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The Anticancer Activity of Organotelluranes: Potential Role in Integrin Inactivation

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Organic Te^{IV} compounds (organotelluranes) differing in their labile ligands exhibited anti-integrin activities in vitro and anti-metastatic properties in vivo. They underwent ligand substitution with L-cysteine, as a thiol model compound. Unlike inorganic Te^{IV} compounds, the organotelluranes did not form a stable complex with cysteine, but rather immediately oxidized it. The organotelluranes inhibited integrin functions, such as adhesion, migration, and metalloproteinase secretion mediation in B16F10 murine melanoma cells. In comparison, a reduced derivative with no labile ligand inhibited adhesion of B16F10 cells to a significantly lower extent, thus pointing to the importance of the labile ligands of the Te^{IV} atom. One of the organotelluranes inhibited circulating cancer cells in vivo, possibly by integrin inhibition. Our results extend the current knowledge on the reactivity and mechanism of organotelluranes with different labile ligands and highlight their clinical potential.

Introduction

Tumor metastasis is a dynamic process involving a number of complex interactions between tumor cells and their environment.[1] The progression of a tumor and its metastasis depend on factors such as cellular adhesion molecules, proteins of the extracellular matrix (ECM), and proteases (e.g., matrix metalloproteinases, MMPs). Specifically, integrins play important roles during metastasis.[2]

Integrins are heterodimeric membrane glycoproteins comprised of two noncovalent subunits (α and β) that promote cell adhesion and migration on the surrounding ECM. Integrins expressed on tumor cells contribute to the metastatic process by increasing tumor cell migration, invasion, proliferation, and survival. Adhesion of a tumor cell to the ECM by its integrins is often regulated by the expression and secretion patterns of various ECM ligands. For example, the fibrillar cell-adhesion protein fibronectin (FN) is a ligand for various integrin receptors; at least ten different receptors have been recognized to bind FN.[3] Thus, FN is involved in many biological processes, such as tumor formation and metastases.

Although a wealth of evidence[4] shows that "inside-out" signaling (activation of the ligand binding function) can control integrin activation, it has been postulated that specific integrin function, at least, might be directly affected by redox rearrangements within the cysteine-rich domain of the extracellular integrin regions. Thus, a disulfide bond-reshuffling mechanism in which resting and active integrins differ in the number and positions of unpaired cysteine residues has been proposed.[5] We found that integrins are key targets for inorganic tellurium compounds. Based on the unique Te^{IV}–thiol chemistry,[6] we have shown that the inorganic Te^{IV} compounds ammonium [trichloro(dioxoethylene-*O*,*O*′-)tellurate] (AS-101) and octa-*O*-bis-(*R*,*R*)-tartarate ditellurane (SAS; Scheme 1), synthesized and studied by us,[7] interact with specific thiol-containing integrins by redox modulation, thereby affecting their conformation and inhibiting their physiological activity. This phenomenon was specifically relevant to integrin $\alpha_4\beta_1$ (very late antigen 4; VLA-4),[8] inhibition of which has consequences both in vitro and in vivo. B16F10 melanoma cells abundantly express VLA-4,[9] which interacts with VCAM-1 (vascular cell adhesion molecule 1) to enhance transendothelial migration of melanoma cells.[10]

However, prior to redox modulation of integrins by either AS-101 or SAS, ligand substitution reactions occur: substitution of labile ligands covalently bound to the tellurium atom (i.e., good leaving groups) with cell-membrane cysteines.[11,12] Based on our knowledge regarding the different labile ligands responsible for the reactivity of the inorganic Te^{IV} compounds AS-101 and SAS, we hypothesized that synthetic Te^{IV} organotellurane compounds bearing labile ligand moieties similar to

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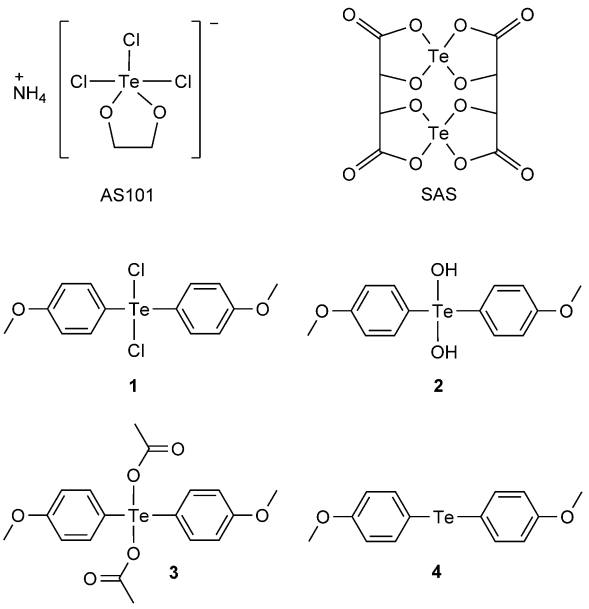


Scheme 1. Inorganic tellurium compounds AS-101 and SAS, and the synthetic organotellurium compounds.

those of AS-101 and SAS are likely to exhibit biological activity in vitro, possibly associated with integrins.

Organotelluranes, a sub-family of organotellurium compounds, have at least one Te–C covalent bond. They have been studied as antioxidants,[13] as inducers of the Ca^{2+}-dependent MPTP (mitochondrial permeability transition pore) opening,[13] as anti-leishmanial agents,[14] and as antiepileptogenic agents.[15]

Previously, we evaluated a few inorganic Te^{IV} compounds as specific inhibitors of the cysteine proteases papain and cathepsin B.[12] We showed that ligand substitution takes place between the labile ligands of AS-101 or SAS upon reaction with four equivalents of L-cysteine (per one tellurium atom), thereby resulting in a stable Te–Cys_4 complex. Since then, organotellurane compounds have also been demonstrated to inhibit cysteine proteases.[16] Cunha et al. evaluated different organotelluranes as inhibitors of the cysteine proteases cathepsins B, L, S, and K;[17] however, the authors suggested that the different reactivity patterns were attributable to a combination of the tellurium labile groups and the organic side chain, which was manipulated to fit the cathepsin allosteric sites. Moreover, whether these biological activities allow the formation of a stable Te–Cys complex, as seen for inorganic Te^{IV} compounds, is unknown.

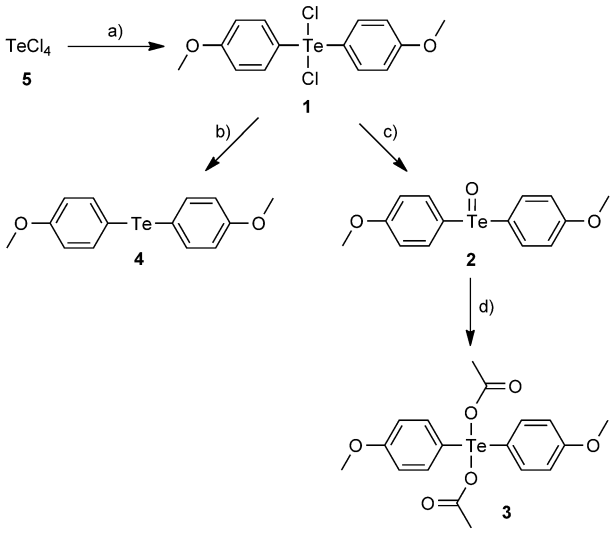
As cathepsins are involved in malignancy progression, antitumor activity was also expected for organotelluranes. Indeed, a new organotellurane molecule, RT-04, had cell-killing effects in the human promyelocytic leukemia cell line HL60,[18] by triggering apoptosis (DNA fragmentation and caspase-3, -6, and -9 activation).

Nevertheless, the role of the labile ligand moieties of organotelluranes has been poorly studied, and to the best of our knowledge, no structure–activity relationship (SAR) experiments with integrins (the suggested target) have been reported. This led us to assess whether organotelluranes have anti-integrin activity in cancer and to elucidate the role of the labile ligands bound to Te^{IV}. Also, we addressed several mechanistic questions regarding the interaction between organotelluranes and cysteines in vitro, and investigated the clinical potential of one organotellurane compound in vivo.

Results

Chemistry

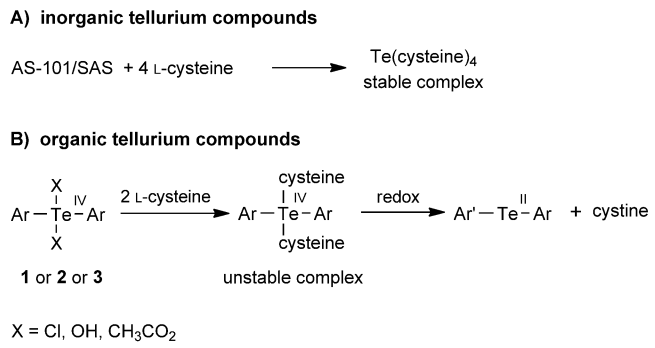
Design and synthesis of organotelluranes: A few organotelluranes of the form $TeAr_2X_2$ (**1**, **2**, and **3**, Scheme 1) were designed such that each shared the same core structure but had only one type of labile ligand (leaving group). The chlorides and carboxylates in **1** and **3** mimic the leaving groups of the highly reactive compounds AS-101 and SAS, respectively (Scheme 1). The oxide moiety (or its hydrate form) of **2** imitates a possible hydrolyzed oxide product of AS-101 and/or SAS, as we previously reported.[12] The corresponding Te^{II} analogue **4** (Scheme 1), which bears no labile ligand moiety, was used to evaluate the effect of the leaving group on integrin inhibition and to study the mechanism of the reaction between our synthetic organotelluranes and L-cysteine. In order to identify the specific effect of the leaving group and to exclude any effects of the interactions between the organotellurane side chain and the target protein, we used the same methoxybenzene aryl moiety in all four compounds. All were synthesized by known procedures and were obtained in reasonable overall yields (Scheme 2).[19–22] Briefly, tellurium tetrachloride (**5**) was dissolved in 6 equivalents of anisole, and the mixture was refluxed for 6 h under dry argon, thereby allowing a Friedel–Crafts tellurization reaction to occur by substituting two chlorines for two aromatic anisole rings. Solvent evaporation and hot filtration in boiling acetonitrile afforded **1** in 47% yield as



Scheme 2. Synthetic pathways for organotellurium compounds **1**–**4**. a) anisole, reflux, 6 h, 47%; b) sodium ascorbate, H_2O/MeOH, RT, 27 h, 93%; c) NaOH (2 M), reflux, 3 h, 58%; d) Ac_2O, $CHCl_3$, RT, 90 min, 65%.

pure crystals. Next, **1** was refluxed in NaOH (2 M) for 3 h. Ethanol was then added under reflux, thereby resulting in full dissolution of the reaction mixture, which was then cooled to 4 °C to allow formation of pure crystals of **2** in 58% yield. For the synthesis of **3**, **2** was dissolved in chloroform and treated with one equivalent of acetic anhydride at room temperature for 90 min. Next, the unreacted acetic anhydride was evaporated under vacuum, and the crude product was crystallized from hexane/chloroform (3:1) to afford pure crystals of **3** in 65% yield. For the synthesis of **4**, **1** was dissolved in acetone, and the mixture was added to a suspension of sodium ascorbate in MeOH/H_2O (8:2), and the resulting clear reaction mixture was stirred for 27 h. The product was extracted with dichloromethane, dried over $CaCl_2$, and evaporated under vacuum to afford **4** in 93% yield.

NMR studies: We characterized the chemical reaction between the synthetic organotelluranes and L-cysteine (a thiol model compound) by applying ^{125}Te and ^{13}C NMR to follow the reaction products. Upon addition of two equivalents of L-cysteine, **1**, **2**, and **3** were immediately and completely reduced, in the NMR tube, to yield the corresponding diorganotelluride **4** (Scheme 3B; resonates at 639 ppm, see Figure S4C in the Supporting Information). ^{13}C NMR data support the formation of disulfide cystine, the product of the oxidation of two cysteine molecules, in comparison to a blank (not shown). To our surprise, no complex of the form $TeAr_2X_mCys_{2-m}$ was detected, thus suggesting a different mechanism of reaction and/or kinetics for organotelluranes in comparison to the inorganic Te^{IV} compounds AS-101 and SAS.[12]



Scheme 3. Reactions of tellurium compounds with L-cysteine. A) The ligand substitution reaction of the inorganic tellurium compounds AS-101 and SAS with L-cysteine leads to the formation of a Te–Cys_4 complex, as previously reported.[12] B) Suggested scheme of reactions between organotelluranes of the form $TeAr_2X_2$ and L-cysteine, thereby leading to the reduced organotellurium(II) product.

Organotellurane **1** was further reacted with two equivalents of a different thiol compound, *N*-acetylcysteine. ^{125}Te NMR of the reaction solution revealed, in addition to the reduced diorganotelluride **4**, a new signal at 921 ppm, thus indicating the formation of a Te^{IV} complex of the form $TeAr_2X_m$(*N*-acetylcysteine)$_{2-m}$ (Figure S5).

Biological evaluation

In vitro assays

VLA-4 integrin is a target for organotelluranes 1–3: *Adhesion screening assays*. In order to evaluate the biological activities of our synthetic organotelluranes and to assess whether integrins are also a target for organotelluranes, we used adhesion assays. Adhesion of integrin-expressing cells to ligand-

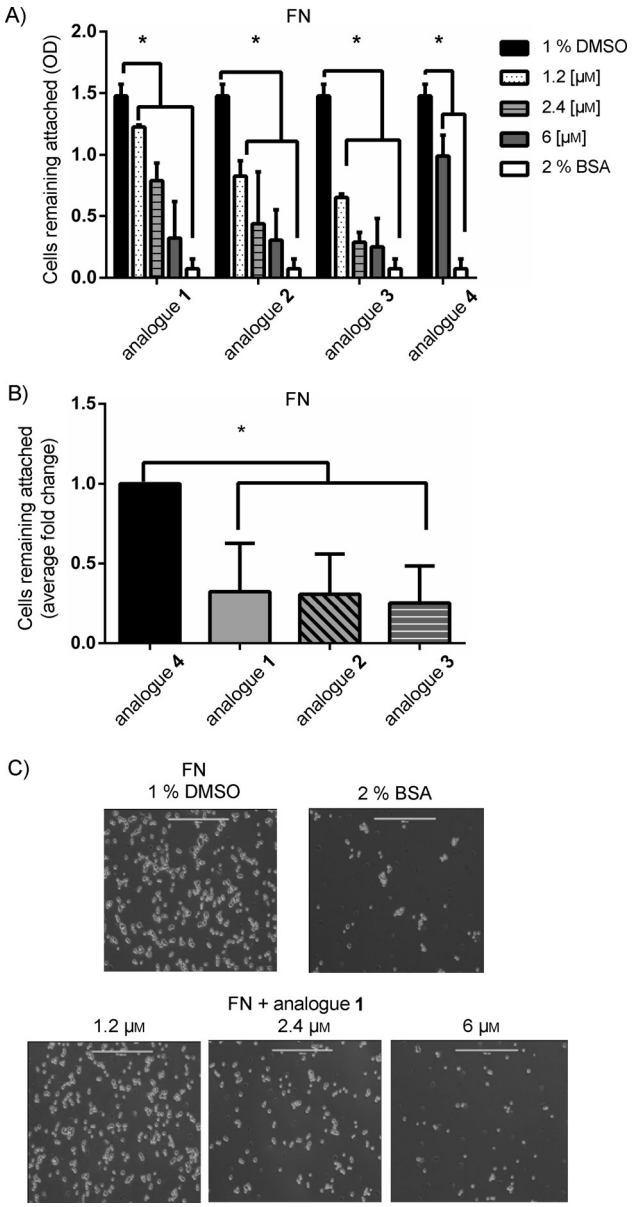


Figure 1. Adhesion of synthetic organotellurium compounds in vitro. A) Cells were seeded in a 96-well plate onto an FN- or BSA-coated surface, together with organotellurane or the diorganotelluride (0, 1.2, 2.4, or 6.0 μM) for 1 h. The cells were then washed, and the attached cells were subjected to an XTT assay (450 nm). Data are mean ± SD ($n = 3$); * $p < 0.05$ in comparison to 1% DMSO control (FN). B) Fold change of the adherence for 6 μM **1**–**3** in comparison to **4**; * $p < 0.05$. C) Representative images of the adherence assay. Cells were seeded in a 6-well plate onto an FN- or BSA-coated surface, together with **1** (1.2, 2.4, and 6.0 μM) for 1 h. The cells were then washed, and the cells remaining attached were photographed with ×10 magnification.

coated plates is an established method to study integrin function, and we have previously applied this for the study of various integrins and ligands.[8] First, we quantified B16F10 cells adhered to either FN or to bovine serum albumin (BSA, a non-specific ligand), after treatment with **1**, **2**, **3**, or **4** (1.2, 2.4, and 6.0 μM). At all concentrations, **1**–**3** were biologically active (17–83% adhesion inhibition), and exhibited dose-dependent reactivity (Figure 1A). As expected, **4** inhibited adhesion of B16F10 cells to FN to a significantly lower extent (34% at 6 μM) than **1**, **2**, and **3** (80–83% at 6 μM; Figure 1A). Next, we assessed whether our organotelluranes specifically target integrin, by quantifying B16F10 cells adhered to VCAM-1 (Figure 2) after

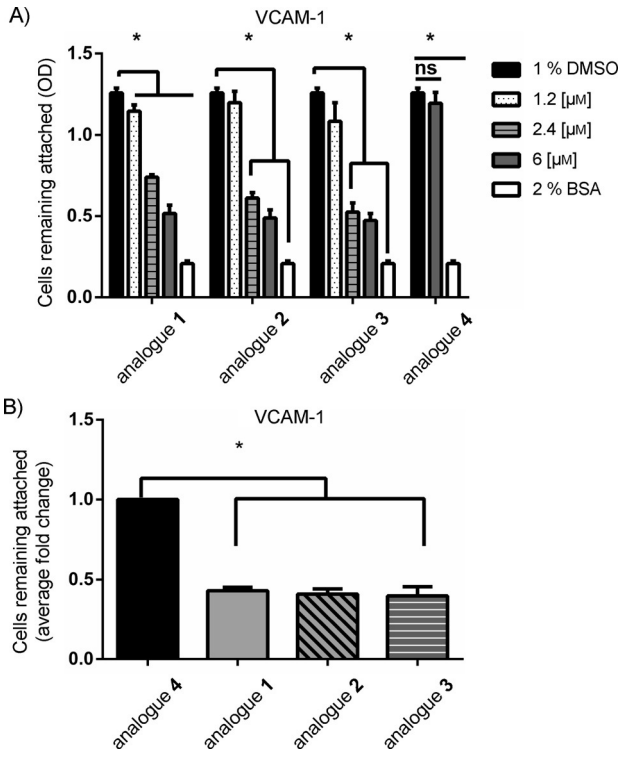


Figure 2. Synthetic organotellurium compounds inhibit VCAM-1 in vitro: adhesion assay. A) Cells were seeded in a 96-well plate onto a VCAM-1- or BSA-coated surface, together with organotelluranes or diorganotelluride (0, 1.2, 2.4, or 6.0 μM) for 1 h. The cells were then washed, and the attached cells were subjected to an XTT assay (450 nm). Data are mean ± SD ($n = 3$). $^*p < 0.05$ in comparison to 1% DMSO control; n.s.: not significant. B) Fold change of the adherence assay for 6 μM **1**–**3** in comparison to **4**; $^*p < 0.05$.

treatment with **1**, **2**, **3**, or **4** (1.2, 2.4, and 6.0 μM): **1**, **2**, and **3** inhibited adhesion (6–62% inhibition, Figure 2A), whereas **4** did not cause significant inhibition (5% at 6 μM, Figure 2). Furthermore, B16F10 cells did not adhere to the α4β7 ligand MadCAM (not shown). These data imply that **1**–**3** specifically inhibit the activity of the α4β1 integrin, which is abundantly expressed on B16F10 melanoma cells; they do not inhibit non-specifically other α4 integrins (e.g., α4β7) that are not expressed on these cells.

In vitro toxicity, cell migration, and zymography. In vitro biological studies with **1** aimed to study the interaction between organotelluranes and cell-membrane cysteines and to confirm integrins as a target for organotelluranes.

In order to support our adhesion findings and to exclude cytotoxic effects on B16F10 cells, we incubated **1** (2.4 and 6 μM) with cells for 1 h and assessed the results with propidium iodide (PI). FACS analysis revealed that **1** is non-toxic at either of these concentrations (Figure 3A; positive control: etoposide). In order to confirm this result, we incubated cells with **1** (6 μM) for a few hours, and quantified the number of living cells by using the XTT colorimetric assay. No toxic effects were observed for **1** (Figure 3B; positive control: cycloheximide). Taken together, these results suggest that the inhibition of adhesion by **1** can be attributed to specific inhibition of VLA-4 rather than to cytotoxicity.

Next, we tested the ability of **1** to inhibit the migration of B16F10 cells; migration can also be induced by integrins.[23] Organotellurane **1** significantly inhibited migration only when the cells were incubated in the presence of FN (67–76%, Figure 4A), whereas cells that were incubated with the nonspecific ligand BSA (as a negative control) did not demonstrate any migration. These results suggest a possible ligand-dependent activation of B16F10 integrins and their further inhibition, in accordance with the adhesion screening assay results (Figure 1 and Figure 2). Further to our observation that **1**–**3** do not form a stable $TeAr_2X_mCys_{2-m}$ complexes when treated with L-cysteine (but are immediately reduced), we wished to address the biochemical interaction between organotelluranes and cell-membrane cysteines. For this, we applied the impermeable cysteine modifier pCMBS,[24] which irreversibly binds cysteine thios, and followed its ability to inhibit cell migration. No impaired migratory activity was observed, compared with **1**-treated cells (at 2.4 and 6 μM, Figure 4A), thus pointing to a different mechanism of interaction between pCMBS and cell-membrane cysteine thiols.

Next, we wished to evaluate the clinical potential of organotelluranes. For this, we first assessed the anti-invasionary properties of **1**. Specifically, we followed the enzymatic activities of secreted proMMP-2 and -9, both of which are involved in metastasis[25] and regulated by VLA-4. Interestingly, **1** highly suppressed the secretion of proMMP-9 and significantly suppressed the secretion of proMMP-2 (Figure 4B).

In vivo activity

We wished to test the ability of **1** to inhibit metastasis in vivo. First, we evaluated the toxicity of **1** by applying an acute toxicity model. Organotellurane **1** was injected intraperitoneally (IP) into C57Bl/6 male mice every other day, for a total of three weeks per dose. Because of the limited solubility of **1** in the vehicle, the highest administered dose was 5.4 mg kg^{-1}. Encouragingly, no severe weight loss (< 10%) was observed at any of the administered doses following each three-week experiment (Table 1). Most importantly, not even mild behavioral clinical symptoms pointing to deterioration in the condition of the mice were observed at any dose.

Finally, as a proof of concept, we studied the effect of **1** on the migratory properties of B16F10 murine melanoma cells in

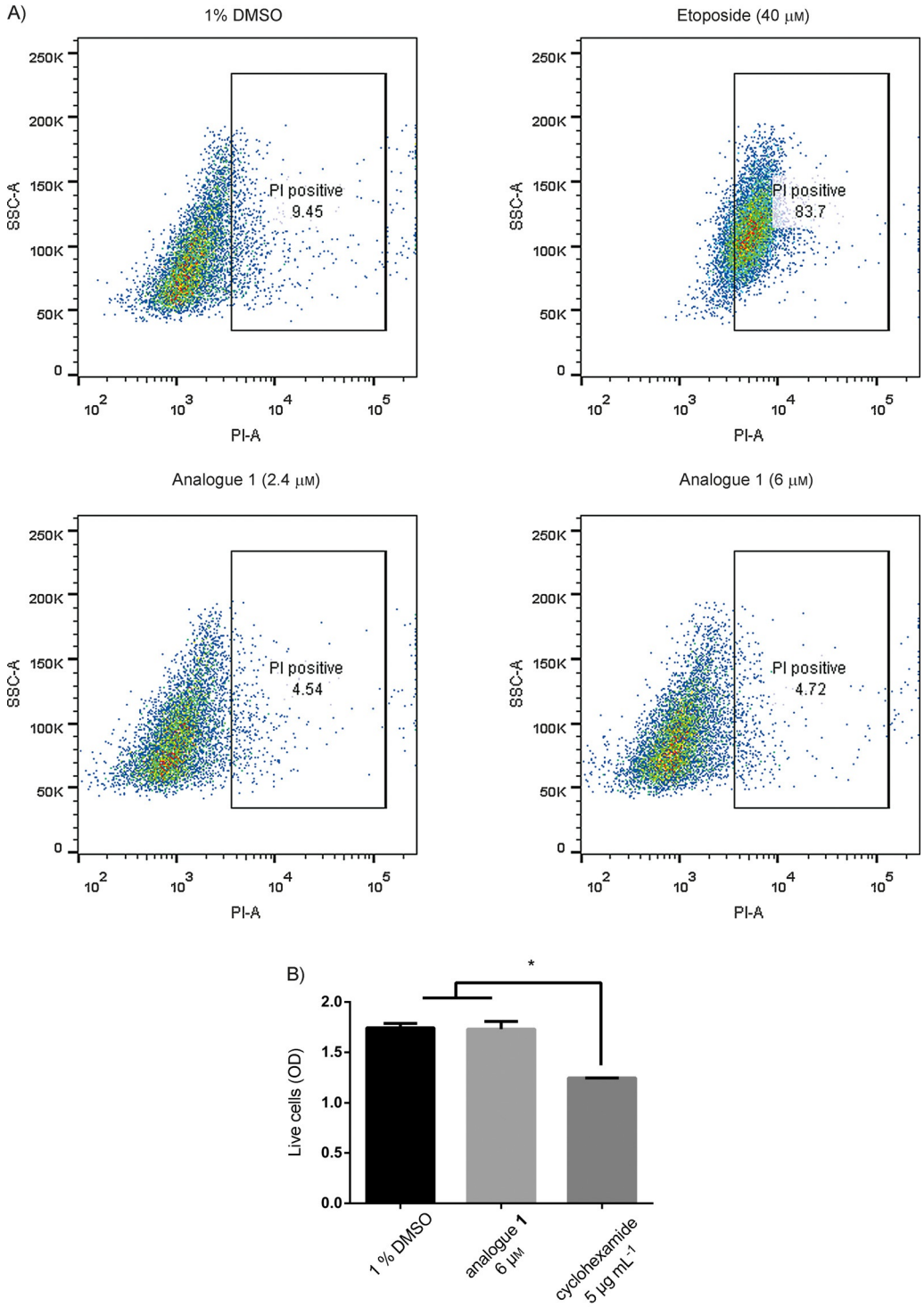


Figure 3. Organotellurane **1** is non-toxic in vitro. A) Cells were cultured to 60% confluence. Upon medium replacement, cells were incubated with either **1** (2.4 or 6 μM) or DMSO (1%) for 1 h, or overnight with etoposide (40 μM). Floating cells were collected together with trypsinized cells, centrifuged at 394 *g* and suspended in 1 mL of PBS with 1 μL of propidium iodide (PI). PI-positive and -negative cells were quantified by flow cytometry (representative images from two experiments). B) Cells were incubated for 6 h in a 96-well plate with either **1** (6.0 μM) or controls, DMSO (1%) or cycloheximide (5 μg mL^{-1}). The cells were subjected to an XTT assay (450 nm). Data are mean ± SD ($n = 3$); *$p < 0.05$ in comparison to cycloheximide.

vivo, by using an experimental metastasis model.[26] In C57Bl/6 mice injected with B16F10 cells into the tail vein and IP treated with **1** (0.25, 0.9, or 1.8 mg kg^{-1}) or vehicle (1% DMSO, control), **1** inhibited the formation of liver metastases. Quantification revealed that **1** significantly inhibited infiltration of B16F10 cells into the liver of C57Bl/6 mice (55% at 0.9 mg kg^{-1}; Figure 5A). Representative images of livers were taken from both treated and untreated mice (Figure 5B).

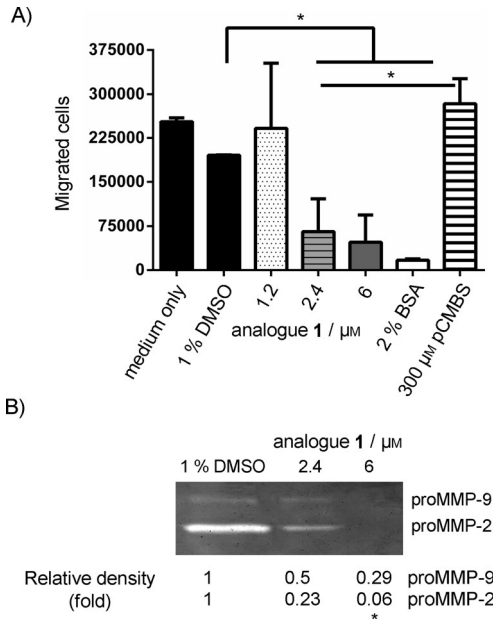


Figure 4. Organotellurane **1** inhibits cell migration and proMMPs secretion, in vitro. A) Cells were incubated for 30 min at 37 °C with **1** (0, 1.2, 2.4, or 6.0 μM) or pCMBS (300 μM), in the presence of mobilized FN and $MnCl_2$. Cells incubated with mobilized BSA served as a negative control. Next, the cells were subjected to a migration assay. The migrated cells were collected the following day and quantified. Data are mean ± SD ($n = 3$). *$p < 0.05$ in comparison to control. B) Cells were incubated for 72 h on FN-coated plates with 0.1 % BSA-containing medium with **1** (0, 2.4, or 6.0 μM). The conditioned medium was collected and subjected to zymography. A representative image and quantification (densitometric analysis) of proMMP-2 and -9 on a zymography gel ($n = 3$); *$p < 0.05$ in comparison to control

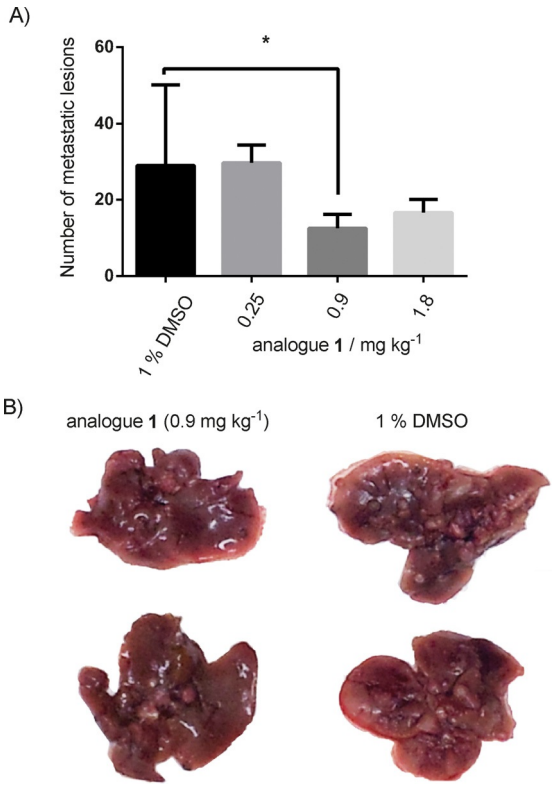


Figure 5. Organotellurane **1** inhibits circulating B16F10 cells in vivo. A) B16F10 cells were injected into the tail vein of C57Bl/6 mice, which were divided into three groups ($N = 10–14$). Each group was treated with a different dose of **1** (0.25, 0.9, or 1.8 mg kg^{-1} or 1 % DMSO as control), injected IP for three consecutive days and then every other day. Following 18 days, the mice were sacrificed and subjected to liver analysis. The number of nodules (metastatic lesions) was quantified. B) Representative images of livers from untreated (1 % DMSO, right) and treated (0.9 mg kg^{-1}, left) mice. Data are mean ± SD ($N = 10–14$ mice). *$p < 0.05$.

Table 1. Average weights of mice in acute toxicity assay with **1**.

Administered dose [mg kg^{-1}]	Weight [g]	Administered dose [mg kg^{-1}]	Weight [g]
(1 % DMSO)	23.81 ± 0.83	4.00	23.55 ± 0.67
0.90	22.55 ± 0.61	5.40	22.27 ± 1.69
2.25	22.78 ± 0.26		

Discussion

Over the last few years, the inorganic biologically active Te^{IV} molecules AS-101 and SAS were shown to exhibit diverse biological activities.[11,27,28] Many were attributed to the pivotal tellurium(IV) atom and its high affinity for thiols.[6]

Following our previous observations that the anti-metastatic activities of AS-101[8] and SAS are by integrin inhibition, we wished to evaluate the potential of organotelluranes as anti-metastatic agents, and to address several mechanistic questions regarding their interactions with thiols. Here, we synthesized and characterized organotelluranes **1**–**3** and diorganotelluride **4**, which lacks the leaving group moieties.

The inorganic compounds AS-101 and SAS are highly reactive towards L-cysteine, and yield stable, NMR-detectable complexes of $TeCys_4$ (Scheme 3 A).[11,12] However, whether the chemistry behind these reactions is different for organotelluranes and whether a stable and detectable complex of the form $TeAr_2X_mCys_{2-m}$ can also be formed when reacting organotelluranes of the form $TeAr_2X_2$ with L-cysteine, was unknown.

To our surprise, the chemical interaction between the organotelluranes and L-cysteine was substantially different to that for the inorganic tellurium compounds. Both AS-101 and SAS yielded the stable ligand-substituted complex $TeCys_4$ (1807 ppm, ^{125}Te NMR) when separately reacted with four equivalents of L-cysteine (Scheme 3 A).[11,12] These results are in accordance with recent experiments where we identified selective affinity of AS-101 towards vicinal thiols and possible formation of an S-Te-S intermediate.[8] In contrast, upon reacting our organotelluranes with L-cysteine, an immediate redox reaction yielded the Te^{II} derivative **4** (Scheme 3 B), which was also independently synthesized and characterized (Scheme 2). The fact that no stable complex of the form $TeAr_2X_mCys_{2-m}$ (X = Cl, CH_3COO, OH) was detected excludes the formation of a stable S-Te-S intermediate. Our results are also in agreement with a study that demonstrated that the number of labile groups in each organotellurane compound corresponds to the number of consumed cysteines.[17] Thus, an organotellurane composed

of two chlorines consumes only two cysteine equivalents, as was also observed for **1**–**3**, which rapidly consumed two cysteine equivalents and were reduced to the corresponding Te^{II} analogue **4** (with no trace of starting material). In contrast, reacting **1** (as a representative) in the NMR tube with two equivalents of *N*-acetylcysteine resulted in the formation of a new Te^{IV} complex at 921 ppm (Figure S5), in addition to **4** and the starting material. This suggests that even in the protein-free "clean" NMR environment, the chemistry between organotelluranes and thiols can be controlled by stereoelectronic factors.

In order to assess the anti-cancer activity of our synthetic organotelluranes and to determine whether the cysteine-rich domain of extracellular integrin regions is a possible target for organotelluranes, we conducted adhesion screening experiments in which we followed the ability of **1**–**3** to inhibit the adhesion of murine melanoma B16F10 cells to the abundant integrin ligand FN. In addition, in order to evaluate the importance of the labile ligand moiety to integrin inhibition, we also studied the diorganotelluride **4**, which bears no labile ligands. All three organotellurane compounds inhibited adhesion of B16F10 cells to FN (Figure 1A) in the low-micromolar range (partial inhibition (17–56%) at 1.2 μM; almost complete inhibition (80–83%) at 6 μM). In comparison, **4** inhibited adhesion of B16F10 cells to FN to a significantly lower extent at 6 μM (33%, Figure 1A and B). Although less probable, we cannot exclude the possibility of substitution reactions between **4** (at its methoxy phenyl carbons) and integrin membrane thiols. However, other studies suggest that diorganotellurides work by other mechanisms.[29]

In order to assess whether our synthetic organotelluranes specifically target integrin, we quantified B16F10 cells adhered to VCAM-1 (Figure 2) after treatment with **1**, **2**, **3**, or **4**. Derivatives **1**, **2**, and **3** inhibited adhesion (6–62% inhibition, Figure 2A), thus suggesting high specificity toward VLA-4, whereas **4** did not significantly inhibit adherence (5% at 6 μM, Figure 2A and B).

These results point again to the important role of the labile ligand of the organotelluranes in relation to integrin inhibition and confirm our previous observations (Figure 1B).

B16F10 cells did not adhere to the $\alpha4\beta7$ ligand MadCAM (not shown). These data imply that **1**–**3** specifically inhibit the activity of the $\alpha4\beta1$ integrin, abundantly expressed on B16F10 melanoma cells and do not merely inhibit nonspecifically other $\alpha4$ integrins (e.g., $\alpha4\beta7$) that are not expressed on these cells.

In light of these results, we suggest that **1**–**3** participate in ligand substitution reactions with cell-membrane integrins, specifically VLA-4 (Figure 2). We conclude that the different labile ligands do indeed play a significant role in the biological activity of organotelluranes. However, we cannot exclude the possibility that all the analogues were partly hydrolyzed in the presence of aqueous biological solutions to the same active metabolite. Nevertheless, after establishing the role of the labile ligands, the similar range of activity of the synthetic organotelluranes might be a good reference point for further manipulation of the aryl side chain for the development of more-potent Te^{IV} organotelluranes.

We selected **1** for subsequent in vitro and in vivo experiments aimed at studying the chemistry between organotelluranes and cell-membrane thiols, and to confirm integrins as a target for organotelluranes. As cell migration and tissue invasion are essential for metastasis, we evaluated **1** for its ability to inhibit the migratory activity of the highly metastatic B16F10 cells and to inhibit proMMP secretion, two key metastasis activities that are known to be regulated by integrin receptors such as VLA-4..[30,31] Thus, having demonstrated its ability to inhibit adhesion (Figures 1 and 2) in a specific manner rather than by cytotoxic effects (Figure 3), we expected that **1** would inhibit the migratory activity of B16F10 cells. Indeed, **1** significantly inhibited the migratory activity only of B16F10 cells (6–76%, Figure 4A) that were incubated in the presence of FN. In comparison, no migration activity was observed for cells that were incubated in the presence of the nonspecific ligand BSA, thus pointing to specific inhibition by **1**, possibly at FN-activated integrins. Importantly, incubation of the cells with the impermeable cysteine modifier pCMBS did not have any effect on the migratory activity of B16F10 cells. This suggests that in order to inhibit the migratory activity of B16F10 cells, oxidation of cysteines and formation of disulfides are necessary. This further corroborates our NMR experiments (Scheme 3), in which we observed a redox-based mechanism for **1**–**3** when reacting with L-cysteine.

Integrins also contribute to the invasion properties of tumor cells by regulating the localization and activity of matrix-degrading proteases, such as MMP-2 and -9.[32] Cultured melanoma cells have been shown to produce at least MMP-1, -2, -3, and -9, and their activity correlates with melanoma invasion.[33] Indeed, our cultured B16F10 murine melanoma cells were found to secrete high levels of proMMP-2 and to a lesser extent proMMP-9, in agreement with previous findings (Figure 4B, untreated controls). Inhibited secretion of proMMP-9 and proMMP-2 (Figure 4B) by **1** suggests a further mechanistic link for the inactivation of VLA-4 and possibly other FN-activated integrin receptors.

In light of the ability of **1** to inhibit in vitro cell adhesion to VCAM-1, migration, and MMP secretion, we wished to assess its activity in vivo. However, because preclinical toxicology studies play an important role in drug development, and because of the scarcity of data regarding the toxicity of tellurium compounds (and specifically of organotelluranes), we wished to evaluate the acute toxic effects of **1** on C57Bl/6 mice. No severe changes ($<10\%$) in the average weights of the mice were observed in response to administered doses of **1** (Table 1), neither was there any acute toxic clinical symptom. Therefore, we conclude that intraperitoneal administration of **1** at 0.9–5.4 mg kg^{-1} is safe in vivo. As a proof of concept for the ability of **1** to inhibit the infiltration of B16F10 cells into organs/tissues (a process that is known to involve integrins),[10] we applied an experimental liver metastasis model. Hepatic metastases are most frequent, and once diagnosed they are often associated with poor prognosis.[34] We showed that **1** significantly inhibited the formation of liver metastases in C57Bl/6 mice that were directly injected with B16F10 melanoma cells through the tail vein (55% inhibition at 0.9 mg kg^{-1}; Fig-

ure 5A), thus validating **1** as an in vivo bio-active compound, possibly by the inactivation of integrins.

Conclusion

We have studied the anti-cancer activities of organotelluranes. Our NMR experiments indicate an immediate redox reaction between organotelluranes (at their labile ligands) and L-cysteine. These findings are substantially different from the previous reported behavior of inorganic tellurium compounds. This study demonstrates for the first time, to the best of our knowledge, that VLA-4 is a target for organotellurane compounds. The labile ligands of organotelluranes play a significant role in cancer cell adhesion to the abundant integrin ligand FN and to the specific VLA-4 integrin ligand VCAM-1.

The migration assay supports our NMR findings and points to a different mechanism of interaction between **1** and cell-membrane cysteines, compared to the well-characterized interaction between pCMBS and cell-membrane cysteines. Organotellurane **1** was further established as a potential non-toxic and anti-metastatic Te^{IV}-based drug for the treatment of metastatic melanoma.

Experimental Section

General procedures: Solvents were of high purity. Anhydrous solvents were used as received. Commercial compounds were used without further purification. All synthetic compounds have been reported earlier.[19–22] ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker DPX-300, Avance-400, and DMX-600 spectrometers. ^{125}Te NMR spectra were obtained on a Bruker DMX-600; chemical shifts are reported in ppm relative to diphenyl telluride as an internal reference. Mass spectra were recorded in CI, ESI, and MALDI-TOF modes with methane as the reagent gas. The purities of all synthetic compounds were >95% as determined by elemental analyses on a FlashEA 1112 analyzer (Thermo Fisher Scientific). ICP analyses were made in an ULTIMA 2 spectrometer (Jobin Yvon Horiba); classical calibration with standard solutions was used to analyze tellurium.

Cells: Murine melanoma B16F10 cells were cultured at 37 °C in DMEM containing glucose (4.5 g L^{-1}) and FCS (10%) under CO_2 (5%). The cells were used for up to 20 passages.

Mice: Male C57Bl/6 mice (8–12 weeks) were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were kept in a specific pathogen-free environment and were fed a standard pellet diet and tap water. Mice were allowed to acclimate for seven days before the experiments. Animal experiments were performed in accordance with confirmed institutional protocol and were approved by the Institutional Animal Care and Use Committee (researcher license number: BS 20A2000).

Chemistry

Synthesis of organotellurium compounds: **Dichlorobis(4-methoxyphenyl)-λ^4-tellane (1):** $TeCl_4$ (5.28 g, 19.6 mmol) was placed in a 50 mL three-neck flame-dried flask. Anisole (12.74 g, 117.6 mmol) was added, and the mixture was stirred for 6 h at reflux (160 °C) under argon. The mixture was cooled to RT, and the solvent was evaporated to dryness under vacuum. Next, boiled acetonitrile (45 mL) was added, and the mixture was filtered. The filtrate was cooled, left at 4 °C for 30 min, then left to precipitate at −20 °C overnight. The collected product was obtained in 47% yield. ^{1}H NMR ([D$_6$]DMSO): δ = 7.87 (d, J = 9.0 Hz, 4H; H-3), 7.11 (d, J = 9.0 Hz, 4H; H-2), 3.81 (s, 6H; CH_3); ^{13}C NMR ([D$_6$]DMSO): δ = 161.0 (q, C1), 136.1 (CH, C3), 128.2 (q, C4), 114.8 (CH, C2), 55.4 (CH_3); ^{125}Te NMR ([D$_6$]DMSO): δ = 994.8; elemental analysis calcd (%) for $C_{14}H_{14}Cl_2O_2Te$: C 40.74, H 3.42; found: C 40.44, H 3.37; HRMS (CI$^+$): *m/z* calcd: 378.9730; found: 378.9735 ([M$^+$-Cl], 100).

4,4′-Tellurinylbis(methoxybenzene) (2): Organotellurane **1** (0.5 g, 1.21 mmol) was stirred in NaOH (2 M, 2 mL) in a 10 mL flask at reflux for 3 h. Then, ethanol (1 mL) was slowly added under reflux, thereby resulting in full dissolution of the reaction mixture, which was then cooled to RT, and then to 4 °C to allow crystallization. The crystals were washed with a small amount of H_2O/EtOH (2:1) to afford purified crystals of the product in 58% yield. ^{1}H NMR (CD_3OD): δ = 7.78 (d, J = 7.6 Hz, 4H; H-3), 7.10 (d, J = 7.6 Hz, 4H; H-2), 3.84 (s, 6H; CH_3); ^{13}C NMR (CD_3OD): δ = 163.6 (q, C1), 135.2 (CH, C3), 126.2 (q, C4), 116.3 (CH, C2), 56.0 (CH_3); ^{125}Te NMR ([D$_6$]DMSO): δ = 1209.2; elemental analysis calcd (%) for $C_{14}H_{14}O_3Te$: C 46.98, H 3.94; found: C 46.63, H 3.98; HRMS (CI$^+$): *m/z* calcd: 344.0056; found: 344.0025 ([M$^+$-O], 100).

Bis(4-methoxyphenyl)-λ^4-tellanediyl diacetate (3): Organotellurane **2** (0.14 g, 0.38 mmol) was dissolved in $CHCl_3$ (5.5 mL) for 1 h. Then, Ac_2O (0.043 g, 0.42 mmol) was added, thereby resulting in a clear mixture, and the reaction mixture was stirred at RT for 90 min. The solvent and unreacted Ac_2O were evaporated under vacuum, and the crude was crystallized from hexane/chloroform (3:1) to afford purified crystals of the product in 65% yield. ^{1}H NMR ($CDCl_3$,): δ = 7.76 (d, J = 8.8 Hz, 4H; H-3), 7.00 (d, J = 8.8 Hz, 4H; H-2), 3.84 (s, 6H; CH_3O), 1.96 ppm (s, 6H; CH_3CO); ^{13}C NMR ($CDCl_3$): δ = 177.5 (q, CO_2), 161.9 (q, C1), 134.9 (CH, C3), 125.7 (q, C4), 115.3 (CH, C2), 55.4 (CH_3O), 22.4 ppm (CH_3CO); ^{125}Te NMR ([D$_6$]DMSO): δ = 983.8 ppm; elemental analysis calcd (%) for $C_{18}H_{20}O_6Te$: C 47.00, H 4.38; found: C 47.04, H 4.31; HRMS (MALDI): *m/z* calcd: 403.0189; found: 403.023 ([M$^+$- CH_3COO^-]).

Bis(4-methoxyphenyl)tellurane (4): Organotellurane **1** (0.2 g, 0.49 mmol) was dissolved in acetone (10 mL), and the mixture was added to a suspension of sodium ascorbate (0.2 g, 1 mmol) in MeOH/H_2O (8:2, 10 mL), which was already pre-stirred for 30 min. The resulting clear mixture was stirred for 27 h. Then, the product was extracted with CH_2Cl_2. The organic phase was dried with $CaCl_2$, vacuum filtered, and evaporated to provide the highly pure product in 93% yield. ^{1}H NMR ($CDCl_3$): δ = 7.61 (d, J = 8.7 Hz, 4H; H-3), 6.74 (d, J = 9.0 Hz, 4H; H-2), 3.74 ppm (s, 6H; CH_3); ^{13}C NMR ($CDCl_3$): δ = 159.8 (q, C1), 139.8 (CH, C3), 115.5 (CH, C2), 104.4 (q, C4), 55.2 ppm (CH_3); ^{125}Te NMR ([D$_6$]DMSO): δ = 639.7 ppm; elemental analysis calcd (%) for $C_{14}H_{14}O_2Te$: C 49.19, H 4.13; found: C 50.38, H 4.51; HRMS (CI$^+$): *m/z* calcd: 342.0038; found: 342.0037 ([M^+]).

NMR studies

General procedure for the reaction of organotelluranes with L-cysteine and N-acetylcysteine: Each organotellurane (0.025 mmol) was dissolved in [D$_6$]DMSO (1.2 mL) and transferred into a 10 mm NMR tube for recording its ^{125}Te NMR spectrum. Next, L-cysteine HCl monohydrate (0.05 mmol) or *N*-acetylcysteine (0.05 mmol) was added to the test tube, which was shaken for a few seconds. The reaction products were followed by ^{125}Te and ^{13}C NMR.

Biology

In vitro assays

Adhesion to fibronectin, VCAM-1, and MadCAM-1: A 96-well plate was coated with FN, VCAM-1, or MadCAM-1 (80 μL, 5 μg mL^{-1}) or with BSA (2%) and left overnight. Cells (10^5 in 200 μL serum-free DMEM) were incubated with various concentrations of organotellurium for 1 h. Next, the wells were washed twice in PBS to remove unattached cells, then serum-free DMEM (200 μL) and XTT mixture (50 μL, Biological Industries, Israel) was added to each well and incubated for approximately 4–6 h. Plates were read at 450 nm for the quantification of live attached cells.

XTT cytotoxicity assay: Cells (10^5 in serum-free DMEM (200 μL)) were incubated in a 96-well plate with **1** (6 μM), DMSO (1%), or cycloheximide (5 μg mL^{-1}). XTT (50 μL) was added to each well. Plates were read after 6 h at 450 nm for the quantification of live cells.

FACS analysis: B16F10 cells were cultured to 60% confluence. The medium was replaced, and cells were incubated with either **1** (2.4 or 6 μM) or DMSO (1%) for 1 h, or overnight with etoposide (40 μM). Floating and trypsinized cells were collected, centrifuged at 394 *g* and suspended in PBS (1 mL) with PI (1 μL). Cell death was measured with an LSRII flow cytometer (BD Biosciences).

Cell migration. Cells (2×10^5) were incubated for 1 h in the presence of either mobilized FN (13.2 μg mL^{-1}) or BSA (2%), with $MnCl_2$ (0.25 mM) and **1** (1.2–6 μM) or pCMBS (300 μM). The cells were washed twice with PBS, and equal amounts of cells were loaded onto 8 μm polycarbonate membrane inserts. The bottom chambers were filled with DMEM (800 μL) containing FBS (20%) and FN (13.2 μg mL^{-1}) serving as a chemo attractant. Migrated cells were quantified after 24 h by Trypan Blue exclusion test using a hemacytometer.

Zimography: A 24-well plate was coated with FN. Cells (2.5×10^5) were incubated for 72 h in DMEM containing BSA (0.1%) on top of the FN-coated wells. Next, the upper fluid was collected, concentrated in Centricon tubes (>30 kDa; Merck Millipore), and tested for protein concentration by a Bradford assay. For every sample, the same amount of protein was loaded on a zimography gel (Bio-Rad, Israel) containing gelatin (10%). After electrophoresis (30 mA, 1.5 h), the gels were washed and incubated for 30 min with Triton X-100 (2.5%), washed, and incubated in a development buffer to initiate enzyme activity for 30 min. The latter was replaced with a fresh development buffer, and the gels were incubated overnight. Then, the gels were stained with Coomassie Blue for 45 min and washed in a destaining solution (acetic acid/ methanol/water; 5:10:85) until the appearance of transparent white bands. All reagents were purchased from Bio-Rad.

In vivo activity

Acute toxicity studies: Organotellurane **1** (0.9–5.4 mg kg^{-1} in 200 μL PBS containing DMSO (1%)) was injected IP into C57Bl/6 mice (8–12 weeks; $N=10$) every other day, for a total of three weeks. PBS containing DMSO (1%) served as the vehicle and as a control. The mice were monitored for abnormal behavior or death, and each group was measured for average weight once a week. After 3 weeks, the dose of the compound was raised and injected into a new group of C57Bl/6. For each dose, freshly prepared solutions of **1** (dissolved in PBS containing DMSO (1%)) were made every week, and the concentration of **1** was calculated based on ICP analysis.

Experimental liver metastases model: B16F10 cells (5.5×10^5) were injected through the tail vein of C57Bl/6 mice (8–12 weeks old), which were then randomly divided into four groups ($N=10$–14). Each group was treated with a different dose of **1** (0.25, 0.9, or 1.8 mg kg^{-1}) or with DMSO (1%) in PBS (vehicle and control group). Freshly prepared **1** was made each week, and the concentration of the tellurium was determined by ICP analysis. The mice were injected IP with **1** for three consecutive days and then every other day. After 18 days, the mice were sacrificed and subjected to liver analysis.

Statistical analysis: Data are expressed as mean ± SD. Unless otherwise stated, all experiments were performed at least three times on different days. Differences between groups in the adhesion assays, relative to control, were analyzed by a Student's t-test. Differences between groups in the migration assay were analyzed by one-way ANOVA. Differences in average weight between groups and control in the acute toxicity model were analyzed with a Student's t-test. Differences between groups for in vivo metastases were analyzed by one-way ANOVA. $p<0.05$ was considered statistically significant.

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