoechst 33342 (emitting in blue) was used as nuclear counterstain. In A, representative images for each time point are reported, focusing on abnormal mitoses and multinucleated cells. In B, columns report the quantification of the mitotic index, as well as of the percentage of cells characterized by multinucleation or caspase-3 activation (Casp-3a+). Columns, mean (n = 3); bars, SE. *p < 0.05; **p < 0.005. C and D, A549-B480 cultured in the presence of 300 nmol/L patupilone were transfected with the indicated siRNAs for 24 h, followed (−) or not (+) by patupilone withdrawal from the culture medium. After an additional 48 h, cells were subjected to cytofluorimetric quantification of mitochondrial transmembrane potential (∆Ψm) and viability (with propidium iodide). D, A549-B480 cells were incubated with the indicated doses of sagopilone alone (−) or together with 300 nmol/L patupilone (+) for 72 h, followed by cytofluorimetric assessment of apoptosis-associated variables as in C. In C and D, white and black columns depict the percentage of cells that have dissipated their ∆Ψm (ΔΨmlow) and lost plasma membrane integrity (PIlow), respectively. Columns, mean (n = 3); bars, SE. In C, asterisks (*) depict statistically significant (Student’s t test, P < 0.05) differences in patupilone withdrawal-induced death of cells depleted for the indicated proteins, compared with control conditions (untransfected cells or cells transfected with a control siRNA). In D, asterisks (*) depict statistically significant (Student’s t test, P < 0.05) increases in cell death upon sagopilone treatment, compared with control conditions (cells maintained in the presence of patupilone alone), whereas daggers (†) indicate statistically relevant (Student’s t test, P < 0.05) decreases in the patupilone withdrawal–induced death of cells treated with sagopilone, compared with control conditions (cells cultured in the absence of both patupilone and sagopilone).
measuring treated versus control (T/C) ratios (the tumor area of sagopilone-treated xenografts divided by that of untreated xenografts; Table 1). As a T/C ratio <0.5 denotes significant efficacy, results indicate that sagopilone was highly efficient against tumors with a MDR phenotype, such as those derived by HeLa/MaTu/Adr and A2780/AD10 cells. T/C ratios <0.5 were observed in a majority of xenografts treated with sagopilone, including models of breast, ovarian, lung, prostate, and pancreatic cancer. For instance, sagopilone induced the regression of the ovarian cancer xenograft OvarCa-2 (Supplementary Fig. S6C) and strongly inhibited the growth of xenografted NCI/Adr cells (Supplementary Fig. S6D). Sagopilone also reduced tumor growth of the HCT 116 colon cancer xenograft, which is intrinsically resistant to paclitaxel (Supplementary Fig. S6E).

**Discussion**

The antitumor activity of paclitaxel and epothilones is primarily due to mitotic arrest, followed by the induction of apoptosis (1, 5, 11). The cytotoxicity produced by such agents generally correlates with their ability to bind microtubules, stabilize mitotic spindles, and induce the formation of interphase microtubule bundles (14). Paclitaxel binds to tubulin at the same binding site as paclitaxel, yet it induces more rapid generation of microtubule bundles (13). The results presented here indicate that another epothilone (i.e., sagopilone) provokes an even more rapid and potent tubulin polymerization than paclitaxel or patupilone. However, the molecular pathways that link tubulin polymerization to mitotic aberration and mitochondrial apoptosis after sagopilone treatment seems substantially similar to that induced by paclitaxel and other epothilones, with a major involvement of Bel-2 family proteins. Two features of sagopilone may explain why this molecule consistently acts at a lower IC₅₀ than other microtubule-polymerizing agents and why different tumor cell lines exhibit a strong and only moderately variable sensitivity in response to sagopilone. First, sagopilone is more efficient than other microtubule poisons in inducing tubulin polymerization in vitro, reflecting an enhanced intrinsic activity of the molecule compared with similar agents.

**Table 1. Efficacy of sagopilone presented as a ratio of tumor area in the treated versus control groups, in multiple xenograft models of human cancer**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Model</th>
<th>T/C ratio</th>
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<tbody>
<tr>
<td>Breast cancer</td>
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<td>MX-1</td>
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<td>Cervical cancer</td>
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<td>PC-3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Abbreviation: SCLC, small-cell lung cancer.

addition, sagopilone exhibits favorable pharmacodynamic properties because it is rapidly taken up by cells and is not recognized by P-gp efflux pumps. The range of activity shown by sagopilone in vitro correlates with its efficacy against several human cancer xenograft models, including those resistant to common antineoplastic agents. The experiments reported here and elsewhere (21) also indicate that sagopilone is well tolerated in animal models, with a moderate initial weight reduction followed by prompt recovery. This is in contrast to the substantial toxicity previously described for patupilone (12, 17).

Sagopilone exerts potent antineoplastic activities across a wide spectrum of tumor models in vitro, at much lower concentrations than other epothilones, and in vivo with favorable tolerability. Irrespective of the molecular pathways activated by sagopilone, which are partially shared with other microtubule poisons, the clinical application of this novel epothilone might take significant advantages of its pharmacodynamic profile. In clinical settings, this might allow for the use of lower doses compared with those used for classic microtubule stabilizers (and notably for paclitaxel, which is routinely used to treat several human malignancies but is associated with neutropenia, weakness, infection, and allergic reactions; ref. 37), thus reducing the incidence and severity of side effects.

These preclinical results provide a sound basis for examining sagopilone in various clinical settings. In phase I trials, sagopilone has already shown favorable bioavailability, antitumor activity, and good tolerability in heavily pretreated patients (38). Based on these promising results, sagopilone is currently being evaluated in a broad phase II program enrolling patients with a wide range of tumors, including glioblastomas and ovarian, prostate, lung, and breast cancers (39). Thus, our preclinical data signal a considerable potential for sagopilone.

**Disclosure of Potential Conflicts of Interest**

J. Eschenbrenner: Commercial research grant, Bayer Schering Pharma AG; C. D'Adamo: research grant, Bayer Schering Pharma AG; K. Lichtner: Employment, Bayer Schering Pharma AG; G. Kroemer: Commercial research grant and consultant, Bayer Schering Pharma AG. B. Buchmann, W. Skuballa, R.B. Lichtner, and U. Klar are inventors of sapogilone (ZK-EPO). The other authors disclosed no potential conflicts of interest.

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