Calpain-Mediated Integrin Deregulation as a Novel Mode of Action for the Anticancer Gallium Compound KP46

Ute Jungwirth1, Johannes Gojo1, Theresa Tuder1, Gernot Walko2, Martin Holcmann1, Thomas Schöffl1, Karin Nowikovsky3, Nastasia Wittfinger3, Sushilla Schoonhoven1, Christian R. Kowol4, Rosa Lemmens-Gruber5, Petra Heffter1,1,1, Bernhard K. Keppeler5, and Walter Berger1,4

Abstract
On the basis of enhanced tumor accumulation and bone affinity, gallium compounds are under development as anticancer and antimetastatic agents. In this study, we analyzed molecular targets of one of the lead anticancer gallium complexes [KP46, Tris(8-quinolinolato)gallium(III)] focusing on colon and lung cancer. Within a few hours, KP46 treatment at low micromolar concentrations induced cell body contraction and loss of adhesion followed by prompt cell decomposition. This rapid KP46-induced cell death lacked classic apoptotic features and was insensitive toward a pan–caspase inhibitor. Surprisingly, however, it was accompanied by upregulation of proapoptotic Bcl-2 family members. Furthermore, a Bax- but not a p53-knockout HCT-116 dimline exhibited significant KP46 resistance. Rapid KP46-induced detachment was accompanied by downregulation of focal adhesion proteins, including several integrin subunits. Loss of integrin-β1 and talin plasma membrane localization corresponded to reduced binding of RGD (Arg–Gly–Asp) peptides to KP46-treated cells. Accordingly, KP46-induced cell death and destabilization of integrins were enhanced by culture on collagen type I, a major integrin ligand. In contrast, KP46-mediated adhesion defects were partially rescued by Mg2+ ions, promoting integrin-mediated cell adhesion. Focal adhesion dynamics are regulated by calpains via cleavage of multiple cell adhesion molecules. Cotreatment with the cell-permeable calpain inhibitor PD150606 diminished KP46-mediated integrin destabilization and rapid cell death induction. KP46 treatment distinctly inhibited HCT-116 colon cancer xenograft in vivo by causing reduced integrin plasma membrane localization, tissue disintegration, and intense tumor necrosis. This study identifies integrin deregulation via a calpain-mediated mechanism as a novel mode of action for the anticancer gallium compound KP46. Mol Cancer Ther; 13(10); 2436–49. ©2014 AACR.

Introduction
Metal compounds have a long history as therapeutic agents, especially in oncology. However, since the successful clinical approval of cisplatin, platinum compounds have dominated the field of anticancer metal drug research. More recently, other metals have also moved into focus for drug development due to their tumor-targeting properties, redox-based activation mechanisms, and unexpected molecular targeting characteristics (1, 2). Major aims of these attempts are to optimize the therapeutic activity, reduce unwanted adverse effects, and prevent resistance development (3).

The development of gallium compounds for anticancer therapy is especially interesting due to the fact that gallium shares several characteristics with iron. On the basis of the enhanced needs of the rapidly proliferating malignant tissue for iron, gallium compounds harbor intrinsic tumor-targeting properties by preferential uptake via the transferrin/transferrin receptor system (4). Moreover, gallium drugs interfere with several iron-containing enzymes, including ribonucleotide reductase, essential for the maintenance of the malignant phenotype. In addition, gallium nitrate has been approved for the treatment
of cancer-related hypercalcemia and was proved to exert positive effects on bone turnover and osteolysis in patients with multiple myeloma (5) and bone metastases (6).

Currently, the orally bioavailable gallium maltolate and tris(8-quinolinolato)gallium(III) (KP46) are in early clinical development as anticancer agents (7–9). KP46 was designed to optimize hydrolytic stability and membrane penetration abilities. A recent X-ray absorption study proved high stability under physiologic conditions in cell culture media and even in tissue samples of treated mice (10). Antihypercalcemic as well as antimetastatic effects were demonstrated in the Walker carcinosarcoma 256 model in rats (11). A first-in-human phase I study revealed favorable tolerability and preliminary evidence of activity against renal cancer (9). Moreover, gallium compounds, including KP46 and gallium nitrate, preferentially accumulate in the bone (12), reflected by the use of radioactive gallium compounds as PET tracers (13). This enhanced bone accumulation also suggests direct activity against primary bone tumors and metastases.

Although tumor-targeting mechanisms of KP46 are well characterized, the molecular mechanisms underlying cancer cell death induction by KP46 are widely unexplored. In this study, we elucidated for the first time loss of integrin-mediated cell adhesion followed by rapid cell death induction by a caspase-independent but a calpain-promoted mechanism as a novel mode of action for the gallium compound KP46.

Materials and Methods

Reagents

KP46 [tris(8-quinolinolato)gallium(III)] was synthesized according to previously described methods (12) at the Institute of Inorganic Chemistry, University of Vienna (Vienna, Austria). For in vitro studies, compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted into culture media. Maximum DMSO content was 0.3%. All other substances were purchased from Sigma-Aldrich. All solutions were freshly prepared before use.

Cell culture

The following cancer cell lines were used in this study: the human non–small cell lung cancer (NSCLC) cell lines A549, A427, SW1537, and Calu-6; the human colon carcinoma cell lines SW480 and Caco2; and the human osteosarcoma cell lines HOS and MG63; the murine colon cancer cell line CT-26; the human leukemic cell model HL60; the human osteosarcoma cell lines HOS and MG63 (all obtained between 1995 to 2009 from American Type Culture Collection); the human small cell lung carcinoma cell line GLC-4 (generously provided in 1995 by Dr. De Vries, University Groningen, Groningen, The Netherlands; ref. 14); the colon carcinoma cell model HCT-116; and respective sublines with deleted p53 or Bax genes (generously provided in 2005 by Dr. Vogelstein, John Hopkins University, Baltimore, MD). HCT-116 was grown in McCoy’s, SW480 in minimum essential medium (MEM), A427 cells in MEM supplemented with pyruvate and nonessential amino acids, CT-26 in DMEM/F12, and all other cell lines in RPMI-1640. Culture media were supplemented with 10% fetal calf serum (FBS, PAA). All cells were cultured at 37°C in humidified atmosphere and 5% CO2. The cell lines were authenticated in all cases by array comparative genomic hybridization (Agilent; 44k human whole genome DNA arrays) as published previously (15) and/or STR fingerprinting before the start of this study.

Cytotoxicity and clonogenic assays

Impact of drug exposure on cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based vitality assay (EZ4U; Biomedica; ref. 16). Experiments were carried out in triplicate in growth media with 10% FBS and repeated three times. For viability assays with magnesium (Mg2+), the cell line was replaced with serum-free medium (containing standard 0.4 mmol/L Mg2+) or supplemented with Mg2+ to 3.2 mmol/L and cells were exposed to KP46 for 24 hours. For vitality assay with calpain inhibitors, cells were preexposed to PD150606 for 1 hour before KP46 treatment. For clonogenicity assays, 6-well plates were coated with calf skin collagen type I (Sigma) for 24 hours and washed twice with phosphate-buffered saline (PBS). Then, 1,000 cells per well were seeded in uncoated and coated wells, treated for 24 hours with increasing KP46 concentrations and, after drug removal, cultured for 7 to 12 days until clones had reached the optimal size for microscopical evaluation after crystal violet staining as published previously (17). Experiments were performed twice in duplicate.

Annexin V/PI staining

After 24 and 48 hours of KP46 treatment, cells were stained with Annexin V (Annexin V–FITC; BD Biosciences) and propidium iodide (200 ng/ml PI; Sigma-Aldrich). Cells were analyzed according to the manufacturer’s protocol using fluorescence-activated cell sorting (FACSCalibur; Becton Dickinson). CellQuest Pro software (Becton Dickinson and Co.) was used to analyze the data. Experiments were performed three times.

Western blot analysis

Cells were exposed to KP46, and total protein lysates were prepared, resolved by SDS/PAGE, and transferred onto a polyvinylidene difluoride membrane for Western blotting as published previously (16, 18). The p53 antibody was from Thermo Scientific (clone DO-1). The following antibodies were purchased from Cell Signaling Technology: Bid, Bax, Bim, and Bak from the Pro-Apoptosis Bcl-2 Family Sampler Kit (#9942), P-myosin light chain (Ser19) and myosin light chain from the Myosin Light Chain 2 Sampler Kit (#9776), all integrin subunits except β1 from the Integrin Sampler Kit (#4749), all Rho/Rac antibodies from the Rho-GTPase Sampler Kit (#9678), p21Waf1/Cip1 from the Cell Cycle Regulation Sampler Kit (#9932), focal adhesion kinase (FAK; #13009). The antibodies against talin and integrin-β1 were from Sigma (#T3287) and Becton Dickinson (#610467), respectively.
Secondary antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology) were used at working dilutions of 1:10,000. Western blot bands were quantified with QuantiScan software (Biosoft).

Live cell imaging
Time-lapse video microscopy was performed using an AxioObserver Z1 microscope coupled to AxioCam MRm (Carl Zeiss MicroImaging) equipped with phase contrast optics. Cells were plated in 6-well dishes at a density of $1 \times 10^5$ cells per well and kept in the appropriate medium during the whole period of observation. Control and KP46 (10 $\mu$mol/L)-treated cells were monitored in parallel in a PM S1 incubator (Carl Zeiss MicroImaging) at 37°C and 5% CO$_2$ using the “mark and find” module of AxioVision (version 4.8.1) image analysis software (19). Recordings started 20 hours after plating and 1 hour after addition of the drug. Frames were taken with an EC Plan-Neofluar 10×/0.3NA objective lens in 10-minute intervals over a period of up to 24 hours. Images were processed with Zeiss AxioVision 4.8.1 image analysis software and further analyzed with ImageJ (NIH, Bethesda, MD).

Immunofluorescence staining and confocal microscopy
For immunofluorescence staining, cells were treated as indicated. After washing with PBS, cells were fixed with 3% (w/v) paraformaldehyde in PBS followed by permeabilization for 7 minutes with 0.5% Triton-X100 (v/v) in PBS. For mitochondrial staining, MitoTracker Red (Invitrogen) was used according to the manufacturer’s instruction. For membrane staining, rhodamine-labeled wheat germ agglutinin (WGA; Vector Labs) was used. Briefly, cells were incubated with 5 $\mu$g/mL rhodamine-WGA for 10 minutes at 37°C. Mitochondria- and membrane-stained cells were washed with PBS and fixed as described above. FITC-phalloidin was used at a dilution of 1:250 (Sigma). Primary antibodies were diluted in 1% BSA/PBS (w/v) at the following dilutions: $\alpha$-tubulin (1:200; Sigma), talin (1:100; Sigma), and integrin-β1 (1:200; BD; #610467). Anti-mouse IgG-FITC (1:500; Sigma; #F2012) and anti-rabbit-Dylight549 (1:500; Santa Cruz Biotechnology) were used as secondary antibodies. In all cases, controls without or with isotype-specific control antibodies instead of the first antibody were used. After mounting with Vectashield (containing DAPI), samples were examined under a confocal laser scanning microscope (Zeiss Invert Axio Observer.Z1, Two-channel LSM 700 URGB). Digital images were acquired and processed with the Zeiss ZEN (2011) software. Stacks (scan zoom 1.0, z distance 1.5 $\mu$m) were recorded at the lowest focal plane close to the basal cell membrane.

Adhesion assay
Cells were detached with Accutase (Sigma) and allowed to adhere for 1 hour on plates coated with collagen-1 (Sigma; C8919; 0.5 $\mu$g/well) in medium without serum but supplemented with 1% BSA (w/v). In short-term assays, cells were treated with KP46 at the indicated concentration during the period of adhesion. Otherwise, cells were treated with KP46 and PD155066 for 14 hours before adhesion assay. After cell adhesion, wells were washed twice with PBS, fixed with methanol, and stained with crystal violet. Differences in cell adhesion were quantified using ImageJ software.

RGD peptide binding
HL60 cells were treated with indicated KP46 concentrations for 6 hours. Afterward, cells were incubated with FITC-labeled RGD (Arg–Gly–Asp) peptide (Anaspec) in binding buffer (10 mmol/L HEPES, 150 mmol/L NaCl, and 1% w/v BSA) for 20 minutes and washed with PBS. PI was used to exclude dead cells. Binding of the FITC-labeled RGD peptide to the cell surface HL60 leukemic cells was measured by fluorescence-activated cell sorting (FORTESSA; BD Biosciences).

Intracellular calcium levels
HCT-116 cells were loaded with FURA 2AM (Sigma-Aldrich) and an equivalent of pluronic 20% (Molecular Probes) at room temperature for 30 to 45 minutes. After washout, cells were allowed to equilibrate for 30 minutes. KP46 was diluted to the bathing solution in an appropriate concentration and experiments were performed as previously described (20). Ratiometric measurements were realized following background subtraction with the Axon Imaging Workbench 2.2 software (Axon Instruments) averaging two frames. Results are presented as the relative change ($\Delta F/F_0$) of the F340 nm/F380 nm signal.

In vivo experiments
Six- to 8-week-old female SCID/BALB/c mice were purchased from Harlan. For xenograft experiments, animals were kept in a pathogen-free environment and every procedure was done in a laminar airflow cabinet. The experiments were done according to the Federation of Laboratory Animal Science Association (FELASA) guidelines for the use of experimental animals and approved by the Ethics Committee for the Care and Use of Laboratory Animals at the Medical University Vienna and the Ministry of Science and Research, Austria.

Solid tumor xenograft model
For local tumor growth experiments, $5 \times 10^5$ HCT-116 cells were injected subcutaneously into the right flank of mice. Animals were randomly assigned to treatment groups and therapy started when tumors were palpable. Mice were treated with KP46 (15 mg/kg dissolved in 5% DMSO and diluted in medium) for 2 weeks, five times per week, intraperitoneal (i.p.). The control group received 100 $\mu$L solvent. Animals were controlled for distress development daily and tumor size was assessed regularly by caliper measurement. Tumor volume was calculated as $[\text{length} \times \text{width}^2] \times 0.5$; ref. 21. Body weight was determined before drug administration and recorded regularly during the experiment. Tissue sections were
paraffin-embedded and hematoxylin and eosin (H&E) stained by routine procedures. Immunohistochemical staining for integrin-β1 and detection of DNA breaks by terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay were performed as published previously (22). The percentage of mitotic figures and dead cells (TUNEL-positive + TUNEL-negative, nonmitotic cells with condensed chromatin) was determined microscopically in nonnecrotic tumor regions (at least 6 optical fields from 4 different tumors/animals) using DAPI/TUNEL staining.

**Statistical analysis**

Where not mentioned otherwise, data are expressed as mean ± SEM. Results were analyzed and illustrated with GraphPad Prism (version 5; GraphPad Software). Statistical analyses were performed using one- and two-way ANOVA with drug treatment, time, concentration, or cell type as independent variables and conducted with Bonferroni posttests to examine the differences between the different drug treatment regimens and the diverse responses. A *P* value of 0.05 was considered statistically significant.

**Results**

**Anticancer activity of KP46 in vitro: minor impact of the p53 status**

In an initial *in vitro* screen, KP46 proved to be highly active against 22 cell lines from different tumor entities with IC_{50} values for 72-hour drug exposure generally in the low μmol/L range (Supplementary Fig. S1A). Besides cell lines from melanoma (23) and osteosarcoma (manuscript in preparation), also A427 (NSCLC) and HCT-116 p53/wt (colon cancer) cells were among the most sensitive cell models (IC_{50} values of 0.5 and 1.2 μmol/L, respectively). In contrast, the NSCLC cell line A549 and the colon cancer cell line SW480 were relatively KP46 resistant (IC_{50} values above 3.0 μmol/L). Differences of the KP46 IC_{50} values were highly significant for A427 versus A549 and for HCT-116 versus SW480 cells (both Student t test, *P* < 0.001) and not a consequence of different proliferative capacities (data not shown). Thus, we decided to focus on these major tumor entities (IC_{50} values for a cell line panel from these tumor entities are shown in Table 1) and to compare A549 and SW480 as “KP46-resistant” cells in contrast to HCT-116 and A427 as “KP46-sensitive” representatives.

Caspase-mediated apoptosis is not essential for rapid cell death execution induced by KP46

Time-dependency experiments revealed that the number of viable cells was reduced by up to 75% in the sensitive cell lines already after 24 hours of exposure to 2.5 μmol/L KP46, and even the viability of the resistant cell models was inhibited by >50% at 10 μmol/L (Fig. 1A). Surprisingly, however, Annexin V staining revealed an unexpectedly low percentage of apoptotic cells (Fig. 1B). Thus, solely in the highly sensitive cell lines a rather moderate increase in the percentage of Annexin V+ cells

<p>| Table 1. IC_{50} values of KP46 tested against diverse lung and colon cancer cell models |
|-----------------|-----------------|---|---|</p>
<table>
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<tr>
<th>Cell line</th>
<th>Histology</th>
<th>p53 status</th>
<th>IC_{50} KP46 (μmol/L ± SD)</th>
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<td>Lung</td>
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<tr>
<td>A549</td>
<td>NSCLC (AC)</td>
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<td>3.89 ± 0.87</td>
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<td>A427</td>
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<td>wt</td>
<td>0.40 ± 0.23</td>
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<td>SW1573</td>
<td>NSCLC (AC)</td>
<td>wt</td>
<td>1.74 ± 1.15</td>
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<td>NSCLC (AC)</td>
<td>mut</td>
<td>1.97 ± 0.46</td>
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<td>NSCLC (SCC)</td>
<td>mut</td>
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<td></td>
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<td>wt</td>
<td>1.06 ± 0.35</td>
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<td>2.30 ± 0.13</td>
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<tr>
<td>CT-26a</td>
<td>Colon carcinoma</td>
<td>mut</td>
<td>2.17 ± 0.31</td>
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</table>

*aOriginating from mouse, all others human.*
Figure 1. KP46 induces rapid cell detachment and loss of viability without massive apoptosis induction. A, relative viability of KP46-sensitive and -resistant lung and colon cancer cell lines untreated (100%) or following treatment with the indicated KP46 concentrations for 24 hours was determined by microscopical counting in Trypan blue solution. B, induction of apoptosis by KP46 was analyzed by FACS of Annexin V$^+$ cells treated as in A. Mean percentages $\pm$ SD of Annexin V$^+$ (PI$^-$ and PI$^+$) cells were determined from two independent experiments. C, impact of the pan-caspase inhibitor Z-VAD-FMK on the anticancer activity of KP46 exerted by 24 hours of exposure was determined by MTT assay in the indicated KP46-sensitive cell models. (Continued on the following page.)
(both PI+ and PI−) was detectable after 24 hours of KP46 treatment, while the effects in the resistant cell models did not even reach significance. Accordingly, mitochondrial membrane depolarization as an early marker of apoptosis induction was not enhanced in response to KP46 (Supplementary Fig. S1B). In contrast to other anticancer drugs like doxorubicin (data not shown), the pan–caspase inhibitor Z-VAD-FMK did not significantly reduce the cytotoxic activity of KP46 (Fig. 1C). Surprisingly, however, we detected in KP46-treated cells an early upregulation of proapoptotic proteins, such as Bim, Bak, and Bak (Supplementary Fig. S2A), loss of antiapoptotic Bcl-2, and translocation of Bax into the mitochondria (Supplementary Fig. S2B). In case of Bim EL, additionally a KP46-induced shift of the respective protein band could be detected. Accordingly, but in contrast to the p53 knockout, a Bax-deleted HCT-116 subline displayed moderate resistance against KP46-induced cell death (Supplementary Fig. S2C and Table 1). Together, our data suggest that KP46 induces a form of cell death different from classic caspase-mediated apoptosis that is supported by p53-independent activation of proapoptotic Bcl-2 family members.

**Rapid KP46-induced cell death involves cell-detachment by loss of integrin-mediated focal adhesion contacts**

To further characterize this unusual form of cancer cell death, we monitored cell morphology during KP46 treatment for 24 hours using time-lapse microscopy. Pronounced morphologic changes were already detected after short-term exposure to KP46 (Fig. 1D and E). A427 and HCT-116 cells were found to retract cell bodies and round-up rapidly, whereas A549 and SW480 cells detached slowly (Fig. 1F and G). The retraction phase was accompanied by highly dynamic formation and loss of branching filopodia (Fig. 1H). Although the detached cells at this stage were still viable (proved by the lack of PI accumulation; Fig. 1I), the process ended in rapid and massive cell disintegration.

Next, confocal immunofluorescence microscopy was performed to follow dynamics of the cytoskeletal changes during KP46-induced cell death (Fig. 2). Phalloidin-staining of the actin filament network revealed that KP46-treated A427 cells had lost most of the central stress fibers, whereas peripheral actin bundles were unaffected (Fig. 2A and B). Orthographic projections generated from optical sections through short-term KP46-treated cells immunostained for α-tubulin revealed the perikaryal region bulging out of the extremely flattened cell body (Fig. 2C and D). Similar observations were made using WGA as an unspecific membrane stain (Fig. 2E and F). The contracting cells left back several plasma membrane remedies ripped-off from retraction fibers (Fig. 2F).

Rapid cell detachment by KP46 was accompanied by altered expression of cytoskeletal proteins involved in adhesion and contraction. Thus, KP46 treatment distinctly reduced the levels of several integrin subunits with the strongest effect on integrin-β1, especially in KP46-sensitive cell models (Fig. 2G, top). Time-course analyses revealed that downregulation of integrin-β1 and its focal adhesion–specific binding partner talin occurred already at 6-hour KP46 treatment (Fig. 2G, bottom and Supplementary Fig. S3A), paralleling the loss of cell adhesion. Expression levels of integrin-β1 were variable in the different cell lines but KP46-mediated loss was less pronounced in KP46-resistant A549 and SW480 cells (Supplementary Fig. S3A) but massive in KP46-sensitive osteosarcoma cell lines (Supplementary Fig. S3B). In all cases, phosphorylation of the myosin light chain was upregulated by KP46 (HCT-116 cells representatively in Fig. 2G), indicative of increased actin–myosin–mediated cell contraction (25).

Immunofluorescence microscopy proved that KP46-treated A427 cells were devoid of peripheral focal adhesion–associated integrin-β1 (Fig. 2H and I) and talin (Fig. 2J and K) localization. Costaining with F-actin (Supplementary Fig. S3C) again depicted the complete loss of integrin and talin staining on the cell surface. Loss of cell surface integrin exposure was also proved by determining binding of FITC-labeled RGD peptide, the major binding site for several integrins in various extracellular matrix (ECM) proteins (26). KP46 treatment for 6 hours significantly reduced peptide binding to approximately 60% (Fig. 2L). HL60 leukemic cells were used in these experiments to avoid proteolytic cleavage of integrins during trypsinization.

**Culture on collagen sensitizes while Mg2+ ions protect against KP46**

Integrins are the cellular receptors for ECM components resulting in activation of outside-in signaling, promoting survival and proliferation (26, 27). Hence, we investigated whether cultivation of KP46-sensitive cancer cells on collagen type I, a major ligand for integrins, alters sensitivity against KP46. Indeed, plating on collagen resulted in significantly enhanced sensitivity against KP46 in both A427 and HCT-116 cells (Fig. 3A). In addition, we tested expression of a panel of...
integrin-α and -β subunits in cells grown on culture plastic as compared with collagen type I and the impact of 5 hours of KP46 treatment (Fig. 3B, top). Collagen tended to result in upregulated expression of integrin-β5 but distinctly reduced levels of α5 and β1 subunits. Integrin-α5 and several β subunit (β1, β4, β5) levels were markedly reduced by KP46 treatment. This integrin destabilization was, at least in HCT-116 cells, distinctly stronger in cultures on collagen. Also, reduced expression levels of integrin outside-in signaling molecules (28) were detectable after KP46 treatment, including FAK and members of the Rho/Rac GTPase family. Rho
A was downmodulated in both cell lines but FAK only in A427 cells in a collagen-dependent manner. The effect was even more pronounced for Rho B in the HCT-116 cell line. In contrast, the negative cell cycle–regulated p21 was slightly enhanced by KP46 (Fig. 3B, bottom). These data suggest that KP46 treatment leads to a widespread loss of ECM adhesion molecules. Consequently, we decided to determine the impact of KP46 on cell adhesion dynamics. KP46 treatment for 1 hour significantly inhibited the re-adhesion of A427 cells (Fig. 3C). Again, this effect was much stronger in the KP46-sensitive than in the -resistant cell models (A549 in Fig. 3D). Because divalent metal cations are essential for integrin function and facilitate adhesion (29), we tested whether enhanced levels of Mg$^{2+}$ could antagonize adhesion destabilization by KP46. Indeed, addition of Mg$^{2+}$ inhibited the effect of KP46 (Fig. 3C and D, white columns). Supplementation of the serum-free medium with Mg$^{2+}$ also significantly reduced the cytotoxicity induced by short-term KP46 exposure (Fig. 3E and F).
Calpain activity is a driver of KP46-induced cell death

Migratory and invasive functions of cells are strongly linked to turnover of focal adhesion complexes. This process is partially mediated and regulated by calpains and interestingly several cell adhesion and migration-regulating proteins deregulated by KP46 (integrin-β subunits, talin, FA, RhoA) represent calpain substrates (30, 31). Furthermore, calpain activation is connected to increased intracellular calcium levels. In accordance with a previous study (24), also in our experiments KP46 led to a slow but distinct increase of intracellular calcium in HCT-116 cells, in contrast to the rapid increase induced by the Ca²⁺ ionophore ionomycin used as a positive control (Supplementary Fig. S4). Therefore, we further analyzed whether inhibition of calpain influences the anticancer activity of KP46. Indeed, KP46-induced cytotoxicity was distinctly inhibited by the cell-permeable calpain inhibitor PD150606 (50 μmol/L; Fig. 4A and B). In addition, time-lapse microscopy proved that cells cotreated with PD150606 detached later from the cell culture plate surface than cells solely treated with KP46 (Fig. 4C and Supplementary Video S1). In contrast, a weakly membrane-permeable calpain inhibitor (E-64, 20 μmol/L) exerted distinctly minor effects (Supplementary Fig. S5), indicating that cell-associated calpain activity drives KP46-induced detachment. Accordingly, coinubcation with the calpain inhibitor PD150606 completely rescued binding of the labeled RGD peptide to the surface of KP46-treated HL60 cells, indicating restoration of functional integrin complexes at the cell surface (Fig. 4D). To show that calpain is also involved in the KP46-induced cell detachment of adherent cells, re-adhesion assays after 14-hour drug treatment with or without PD150606 were performed. Similar to short time experiments, KP46 strongly inhibited re-adhesion, which was again partially rescued by PD150606 (Fig. 4E). As mentioned above, KP46 treatment weakly enhanced markers of classic apoptosis in the hypersensitive cell models, only. Nevertheless, even the low levels of KP46-induced early and late apoptosis in KP46-hypersensitive cell models could be significantly inhibited by cotreatment with PD150606 (Fig. 4F). Likewise, upregulation of Bim expression and the band shift of Bim EL in Western blot analysis was reduced in the presence of the calpain inhibitor (Fig. 4G).

KP46 is active in vivo: loss of tissue integrity and integrin membrane localization

The in vivo anticancer activity of KP46 was analyzed in the solid human colon cancer xenograft model HCT-116 in SCID mice. Subcutaneous tumor growth was distinctly retarded by KP46 therapy (Fig. 5A), resulting in significantly reduced tumor weight as compared with controls (day 18; Fig. 5B). Histologic sections stained with H&E as well as TUNEL assay for apoptotic cell death induction (Fig. 5C, top and middle, respectively) revealed massive areas of cancer cell necrosis induced by KP46 treatment that stained strongly positive in TUNEL assays. A comparison of viable tumor parts at higher magnification (Fig. 5C, bottom) indicated distinct loss of tissue integrity with reduced cell density in response to KP46. Although mitotic figures were significantly reduced, TUNEL-positive and -negative dead cells (with condensed chromatin in DAPI staining) were dispersed throughout the nonnecrotic parts of KP46-treated tumors (Fig. 5C and D). In accordance with the in vitro data, membrane localization of integrin-β1 immunostaining was distinctly reduced in the remaining viable parts of the KP46-treated HCT-116 xenografts (Fig. 5E), indicating that downregulation of integrin-β1-mediated focal adhesion complexes might also play an essential role in the anticancer activity of KP46 in vivo.

Discussion

Although the (pre)clinical development of the oral gallium compound KP46 has already advanced to a successful phase I study (9), the precise molecular targets in cancer cells are widely unexplored. Here, we describe rapid cancer cell death induction by calpain-mediated focal adhesion deregulation as a novel molecular mode-of-action for KP46. Although promoted by activation of proapoptotic Bcl-2 family proteins, KP46-induced cell death was lacking classic features of apoptosis. Significant in vivo anticancer activity of KP46 against a human colon cancer xenograft was also characterized by reduced integrin-β1 plasma membrane localization, tissue disintegration, and enhanced cell death induction.

One recent study proposed that KP46-mediated cytoxic effects are triggered via Ca²⁺ signaling–mediated p53 activation or a p53-independent activation of FAS-mediated extrinsic apoptosis (24). However, despite confirmation of the slow Ca²⁺ increase by KP46, we found neither an impact of the p53 status on the overall anticancer activity in a larger panel of cell lines from different cancer types nor a significant impact of targeted p53 gene disruption in HCT-116 colon cancer cells. This does not generally preclude a regulatory role of p53 in KP46-mediated cell death, but excludes a key role of the p53 mutation status in determining the overall level of KP46 responsiveness of cancer cells.

In contrast, we were puzzled by a massive loss of viable cells within the first 24 hours of KP46 exposure without appearance of an equivalent amount of apoptotic (or necrotic) cells as would especially be expected from a p53-driven cell death (32). Consequently—at least in the KP46-hypersensitive cell models—another mode of cell death obviously had eradicated the majority of the malignant cells before execution of classic apoptosis. Indeed, time-lapse microscopy revealed an early onset of dramatic morphologic changes upon KP46 treatment with cell body contraction followed by cell detachment. The cell contraction and rounding-up of KP46-treated cells shared similarities with mitotic cell rounding (33). This was corroborated by increased phosphorylation at Ser-19 and, thus,
activation of the myosin light chain (34). However, in contrast to mitosis, no spindle formation, no M-phase arrest (data not shown), or chromatin condensation could be detected. Instead, after a convulsive period with rapid appearance and loss of branching filopodia, cells completely disintegrated, explaining the massive loss of viable cells without appearance of the respective amount of Annexin V$^+$ and/or PI$^+$ cells.
On the basis of this profound impact on cell adhesion and survival, we hypothesized that KP46 might interfere with adhesion-dependent survival signal complexes. Major contributors to cell adhesion, especially to the ECM, are integrins. These families of α and β heterodimeric receptors regulate adhesion predominantly to the ECM and, in turn, cell viability by enabling cells to sense and respond to their chemical and physical environment (26, 27). Integrin deregulation is involved in many pathologic processes such as cardiovascular disease and cancer invasion (26, 27). Consequently, integrins are attractive targets for anticancer therapeutic interventions (35). Several of our observations clearly demonstrate that KP46-mediated cell detachment is critically involving loss of integrin-mediated focal adhesions. First, expression levels of integrin-α5 and several integrin-β subunits, together with the intracellular focal adhesion–specific binding partner talin, were significantly reduced, especially in KP46-hypersensitive cells in a time frame corresponding to cell detachment. Second, downregulation was accompanied by a massive loss of the appropriated integrin-β1 and talin localization to focal adhesion complexes. Third, KP46 treatment led to a reduced FITC-labeled RGD peptide binding of cells mimicking the integrin binding motif.

Figure 5. In vivo anticancer activity of KP46 and impact on integrin-β1 expression. A, impact of KP46 on xenograft growth for HCT-116 p53/wt cells was determined after subcutaneous tumor cell injection in SCID mice. After the tumors were palpable, mice were treated for 2 weeks on 5 consecutive days/week with 15 mg/kg KP46 (N = 5). Data are means ± SEM. Statistical analysis was performed by two-way ANOVA with Bonferroni posttest (*, P < 0.01; **, P < 0.001). B, tumor weights of control and treatment groups on day 18 are shown. Box blot, inner quartile range and median; whiskers, minimum to maximum; †, mean. Statistical analysis was performed with column statistics and t test (**, P < 0.05). C, H&E staining of a representative tumor of the solvent and the treatment group (top) are opposed to photomicrographs of TUNEL staining (middle, TUNEL in red, DAPI counterstain in blue fluorescence). Massive necrotic areas of the treated tumor were strongly reactive of the TUNEL assay (red, middle). Bottom, one representative optical field within the nonnecrotic parts of the tumors depicting mitotic figures in DAPI staining (blue) and apoptotic cells as TUNEL-positive (red). D, mitotic and apoptotic cells in solvent- and KP46-treated tumors were counted in at least eight optical fields from three tumors each (×40 objective; Leica DMX). E, immunohistochemical staining of integrin-β1 in nonnecrotic tumor areas is shown for a control and a KP46-treated tumor representatively.
arg-Gly-Asp) in ECM ligands such as collagen and fibronectin (26). Fourth, culture of cells on the major integrin ligand collagen I sensitized cancer cells against KP46-induced cell death. Fifth, high-dose Mg\(^{2+}\) substitution, a cation essential for integrin–ligand binding (29, 36), reduced the KP46-mediated re-adhesion block and also reduced cytotoxicity of the drug. Sixth, also expression of proteins involved in focal adhesion dynamics downstream of integrin outside-in signaling, such as FAK and members of the small GTPase Rho/Rac family (28), was reduced by short-term KP46 treatment.

Loss of integrin engagement and cell–ECM interactions result in activation of a particular form of apoptotic cell death termed anoikis. This form of programmed cell death is executed either via the intrinsic or extrinsic apoptosis pathways (37, 38). Furthermore, the promotion of BH3-only activators and sensitizers, such as Bim activation, as well as inhibition of Bcl-2 are distinctive for this process (38, 39). Accordingly, we found the levels of Bax, Bim, and Bak to be upregulated after short-term treatment with KP46. In addition, Bax was distinctly translocated to mitochondria paralleled by a decrease in Bcl-2 levels. This corresponds to a mild but significant KP46 resistance in a Bax- but surprisingly not in a p53-deleted HCT-116 subline. However, hallmarks of classic apoptosis were only detected after longer drug exposure (48 hours; data not shown), while the massive viability loss within 24 hours was insensitive to a pan-caspase inhibitor. These data might indicate that KP46 targets an additional cellular factor interfering with a downstream mechanism of KP46-induced anoikis and thus blocking or preceding caspase activation. Interestingly, also for cilengitide, an integrin inhibitor already in advanced stage of clinical development, a comparable rapid cell detachment, massive cell loss within 24 hours, but lack of caspase activation or apoptosis induction has been described recently in human glioma cell lines (40). The rapid cilengitide-induced cell death was connected to autophagy. As enhanced autophagic features were also found after KP46 treatment (data not shown), we are currently aiming to dissect the contribution of this dual cell rescue or cell death–inducing mechanism.

Nevertheless, the question remains how KP46 induces integrin loss and degradation of focal adhesion complexes. The first observation coming into mind is that the ligand 8-hydroxyquinoline is capable of chelating Mg\(^{2+}\) ions. Deprival of this divalent cation would definitely lead to loss of the integrin–ligand interaction. Indeed, Mg\(^{2+}\) overload reduced KP46-induced detachment and cell death. On the contrary, KP46 has been demonstrated to be extremely stable in biologic environments (10, 41), making the presence of substantial amounts of free ligand questionable. In addition, even the formation of small amounts of the 8-hydroxyquinoline Mg\(^{2+}\) complex would not distinctly reduce the extracellular Mg\(^{2+}\) concentration due to the high excess of Mg\(^{2+}\) (400 \(\mu\)mol/L in the culture medium) compared with the used KP46 concentrations in the low \(\mu\)mol/L range. These considerations make a major role for Mg\(^{2+}\) chelation in KP46-induced rapid cell death unlikely.

KP46 treatment, in this study and in a previous one (24), induced a continuous Ca\(^{2+}\) release into the cytosol that has been connected to activation of the Ca\(^{2+}\) channel TRPC6 (42). This led our attention to calpains representing a conserved family of Ca\(^{2+}\)-activated cysteine proteases that regulate cytoskeletal remodeling, cellular signaling, apoptosis, and cell survival by controlled proteolysis. Also cell migration is a complex process including integrin-mediated (de)adhesion and actin-based membrane protrusion, which involve the activity of calpains (30). Calpain is associated with the control of focal adhesion turnover by cleavage of integrins and talin but also of integrin-recruited FAK and members of the Rho/Rac small GTPase family (30, 31). Moreover, calpain activity has been connected with the cleavage of Bcl-2 family members (43). Indeed, the presence of the specific and cell-permeable calpain inhibitor (PD150606) reduced KP46-induced cell rounding and rapid cell death. In addition, a KP46-induced band shift in the longer splice variant of Bim (Bim EL) was reduced by calpain inhibition. The longer isoforms such as Bim EL are inactive by association with microtubules and the dynein light chain 1 (DLC-1) and released by certain stress stimuli to activate apoptosis (44). In another study, calcium-mediated calpain activation led to focal adhesion disassembly and detachment, which could be blocked by lanthanum (La\(^{3+}\); ref. 45), a calcium channel blocker (46, 47). Supplementation of the medium with lanthanum (La\(^{3+}\)) also in our experiments inhibited the anticancer activity of KP46 (unpublished data). Together, these observations suggest a central role of Ca\(^{2+}\) release to the cytoplasm and, in turn, hyperactivation of calpain in KP46-induced cell adhesion loss and rapid cell death induction.

In summary, this study elucidates disruption of integrin-mediated cell adhesion via a calpain-regulated process as a novel and targeted mode of action contributing to the anticancer activity of the gallium compound KP46 currently in early clinical development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acknowledgments

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Ute Jungwirth, Johannes Gojo, Theresa Tudor, et al.


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