Arsenic-based pharmaceuticals have been used in traditional medicine for centuries. Because of the successful use of arsenic trioxide (As$_2$O$_3$) for the treatment of acute promyelocytic leukemia (APL) in China, arsenicals are experiencing a revival in modern cancer medicine (1–3). Arsenic exists in trivalent and pentavalent oxidation states as chemically unstable sulfide or oxide or as a salt of sodium, potassium, or calcium. Trivalent arsenicals, including sodium metaarsenite (NaAsO$_2$) and arsenic trioxide, inhibit many enzymes by reacting with biological ligands that possess available sulfur groups (1, 2, 4, 5). Despite many medical uses, chronic exposure to arsenic has been associated with an increased incidence in cancers of the skin, lung, bladder, liver, and kidney in humans (6). However, the treatment of APL patients with arsenic trioxide is generally well tolerated and shows neither bone marrow depression nor other severe clinical side effects. Moreover, no increase in the incidence of secondary malignancies has been observed with up to 10 years of follow-up in APL patients who received arsenic trioxide in the initial studies. It is clear that the use of arsenite for anticancer therapy is safe and effective in both short-term and long-term (3, 7).

In vitro and in vivo studies have shown that arsenicals cause chromosome abnormalities, including an increase in the frequency of micronuclei, sister chromatid exchanges, chromosome aberrations, and gene amplification. The compounds have also been reported to induce DNA and chromosome damage, inhibit DNA repair, and modulate DNA methylation in mammalian cells (8–10). It has been suggested that the latter effects are a result of the generation of reactive oxygen species (ROS; ref. 11).

Recent studies, however, have shown that arsenic trioxide can alter telomere length (12), telomerase activity (13), and telomere binding proteins (14), indicating that the telomere/telomerase complex might be a direct target of arsenic trioxide.

KML001 Cytotoxic Activity Is Associated with Its Binding to Telomeric Sequences and Telomere Erosion in Prostate Cancer Cells

Purnima Phatak, Fangping Da, Melody Butler, M.P. Nandakumar, Peter L. Gutierrez, Martin J. Edelman, Hans Hendriks, and Angelika M. Burger

Abstract

Purpose: KML001 (sodium metaarsenite) is an orally bioavailable arsenic compound that has entered phase I/II clinical trials in prostate cancer. In this study, we elucidated the mode of action of KML001 and investigated its effects on telomerase and telomeres.

Experimental Design: We compared telomere length to KML001 cytotoxic activity in a panel of human solid tumor cell lines. Duration of exposure and concentrations of KML001 that affect telomerase and telomeres were evaluated in relation to established mechanisms of arsenite action such as reactive oxygen species–related DNA damage induction. Binding of KML001 to telomeres was assessed by matrix-assisted laser desorption/ionization mass spectrometry.

Results: We established a significant inverse correlation ($r^2 = 0.9$) between telomere length and cytotoxicity. KML001 exhibited activity in tumor cells with short telomeres at concentrations that can be achieved in serum of patients. We found that telomerase is not directly inhibited by KML001. Instead, KML001 specifically binds to telomeric sequences at a ratio of one molecule per three TTAGGG repeats leading to translocation of the telomerase catalytic subunit into the cytoplasm. In prostate cancer cells with short telomeres, KML001 caused telomere-associated DNA damage signaling as shown by γ-H2AX induction and chromatin immunoprecipitation assays as well as a rapid telomere erosion shown by metaphase fluorescence in situ hybridization. These effects were not seen in a lung cancer cell line with long telomeres. Importantly, arsenification of telomeres preceded DNA lesions caused by reactive oxygen species production.

Conclusions: Sodium metaarsenite is a telomere targeting agent and should be explored for the treatment of tumors with short telomeres.
In a telomerase RNA component knockout mouse model, arsenic-induced "oxidative stress" promoted telomere attrition, chromosome end-to-end fusion, and apoptotic cell death (15).

Arsenic has also been found to be a potent inhibitor of the transcription of the human telomerase reverse transcriptase (hTERT). This effect appears at doses comparable with or lower than those clinically achievable. The effect may result from a diminished level or function of two transcription factors, c-Myc and Sp1, which are both important for hTERT expression (9).

KML001, sodium metarsenite, is an orally bioavailable, water-soluble, trivalent arsenical. KML001 has shown potent cytotoxic activity in solid human tumor cell lines and xenografts and is currently undergoing phase I/II clinical trials for the treatment of human prostate cancer (ref. 16 and investigators' brochure). Although little is known about the mechanism(s) of action of KML001, in a study involving both KML001 and arsenic trioxide no major differences were seen with respect to growth inhibition of leukemia cell lines and in assays assessing the induction of apoptosis or effects on hTERT transcription. It was observed that the induction of apoptosis was accompanied by down-regulation of hTERT mRNA in leukemia (17).

In this article, we report the effects of KML001 on telomerase enzyme activity and hTERT expression at the mRNA and protein levels as well as on telomeres and telomeric sequences. In a panel of solid cancer cell lines, we compared telomere length to the sensitivity of the cells to KML001. By using KML001-responsive prostate cancer cells, we carefully examined the dose levels and the time course/sequence at which these effects occurred and correlated them to that of established mechanisms of arsenite action such as oxidative stress induction. Our results indicate that telomerase activity is not directly inhibited by KML001 and that ROS production is not the cause of telomere erosion; instead, our data suggest that KML001 blocks the replication of telomeric repeats and induces rapid telomere loss through direct telomere binding.

**Materials and Methods**

**Cell culture and drugs.** Human prostate (PC-3 and DU145), colon (HT29), lung (H1838), and human breast cell lines (MCF7 parental) were purchased from the American Type Culture Collection. The ovarian cancer lines A2780 and A2780cis (cisplatin-resistant) were from the European Collection of Cell Cultures; the osteosarcoma cell line HT29, lung (H1838), and human breast cell lines (MCF7 parental) was first reverse transcribed (10 min at 60°C) and amplified by a 198-bp fragment of the generated cDNA in 40 PCR cycles (0.5 s at 95°C) and amplification of the TRAP assay, the TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics) was used as reported previously (20). The TRAPEZ kit (Intergen) was employed to measure KML001 in situ effects on PC-3 cell lysates. Total cellular protein (250 ng) was used for each PCR. The PCR products were separated on a 1% agarose gel as described (20).

**Quantitative hTERT real-time PCR.** Total RNA was extracted from cells treated for 8 and 24 h with IC50 and IC100 concentrations of KML001 and vehicle control (PBS) by using the Qiagen RNeasy mini kit (Qiagen). Quantitative detection of hTERT mRNA was done with the commercially available LightCycler TeloTAGGG hTERT Quantification kit (Roche Diagnostics) using the LightCycler instrument (Roche Molecular Systems). The real-time procedure was essentially done according to the manufacturer's instructions: hTERT-encoding mRNA was first reverse transcribed (10 min at 60°C) followed by denaturation (30 s at 95°C) and amplification of specific primers in a one-step real-time PCR. PCR was done with 100 ng/μl total RNA using primers for hTERT and primers for the housekeeping gene pshophobrinogen deaminase. The phosphobrinogen deaminase reaction product served as a control for real-time PCR and as a reference for normalization and relative quantification of hTERT mRNA. To establish an external standard curve, all experiments included five standards with a 10-fold range as well as total RNA purified from a hTERT mRNA-expressing cell line supplied by the detection kit. Nuclease-free water without template was employed as negative control. Results were expressed as hTERT copy number relative to porphobilinogen deaminase.

**Immunofluorescence and immunoblotting.** The phosphorylated histone 2AX (γ-H2AX; Se15) antibody was purchased from Upstate, anti-hTERT was purchased from Novusab, and anti-8-hydroxy-2-deoxyguanosine (8-oxo-dG) was purchased from Trevigen (clone 4E9). All primary antibodies were mouse monoclonal; mouse monoclonal FITC-labeled secondary antibodies were from Sigma. hTERT and γ-H2AX staining was done as described before (21). For 8-oxo-dG staining, the fixed cells were denatured with 4 N HCl for 5 min followed by neutralization in 50 mmol/L Tris for 5 min. After neutralization, cells were blocked for 30 min in 10 mmol/L Tris-HCl with 10% FCS followed by 1-h incubation with primary antibody (1:300 dilutions) and 30-min incubation with secondary antibody (1:100) at room temperature. Slides were washed with PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Photomicrographs were taken at ×63 magnification using a Leica DM4000 microscope and 5.0 Openlab ImageProess software to document and quantify signals. Quantification
of γ-H2AX was done by measuring mean fluorescence intensity of the nuclei using a binary layer as a mask. Briefly, the region of interest was drawn around every nucleus in a given field of view to make the binary layer and objects were measured by Boolean operation. At least 100 nuclei per experiment were evaluated. The averages for untreated and treated cells were calculated separately from the measured objects and the mean intensity was presented in the form of a bar diagram.

Immunoblotting for γ-H2AX was done as described by us before (21).

**KML001 binding to artificial telomeres.** Measurement of KML001 binding to 5'-TTAGGG)n-3' (artificial telomeres) was done by adopting the method described by Ishibashi and Lippard for cisplatin telomere binding (22). Instead of atom absorption spectroscopy, high-resolution matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was used. Mass spectra were obtained with an Axima MALDI time-of-flight mass spectrometer (Kratos/Shimadzu).

Telomeric and mutant sequences were synthesized by our Biopolymer Core Facility. Cisplatin (1 mg/mL; Novapluvs) was used as a positive control for the telomeric DNA-binding assay. In brief, oligonucleotides and KML001 were incubated for 24 h at 37°C for a fixed molar ratio of 1:10 in 100 μL aqueous medium. The TTAGGG sequences were incubated with drug for additional time points at 48 and 72 h and then dried using a Speed Vac system and the pellet was resuspended in 2.5 μL 3-hydroxypropionic acid matrix (Sigma-Aldrich) before MALDI was done.

**Metaphase spreads and telomere fluorescence in situ hybridization.** Cells were grown to 70% confluency and treated with 0.23 and 9 mmol/L (for PC-3) or 14 and 25 mmol/L (H1838) KML001 or PBS (vehicle control) for 24, 48, and 72 h in a T25 tissue culture flask. Supernatants were then replaced with medium containing 10 μg/ml colcemide (Invitrogen) and incubated for 90 min at 37°C. Cells were processed and slides were prepared as described before (21). Slides were dried at room temperature for at least 3 h before the hybridization procedure.

All human telomere probes labeled with a rhodamine fluorophore (PAHT1000R, Q-Biogene) were used for hybridization to metaphase preparations of PC-3 cells following a protocol provided by the manufacturer. The chromosomes were counterstained with DAPI (blue; Sigma). Images were captured with a rhodamine/DAPITFTC triple-band excitation filter (excitation wavelengths: 535-560, 395-410, and 490-505 nm) allowing the dual detection of red (rhodamine) and blue (DAPI) at a ×100 magnification with an A70 Olympus fluorescence microscope. Telomere signal was quantified by counting detectable telomere repeats (rhodamine red) in metaphase chromosomes and was expressed as percent of total chromosomes counted. At least 100 chromosomes were assessed and the experiments repeated three times.

**Chromatin immunoprecipitation assay.** Logarithmically growing PC-3 cells (10^6) were treated with KML001 at the IC_{50} concentration (9 mmol/L) for 2, 8, and 24 h. Control cells were treated for 24 h with vehicle (PBS). Chromatin immunoprecipitation (ChIP) assays for studying the specific association of γ-H2AX with telomeric repeat sequences were essentially done as described by d’Addadi Fagagna et al. (23). In brief, cells were washed twice with PBS, fixed in 1% formaldehyde in PBS for 10 min at 37°C, washed with ice-cold PBS, and lysed in 1% SDS, 50 mmol/L Tris-HCl (pH 8.0), and 10 mmol/L EDTA at a density of 10^7/mL. Lysates were sonicated to shear the DNA and centrifuged for 10 min at 4°C. Lysates (200 μL) diluted with 1.8 mL of 0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl were preclared with a protein G plus/Protein A agarose suspension (Calbiochem)/salmon sperm DNA (Sigma). Lysates (2 μg/500 μL) of control IgG (mouse; Santa Cruz Biotechnology), mouse monoclonal anti-phospho-H2AX (Upstate; clone JBW301), or telomere repeat binding factor 1 antibodies (Santa Cruz; clone C-19) were added and incubated overnight at 4°C. Immunoprecipitated pellets were washed with 0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA (pH 8.0), and 20 mmol/L Tris-HCl (pH 8.0) containing 150 mmol/L NaCl in the first wash and 500 mmol/L NaCl in the second wash. Further washes were with 0.25 mol/L LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mol/L EDTA (pH 8.0), and 10 mmol/L Tris-HCl (pH 8.0) and with 10 mmol/L Tris-HCl (pH 8.0) and 1% SDS and 0.1 mol/L NaHCO_3. After addition of 20 μL of 5 mol/L NaCl, cross-links were reversed for 4 h at 65°C. Samples were then treated with 20 μL of 1 mol/L Tris-HCl (pH 6.5), 10 μL of 0.5 mol/L EDTA, and 20 μg proteinase K (Qiagen) and incubated at 45°C for 1 h. Phenol-chloroform extractions were done and the DNA was precipitated overnight at -80°C. The precipitate was dissolved in 10 μL water and denatured at 95°C for 10 min, and 250 ng DNA was spotted onto Hybond membranes (GE Healthcare) with a Schleicher & Schuell apparatus (Whatman). Membranes were then developed using the TeloTAGGG telomere length assay kit (Roche). Signals were quantified using the NIH ImageJ software by measuring the mean signal intensity (gray scale) value and its integrated density. Resulting absolute intensities were determined and a relative intensity value was obtained by dividing the absolute intensity by the absolute intensity of the control sample.

**Statistical tests.** Statistical significance between two treatment groups was evaluated using the Student’s t test. P < 0.05 was considered significant. The software packages used were SPSS 2000 SigmaPlot and SYSTAT version 10 (SYSTAT Software).

**Results**

**Cytotoxic activity of KML001 in human cancer cell lines is correlated to their telomere length.** We tested KML001 in a panel of 10 cancer cell lines and the embryonic kidney cells HEK293T (Table 1). The cancer cell lines included the osteosarcoma Saos-2, a cell line without telomerase that maintains its telomeres through an alternative lengthening mechanism. The cytotoxicity of KML001 in these cells was compared to their telomere length (Table 1; Fig. 1A and B). Among prostate, ovarian, lung, breast, and colon cancer cell lines, the prostate cancer cell line PC-3 was the most sensitive to KML001 (IC_{50} 0.23 μmol/L) and also had the shortest telomeres (2.5 kb). The alternative lengthening mechanism cell line Saos-2 was the least responsive to KML001 (IC_{50} 25 μmol/L) and had long telomeres (TRF length, 13.5 kb).

There was an >100-fold difference in the IC_{50} values (IC_{50} ratio, 108.7) between these two cancer cell lines (Table 1; Fig. 1). The human lung cancer cell line H1838 (13 kb) with a TRF length similar to that of Saos-2 (Fig. 1B) showed also a high IC_{50} ratio (60.8) relative to PC-3. HEK293T normal kidney cells despite having the longest telomeres were relatively sensitive to KML001 (IC_{50} ratio, 13.5; Table 1; Fig. 1A and B). In general, lower IC_{50} values correlated with shorter telomeres and vice versa (Table 1); when we applied the Spearman rank correlation coefficient test to the cytotoxicity and telomere length data, a significant correlation coefficient of r^2 = 0.9 was found, indicating an association between telomeres and cytotoxicity of KML001 (Fig. 1C). The correlation was most pronounced among the breast and prostate cancer cell lines (Table 1).

The prostate cancer cell lines PC-3 and DU145 were selected for the subsequent molecular studies because of ongoing phase II clinical trials in prostate cancer and their short telomeres. The H1838 lung cancer cell line was used as a cell line with long telomeres and to contrast KML001 effects in short telomere cells.

**KML001 effects on telomerase activity and hTERT.** To test whether KML001 has an effect on the enzymatic activity of telomerase or its catalytic subunit hTERT, we made telomerase extracts from PC-3 cells and added KML001 at various
Table 1. Correlation between TRF length and growth inhibition by KML001

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean ± SD IC50 (μmol/L)</th>
<th>Fold ratio IC50</th>
<th>Rank IC50</th>
<th>Mean ± SD TRF length (kb)</th>
<th>TRF rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3 (prostate)</td>
<td>0.23 ± 0.04</td>
<td>1.0</td>
<td>1</td>
<td>2.5 ± 0.21</td>
<td>1</td>
</tr>
<tr>
<td>DU145 (prostate)</td>
<td>2 ± 0.43</td>
<td>8.7</td>
<td>3</td>
<td>4.26 ± 0.14</td>
<td>3</td>
</tr>
<tr>
<td>MCF7 parental (breast)</td>
<td>2 ± 0.35</td>
<td>8.7</td>
<td>3</td>
<td>5.5 ± 0.28</td>
<td>4</td>
</tr>
<tr>
<td>MCF7 wt hTERT</td>
<td>2.33 ± 0.43</td>
<td>10.1</td>
<td>4</td>
<td>5.5 ± 0.21</td>
<td>4</td>
</tr>
<tr>
<td>MCF7 mt hTERT</td>
<td>0.9 ± 0.04</td>
<td>3.9</td>
<td>2</td>
<td>3.1 ± 0.32</td>
<td>2</td>
</tr>
<tr>
<td>A2780 (ovarian)</td>
<td>2.5 ± 0.23</td>
<td>10.9</td>
<td>5</td>
<td>6.2 ± 0.28</td>
<td>5</td>
</tr>
<tr>
<td>A2780 cis</td>
<td>2.6 ± 0.14</td>
<td>11.3</td>
<td>6</td>
<td>10 ± 0.35</td>
<td>7</td>
</tr>
<tr>
<td>HEK293T (embryonic kidney)</td>
<td>3.1 ± 2.3</td>
<td>13.5</td>
<td>7</td>
<td>16 ± 2.47</td>
<td>10</td>
</tr>
<tr>
<td>Saos-2 (osteosarcoma, ALT)</td>
<td>25 ± 5</td>
<td>108.7</td>
<td>10</td>
<td>13.5 ± 0.70</td>
<td>8</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>3.5 ± 0.7</td>
<td>15.2</td>
<td>8</td>
<td>8.6 ± 0.42</td>
<td>6</td>
</tr>
<tr>
<td>H1838 (lung)</td>
<td>14 ± 4.3</td>
<td>60.8</td>
<td>9</td>
<td>13 ± 0</td>
<td>9</td>
</tr>
</tbody>
</table>

NOTE: IC50 ratio, IC50 value of a cell line divided by the IC50 value of PC-3, the cell line with the shortest telomeres and highest sensitivity to KML001.

Abbreviation: ALT, alternative lengthening of telomeres.

concentrations to the TRAP assay reaction (Fig. 2A). We also measured telomerase activity in lysates of prostate cancer cells that were treated with KML001 at IC50 and IC100 concentrations, respectively, for 8 and 24 h before isolation of telomerase (Fig. 2B; Supplementary Fig. S1A). The expression of hTERT was further assessed at the mRNA level (Fig. 2D).

We found that KML001 had no direct effect on telomerase activity even at concentrations as high as 20 μmol/L (Fig. 2A). Similarly, little effect on telomerase activity was seen in PC-3 or DU145 cells that were treated with KML001 for 8 h before extraction of telomerase (Fig. 2B). At 24 h, a small (not significant; P ≥ 0.05), dose-dependent inhibition of telomerase activity was evident at the IC50 and IC100 concentrations of KML001 (Fig. 2B; Supplementary Fig. S1A). In line with this, telomerase protein, as exemplified by the catalytic subunit hTERT, was found relocalized from the nucleus into the cytoplasm at 24 h, with a more pronounced effect seen at the IC100 in prostate cancer cells with short telomeres (Fig. 2C; Supplementary Fig. S1B). The displacement of hTERT from the telomeres renders the enzyme nonfunctional and might contribute to the inhibition of enzyme activity seen in the TRAP assay after 24 h (Fig. 2B). In lung cancer cells with long telomeres, a translocation of hTERT from the nucleus into the cytoplasm was not evident within 24 h (Fig. 2C).

Another possible explanation for the inhibition of telomerase activity by KML001 in intact cells (Fig. 2B) but not in telomerase extracts (Fig. 2A) could be the down-regulation of hTERT expression at the transcriptional level. To assess this possibility, we did real-time PCR analyses with RNA extracted from KML001-treated PC-3 cells and observed that hTERT mRNA expression was indeed significantly inhibited at the IC100 after 8 h (P = 0.04) and the IC50 and IC100 after 24 h (P = 0.002 and 0.003, respectively; Fig. 2D). These observations are consistent with prior reports on arsenic trioxide (9, 17). Together, the data suggest that telomerase activity is inhibited predominantly as a result of effects of KML001 on telomerase transcription and perhaps the displacement of hTERT from telomeres as well.

**Direct binding of KML001 to telomeric sequences.** It is well established that metal species have high affinity for the central guanine in GGG triplets of duplex DNA (22, 24, 25). For example, cis-diamminedichloroplatinum (cisplatin) is known to form N7-Pt-N7 GG intrastrand cross-links. The G-rich sequence of telomeres (TTAGGG)n, which extends beyond the C-rich strand in human telomeres for ~130 to 210 bp has been shown to react with cisplatin (22, 24, 25). It has been proposed that cisplatin might specifically poison telomeres because no transcription products from telomeres exist. Thus, damage to telomeric DNA will not be repaired by the transcription-coupled nucleotide excision repair system. On the other hand, double-stranded coding DNA will be repaired (22). Arsenic trioxide was also reported to interact with DNA and RNA at G-C, A-T, and A-U bases (26). To test whether the trivalent arsenic compound KML001 can bind to telomeric sequences, a possibility that could explain the hTERT displacement data, for example, in Fig. 2C, we incubated artificial telomeric sequences ([TTAGGG])3 and ([TTAGGG])6 with KML001 and used cisplatin as a positive control. KML001 or cisplatin and ([TTAGGG])6 were incubated at a 1:10 ratio for 24, 48, and 72 h as shown for the 24-h incubation at 37°C in Fig. 3A. The addition of KML001 to telomeric sequences led to a shift in molecular weight of ~130 m/z (mass-to-charge ratio) by high-resolution MALDI mass spectrometry. This is consistent with the addition of one molecule of KML001 (molecular weight, 129.91) per triple telomeric repeat (Table 2; Fig. 3A). A longer incubation time did not change the number of molecules binding to the triple TTAGGG repeat sequence but showed consistently the addition of one KML001 molecule (data not shown). When we used six telomeric repeat sequences ([TTAGGG])n, two molecules of KML001 were found to be bound (Table 2). Mutation analyses revealed that guanine and the vicinity of a thymine base to the guanine triplet are important for KML001 binding (Table 2). Hexanucleotide repeats consisting of all guanines bound four molecules of KML001. TTT and three consecutive guanines bound 2 molecules of KML001, whereas AAAAGGG showed no KML001 binding, suggesting that the presence of an adenine in the TTAGG repeat prevents a higher arsenification (Table 2). Likewise, all A and all T hexanucleotides did not bind KML001. When cisplatin was added to ([TTAGGG])3 repeats, three molecules were bound, indicating that one Pt interacts with a GGG triplet (data not shown) as reported before (22, 25).
To evaluate whether KML001 binding to telomeric sequences has biological consequences for telomere maintenance in cancer cells and whether an effect would be telomere length dependent, we exposed PC-3 and H1838 cells to KML001 IC$_{50}$ and IC$_{100}$ concentrations for 24, 48, and 72 h and did fluorescence in situ hybridization analysis with metaphase preparations and all human telomere probes (chromosomes in blue and telomeres in red in Fig. 3B-D). Although IC$_{50}$-treated PC-3 metaphases showed little difference compared with control cells at any time point (Fig. 3B), we observed a relatively rapid erosion of entire telomeres starting at 48 h of exposure to KML001 at the IC$_{100}$. At this time point and drug concentration, each chromosome in PC-3 metaphases had lost two of their four telomere signals in a random fashion that seemed not related to a specific erosion at the long or short arms of chromosomes (Fig. 3C, b). Counting of detectable telomeric repeat signals in 300 metaphase chromosomes revealed that 39 $\pm$ 2.8% of the telomere signal was lost (Fig. 3B; $P = 0.0014$). Seventy-two hours after KML001 exposure, very little telomere signal remained (16.5 $\pm$ 4.9%) and most of the chromosomes had almost completely lost their telomeres (Fig. 3C, c; $P = 0.0011$). This is also evident from interphase nuclei as shown in Fig. 3C (blue boxed inserts). In contrast, in H1838 cells (TRF, 13 kb), even a 72-h exposure to KML001 IC$_{100}$ concentrations did not cause loss of telomere signal compared with control cells (Fig. 3B and D, a-b). These data support the telomere binding/poisoning hypothesis outlined above. Effects, however, appear to be KML001 concentration dependent and telomere length dependent.

Specific induction of telomere-associated DNA damage by KML001. The “uncapping” signal for growth arrest, which is triggered when telomere-mediated chromosome end protection becomes insufficient due to reduction in telomere length and/or damage to telomere structure, has recently been elucidated (27, 28). It activates the double-strand break–mediated DNA damage response pathway, because a short, dysfunctional telomere can resemble a double-strand DNA break (28). Phosphorylation of $\gamma$-H2AX at Ser$^{139}$ is a very early event of double-strand break DNA damage signaling and has been described as a marker for telomere-initiated DNA damage checkpoint response (21, 28). We have therefore examined whether telomere binding and loss of nuclear hTERT following KML001 treatment would lead to a concomitant induction of $\gamma$-H2AX in cells and found that this is indeed the case (Fig. 4A-D). $\gamma$-H2AX is phosphorylated in a concentration-dependent manner; more $\gamma$-H2AX foci were detected in PC-3 and DU145 prostate cancer cells treated with IC$_{100}$ concentrations compared with IC$_{50}$ concentrations of KML001 ($P < 0.0001$; Fig. 4A-B; Supplementary Fig. S1C). Induction of $\gamma$-H2AX was seen as early as 2 h after treatment of PC-3 cells with KML001 at both IC$_{50}$ and IC$_{100}$ concentrations by immunoblotting (Fig. 4C).

H1838 lung cancer cells, however, did not show any evidence of $\gamma$-H2AX focus formation at 24 h and IC$_{100}$ concentrations of KML001 (Fig. 4A).

To assess whether KML001 treatment causes a specific association of phosphorylated H2AX with telomeres in responsive prostate cancer cells, we did ChIP assays with PC-3 cells and anti-$\gamma$-H2AX antibodies and probed the precipitated protein/DNA complexes with telomeric sequences [TTAGGG]$_{n}$, Fig. 4D]. We found a progressive accumulation of $\gamma$-H2AX-bound telomeric repeats consistent with the Western blot data in Fig. 4C for $\gamma$-H2AX expression. Marked induction of telomere-associated $\gamma$-H2AX protein (2-fold; signal intensity, 1.7) was found 2 h after exposure to KML001 and was further increased to 5-fold (signal intensity, 10.4-10.7) over control after 8 and 24 h (Fig. 4D). ChIP with the telomere-associated protein telomere repeat binding factor 1 (used as a positive control) detected a weak TTAGGG repeat signal in control-treated PC-3 cells, whereas the use of mouse IgG (negative control) for the immunoprecipitation did not pull-down any telomeric sequences (Fig. 4D), suggesting that specific telomere-associated DNA damage occurs. The latter was compared with DNA damage caused by arsenite-induced ROS production. The formation of 8-oxo-dG in response to ROS is well established and 8-oxo-dG is commonly used as a marker of oxidative stress. We have stained PC-3 and DU145 cells with anti-8-oxo-dG antibodies in parallel to the $\gamma$-H2AX...
immunofluorescence experiments and found that 8-oxo-dG lesions occur much later and at higher concentrations of KML001 (Fig. 4E; Supplementary Fig. S1D). A careful titration of drug concentrations (IC_{50}, IC_{70}, IC_{90}, and IC_{100}) and a time course over 24 h revealed that ROS-induced 8-oxo-dG appears not before 24 h and exclusively at the IC_{100} of KML001 in PC-3 cells. DU145 cells formed very little 8-oxo-dG even at the IC_{100} of KML001 (Fig. 4E; Supplementary Fig. S1D).

**Discussion**

Arsenic is one of the oldest drugs in the world and one of the best known poisons. Interestingly, it also has proven useful for anticancer therapy and is a prime example of the paradigm that “poisons in small doses are the best medicines and the best medicines in too large doses are poisonous” (29). Although the mechanisms underlying its paradoxical antineoplastic effects remain unclear, several reported observations point toward the involvement of telomeres and telomerase (1, 15, 29). For example, human cells are more sensitive to arsenic trioxide than are cells of rodent origin, and this difference has been attributed to the short telomeres found in human cells compared with the very long telomeres in mouse cells (15). Likewise, a hallmark that distinguishes human normal from cancer cells is the expression of the enzyme telomerase and the short but stable telomere length of cancer cells; this difference may be critical for the anticancer activity of trivalent arsenic (30, 31).

In this study, we provide clear evidence that sodium metaarsenite cytotoxicity is telomere length dependent in that cells with short telomeres are more sensitive to the drug than...
those with longer telomeres (Table 1). Saos-2, a cell line that has long telomeres and regulates its telomere length independent of telomerase through alternative lengthening mechanisms (e.g., telomere exchange), was >100-fold “resistant” to sodium metaarsenite compared with sensitive PC-3 prostate cancer cells with short telomeres (2.5 kb; ref. 32). We further show that sodium metaarsenite can bind to human telomeric sequences and lead to rapid erosion of telomeres in prostate cancer cells with short telomeres but not in a lung cancer line with long telomeres (Fig. 3). Entire telomeres were lost as seen by metaphase and interphase fluorescence in situ hybridization with telomeric probes as early as 48 h after addition of IC_{100} concentrations of KML001 to PC-3 cells; at 72 h, most of the chromosomes lacked telomeres but had not undergone end-to-end fusions. In contrast, KML001 did not cause telomere loss in H1838 lung cancer cells even at 72 h. The latter might require a prolonged exposure time or higher than IC_{100} KML001 concentrations for telomere erosion to occur. These data are consistent with a previous study by Liu et al. evaluating the effects of arsenic trioxide in telomerase-deficient (hTR^{−/−}) and age-matched wild-type (wt) mouse embryos. Wild-type embryos with long telomeres showed telomere erosion after 72 to 96 h of exposure to 30 μmol/L arsenic trioxide and this triggered end-to-end fusions at chromosomes where telomeres were no longer visible. Surprisingly, arsenic trioxide did not cause chromosome end-to-end fusions in embryos with shortened telomeres from late generation telomerase-deficient mice. However, telomerase-deficient mouse embryos with shortened telomeres were more sensitive than wild-type embryos to arsenic-mediated inhibition of cell survival (15). These wild-type and telomerase knockout mouse embryo experiments seem to mirror the effects of sodium metaarsenite on human normal and cancer cells reported by us here. Whereas Liu et al. attribute the differential effects of arsenic trioxide on mouse embryos with long and short telomeres to the production of ROS and ROS-related telomere attrition, we have strong evidence that a direct binding of sodium metaarsenite to telomeric repeat sequences leads to telomere erosion (Fig. 3A).
We showed that the arsenification of telomeres induces DNA damage signaling as exemplified by a rapid and dose-dependent induction of H2AX phosphorylation that is associated with telomeres as shown by γ-H2AX ChIP assay and precedes the formation of 8-oxo-dG, a ROS-specific DNA lesion (28, 33). 8-oxo-dG was only induced by the IC100 concentration of KML001 in PC-3 cells and not earlier than 24 h after drug addition, whereas telomere-specific γ-H2AX expression

Table 2. Binding of KML001 to telomeric and mutant sequences

<table>
<thead>
<tr>
<th>Oligonucleotide sequence</th>
<th>Mass of oligonucleotide (m/z) ± SD</th>
<th>Mass of oligonucleotide + KML001 (m/z) ± SD</th>
<th>∆m* ± SD</th>
<th>No. molecules bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TTAGGG)₃</td>
<td>5,840 ± 6.4</td>
<td>5,970 ± 8.0</td>
<td>130 ± 2.3</td>
<td>1</td>
</tr>
<tr>
<td>(TTAGGG)₆</td>
<td>11,416 ± 7.3</td>
<td>11,632 ± 9.8</td>
<td>216 ± 1.15</td>
<td>2</td>
</tr>
<tr>
<td>(AAAGGG)₃</td>
<td>5,762 ± 4.5</td>
<td>5,831 ± 6.5</td>
<td>68 ± 4.0</td>
<td>None</td>
</tr>
<tr>
<td>(TTGGG)₃</td>
<td>5,635 ± 1.2</td>
<td>5,927 ± 21.6</td>
<td>291 ± 22.1</td>
<td>2</td>
</tr>
<tr>
<td>(AAAAA)₃</td>
<td>5,592 ± 5.8</td>
<td>5,602 ± 4.3</td>
<td>10 ± 2.3</td>
<td>None</td>
</tr>
<tr>
<td>(TTTTTT)₃</td>
<td>5,444 ± 9.3</td>
<td>5,458 ± 6.6</td>
<td>14 ± 3.3</td>
<td>None</td>
</tr>
<tr>
<td>(GGGGG)₃</td>
<td>5,869 ± 3.7</td>
<td>6,394 ± 3</td>
<td>525 ± 2.3</td>
<td>4</td>
</tr>
</tbody>
</table>

* ∆m = [(mass of oligonucleotide treated with KML001) - (mass of oligonucleotide)]; m/z = mass/charge; cisplatin was used as positive control and three molecules found bound per (TTAGGG)₃ repeat.

Fig. 4. Telomere-associated versus ROS-associated DNA damage caused by treatment with KML001. A, γ-H2AX foci formation (green) in PC-3 compared with H1838 cells that were treated with vehicle control (top) or KML001 at IC50 (middle) and IC100 (bottom) for 24 h. Nuclei were counterstained with DAPI. Images were captured with a Retiga cooled camera and Improvision Openlab 5.0 software using a ×63 objective (Leica DM4000). Bar, 15 μm. B, mean signal intensity of γ-H2AX foci from images captured in A. At least 100 nuclei per experiment were evaluated (mean of three experiments). *p < 0.05, statistical significance based on a two-tailed Student’s t test. C, Western blot of H2AX phosphorylated at Ser 139. Lanes 1 and 5, vehicle-treated control cells; lanes 2 to 4, PC-3 cells treated with 0.23 μmol/L (IC50) KML001 for 2, 8, and 24 h, respectively; lanes 6 to 8, PC-3 cells treated with 9 μmol/L (IC100) KML001 for 2, 8, and 24 h. β-Actin was used as loading control. D, dot blot of ChIP with γ-H2AX, mouse IgG, and telomere repeat binding factor 1. Genomic PC-3 DNA (250 ng; equal amount of DNA used from the ChIP) was spotted as a positive control for TTAGGG repeats detection. Telomere repeat binding factor 1 ChIP of vehicle-treated PC-3 cells was used as a positive control for the immunoprecipitation of TTAGGG repeats with telomere-associated proteins and mouse IgG as a negative control to confirm the specificity of the ChIP reaction. ChIP with γ-H2AX revealed its very strong association with telomers after KML001 treatment but not in untreated controls. Relative γ-H2AX signal intensity (SI) values were determined and are shown below the individual spots. E, formation of 8-oxo-dG caused by ROS. Top, vehicle-control-treated PC-3 cells followed by a panel of PC-3 cells treated with 0.23 μmol/L KML001 (IC50), 0.75 μmol/L (IC70), and 3 μmol/L (IC100) for 24 h. Only PC-3 cells treated with 9 μmol/L (IC100) KML001 stained positive of 8-hydroxyguanine (green). Nuclei were counterstained with DAPI. Bar, 15 μm.
occurred as early as 2 h after exposure to IC_{50} concentrations of sodium metaarsenite (Fig. 4C). Our data suggest that arsenification of telomeres, and not ROS, causes sodium metaarsenite–associated telomere erosion and DNA damage signaling.

Experiments examining the effects of sodium metaarsenite on telomerase activity in the TRAP assay by treating either isolated enzyme activity directly with KML001, or by extracting enzyme activity from treated cells, showed that the drug does not affect telomerase activity. Nonetheless, hTERT protein was found translocated from the nucleus into the cytoplasm, a phenomenon observed by us for G-quadruplex ligands, a novel class of telomerase inhibitors that were specifically designed to target telomeres by stabilizing the formation of G-quadruplexes by the G-rich 3′ overhang (21, 30, 31, 34). For the G-quadruplex ligand BRACO19, we could show that hTERT when displaced from the telomeres translocates into the cytoplasm and colocalizes with ubiquitin, suggesting its degradation in the ubiquitin-proteasome system (34). Arsenification of telomeric sequences in prostate cancer cells shown in this study appears to have a similar effect.

Whereas sodium metaarsenite had little effects on telomerase protein, effects on hTERT were seen at the transcriptional level. The detectable number of copies of hTERT amplified from mRNA by real-time PCR was significantly reduced at both IC_{50} and IC_{100} concentrations of sodium metaarsenite after 24 h, which is in line with what has been reported by others (9, 17). Based on our telomerase/telomere protein, RNA and DNA data, and a recent literature report, the reduction of hTERT mRNA is likely due to the possibility of the binding of sodium metaarsenite to A-U bases in RNA (26).

The mechanisms by which arsenic trioxide drives APL cells into apoptosis have been studied extensively and the inhibition of expression of hTERT mRNA has been shown (9, 17, 29). One intriguing mechanism, which is favored in APL, is the rapid degradation of the promyelocytic leukemia gene (PML), retinoic acid receptor-α fusion protein (35), which restores promyelocyte differentiation. Arsenic trioxide treatment was shown to cause PML to localize to the nuclear matrix, where it becomes sumoylated and is degraded after recruitment to the proteasome. PML resides in discrete nuclear subdomains, known as PML nuclear bodies (29). The latter have been implicated in alternative telomere lengthening and have been shown to colocalize with telomeric DNA (36). It is tempting to speculate that in APL arsenic trioxide can target telomere maintenance in a dual fashion. First, it binds to telomerase-regulated telomeric sequences and hTERT mRNA, inducing telomere-associated DNA damage signaling and rapid cell death; second, it degrades PML and prevents cells trying to escape the insult by using PML bodies to repair their telomeres via telomere exchange (32).

Thus, the existence and importance of PML in APL might sensitize this tumor type to arsenic therapy. Together, our data and that of others suggest that arsenite exerts effects on telomeres in a telomere length-dependent manner and that the resulting telomere erosion without end-to-end fusion in cells with short telomeres such as cancer cells could help to explain a therapeutic window for anticancer therapy (15).

Based on our cytotoxicity data for a panel of human tumor cell lines (Table 1) and the telomere erosion shown in the PC-3 cell line, sodium metaarsenite could be useful for the treatment of a variety of cancers with short telomeres. Pharmacokinetic studies with sodium metaarsenite have shown peak plasma levels of 15 μmol/L after i.v. infusion and 2.5 μmol/L via oral administration (16). This is a dose range that encompasses most of the IC_{50} and IC_{100} values from our cell line studies, particularly the cell lines with short telomeres.

Importantly, arsenic trioxide and KML001 are identical substances in solution. Because arsenic trioxide is poorly water-soluble, it must be dissolved with sodium hydroxide and then adjusted to physiologic pH, yielding sodium metaarsenite. In fact, investigators that have studied “arsenic trioxide” have directly used sodium metaarsenite for their experiments (10). We have compared the in vitro cytotoxicity of KML001 and that of solubilized arsenic trioxide in the tumor cell lines from Table 1 and found no differences in potency between the two drugs (data not shown), suggesting that they might act very similar. Owing to its oral bioavailability, water solubility, and molecular antitumor effects, KML001 is a candidate for full clinical development.

A recently published meta-analysis of telomere length in cancer literature has revealed that telomere content, a proxy for telomere length, has prognostic relevance (37). In prostate carcinomas, low telomere content/short telomeres predict metastasis and recurrence. In breast cancers, low telomere content predicts poor clinical outcome and a reduced 5-year breast cancer–free survival interval (37–39). Thus, KML001-based combination treatments in these two difficult-to-treat tumor types should be considered, perhaps together with cisplatin, which also binds to telomeres, or more specific telomerase inhibitors such as GRN163L, a hTR antisense oligonucleotide, which has just entered phase I clinical trials (30, 31).

Disclosure of Potential Conflicts of Interest

A. Burger received a major research grant from Komipharma, who sponsored the work. H. Hendriks received honoraria from Rephatox for consulting on KML001.

Acknowledgments

We thank Dr. Nicholas Ambulos for the synthesis of the telomeric and mutant oligonucleotide sequences and for help with interpreting the mass spectrometry data. Dr. Arundhati Ghosh for help with the ChIP assay, and Colette Burgess for technical assistance. MALDI analyses were done by the University of Maryland Marlene and Stewart Greenebaum Cancer Center Proteomics Core Facility under the direction of Dr. Austin Yang.

References

KML001 Cytotoxic Activity Is Associated with Its Binding to Telomeric Sequences and Telomere Erosion in Prostate Cancer Cells


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/14/4593

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2008/07/15/14.14.4593.DC1

Cited articles
This article cites 38 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/14/4593.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/14/4593.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.