In Vivo Anticancer Activity of a Nontoxic Inert Phenolato Titanium Complex: High Efficacy on Solid Tumors Alone and Combined with Platinum Drugs

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Due to the toxicity of platinum compounds used in the clinic as anticancer chemotherapies, titanium serves as a safe and attractive alternative. Lately, we introduced a new family of Ti complexes based on readily available phenolato ligands, demonstrating incredibly high hydrolytic stability, with the lead compound phenolaTi demonstrating wide cytotoxic activity toward the NCI-60 panel of human cancer cell lines, with an average GI$_{50}$ value of 4.7 ± 2 μm. Herein, we evaluated in vivo: a) the safety, and b) the growth inhibitory capacity (efficacy) of this compound. PhenolaTi was found to be effective in vivo against colon (CT-26) and lung (LLC-1) murine cell lines in syngeneic hosts and toward a human colon cancer (HT-29) cell line in immune-deficient (Nude) mice, with an efficacy similar to that of known chemotherapy. Notably, no clinical signs of toxicity were observed in the treated mice, namely, no effect on body weight, spleen weight or kidney function, unlike the effects observed with the positive control Pt drugs. Studies of combinations of phenolaTi and Pt drugs provided evidence that similar efficacy with decreased toxicity may be achieved, which is highly valuable for medicinal applications.

Introduction

Cisplatin and its derivatives have been established as significant chemotherapeutic drugs used in the clinic for a variety of cancers.[1–4] Cisplatin is commonly used in ovarian and lung cancers, mostly in combination with other drugs.[5–11] Oxaliplatin, a derivative of cisplatin, is often used in colon cancer, especially in combination with fluorouracil.[12–14] However, development of resistance to these drugs and the toxicity of the Pt ion led to a search for other metal-based drugs. Among the transition metals tested, two titanium-based complexes—budotitane and titanocene dichloride—reached clinical trials, but failed

due to rapid hydrolysis and the formation of undefined aggregates.[15–29] To overcome these obstacles, our laboratory developed a new family of titanium(IV) complexes based on phenolato ligands.[30–42] In particular, diaminobis(phenolato)-bis(alkoxo)Ti^{IV} (phenolaTi, Figure 1) demonstrated remarkable stability in
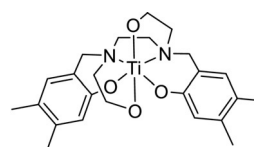

Figure 1. Diaminobis(phenolato)-bis(alkoxo)Ti^{IV} complex, phenolaTi.

aqueous media and an extended shelf life, along with enhanced in vitro cytotoxicity toward various cancer cell types.[43] In previous studies, the phenolaTi complex also displayed synergistic or additive characteristics when combined in vitro with cisplatin or oxaliplatin,[44] and an antitumorigenic effect when tested in vivo in mice inoculated with lymphoma growing as ascites.[43] Furthermore, evaluating this complex on the NCI-60 panel of human cancer cell lines (by the Developmental Therapeutics Program (DTP) of the US National Cancer Institute (NCI)), demonstrated significant cytotoxicity (with an average GI$_{50}$ value of 4.7 ± 2 μm) toward all cell lines tested, particularly colon and lung. Of added significance were our findings that phenolaTi is also active in vitro against cisplatin-resistant, as well as MDR1 (ABCB1) drug-resistant cells, suggesting a distinct mechanism of action.[43] Here, we expand our findings to include the in vivo effect of phenolaTi on different solid tumors,

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of both murine and human origin, and compare these findings, in terms of toxicity and efficacy, with those of the two commonly used platinum-based drugs relevant to the tested cancer types: cisplatin and oxaliplatin. In particular, combination studies enabled the achievement of high efficacy with reduced toxicity.

Results

In vitro cytotoxicity

In vitro cytotoxicity of phenolaTi was tested previously toward several cell lines, including human HT-29 colon cancer cells.[43,44] In an effort to increase solubility of the complex in aqueous media, nanoparticles of phenolaTi complex were obtained as previously described by a rapid conversion of a volatile oil-in-water microemulsion into a dry powder, composed of nanoparticles.[36] The in vitro effect of this emulsion on murine colon CT-26 and lung LLC-1 cancer cell lines is demonstrated in Figure 2. Previous studies also gave evidence that the nanoparticle formulation does not significantly impact the cytotoxicity and is itself inactive.[36,38]
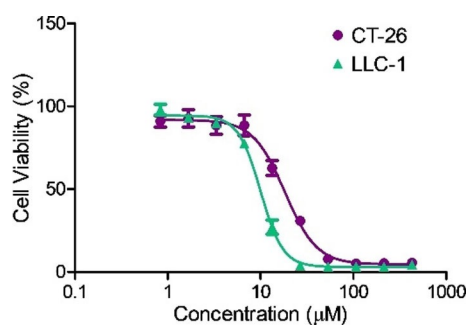

Figure 2. Dose-dependent viability curves of formulated phenolaTi against colon CT-26 ($IC_{50} = 18 \pm 2$ μM) and lung LLC-1 ($IC_{50} = 11 \pm 3$ μM) cancer cell lines.

In vivo toxicity

Balb/c mice were subjected to PBS (control), phenolaTi (1.6 mg kg^{-1}, the highest concentration soluble without formulation), cisplatin (5 mg kg^{-1}), or oxaliplatin (5 mg kg^{-1}) every
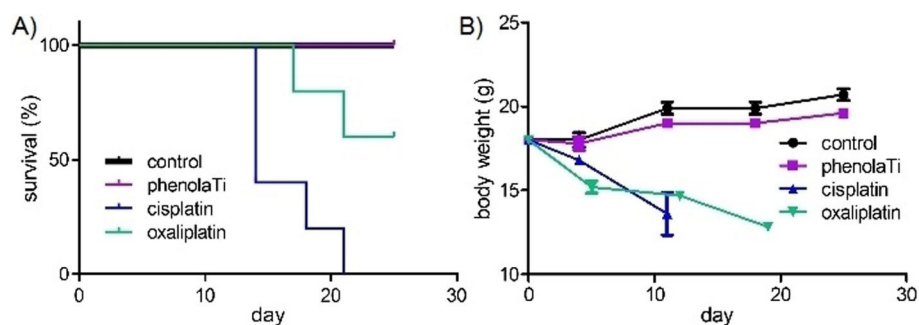
other day for four weeks. Whereas mice treated with cisplatin or oxaliplatin demonstrated a variety of deleterious effects, including decreased body weight and grooming, culminating in diminished survival (Figure 3), mice treated with phenolaTi survived the chemotherapeutic challenge, and did not demonstrate any of these symptoms. Notably, although increasing the phenolaTi concentration to 40 mg kg^{-1} in formulation still did not bring about any signs of toxicity in the treated mice (after five injections; treatment frequency: every other day), increasing it further to 80 mg kg^{-1} caused sudden mortality after the third injection (treatment frequency: every other day).

As cisplatin induces nephrotoxicity, we sought to test whether phenolaTi has a similar effect on the kidney. Interestingly, 72 h following the injection of the drugs (20 mg kg^{-1}, i.p.) marked increases in urine excretion-to-water consumption ratio (Figure 4 A), BUN levels (Figure 4 B), urinary albumin-to-
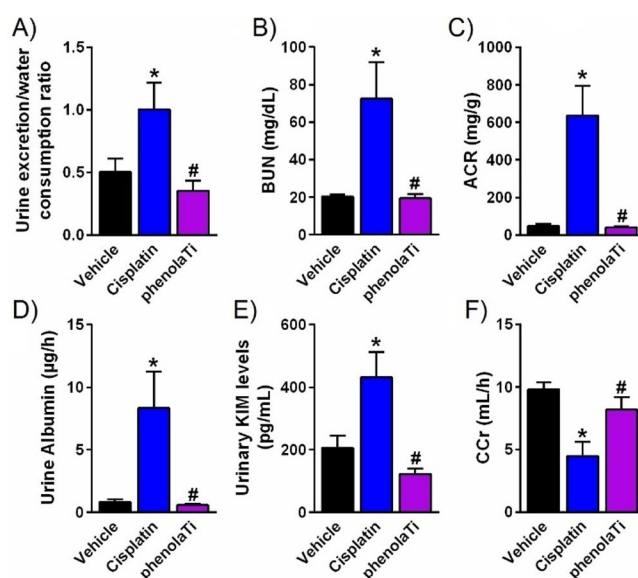

Figure 4. Nephrotoxicity assessment in male C57BL/6 mice 72 h following a single i.p. injection of vehicle, cisplatin (20 mg kg^{-1}) or phenolaTi (20 mg kg^{-1}). Note that only cisplatin resulted in increased urine excretion-to-water consumption ratio (A), blood urea nitrogen (BUN; B), albumin-to-creatinine ration (ACR; C), urine albumin levels (D), and urinary kidney injury marker 1 (KIM-1; E) as well as reduced creatinine clearance (F). Data are the mean ± SEM in 4–5 animals per group. *$P < 0.05$ relative to vehicle-treated group; #$P < 0.05$ relative to cisplatin-treated group.

Figure 3. Comparing the effect of phenolaTi (1.6 mg kg^{-1}), cisplatin (5 mg kg^{-1}), and oxaliplatin (5 mg kg^{-1}) on survival (A) and body weight (B) of Balb/c mice; mice experiencing severe body loss or demonstrating other toxicity profiles were taken out of the experiment for ethical reasons.

creatinine ratio (ACR; Figure 4C), albuminuria (Figure 4D), and KIM-1 levels (Figure 4E) were observed in C57BL/6 mice treated with cisplatin, compared with vehicle-treated control animals. In addition, a significant reduction in CCr (Figure 4F) was also documented in cisplatin-treated mice. None of these changes were found in phenolaTi-treated mice. Taken together, these findings suggest that phenolaTi is practically nontoxic in mice when compared with both cisplatin and oxaliplatin, which encouraged further efficacy studies.

In vivo efficacy

A series of tumor growth inhibition studies by phenolaTi were carried out. PhenolaTi was first tested in comparison with cisplatin on Balb/c and C57BL/6 mice inoculated s.c. with CT-26 and LLC-1 cells, respectively (Figure 5). PhenolaTi (0.5–5 mg kg^{-1}) was employed both directly and in nanoparticles form marked as "phenolaTi F" to increase solubility and enable higher doses, whereby the dose mentioned is the dose of the active agent in the formulated compound. Generally, treatment started immediately following detection of tumors, and grouping the animals uniformly. Whereas both drugs demonstrated a similar in vivo efficacy on both models, phenolaTi demonstrated no decrease in body weight relative to cisplatin. Interestingly, the presence of the formulation only slightly impacted the efficacy, whereby increasing the dose of active drug in formulation did not increase the efficacy of phenolaTi. Additionally, in another experiment using phenolaTi, phenolaTi F, and cisplatin on Nude mice inoculated with cisplatin-resistant A2780-CP human ovarian cancer cells, a markedly decreased efficacy was observed for the cisplatin-treated relative to the phenolaTi-treated groups (Figure S1, Supporting Information).
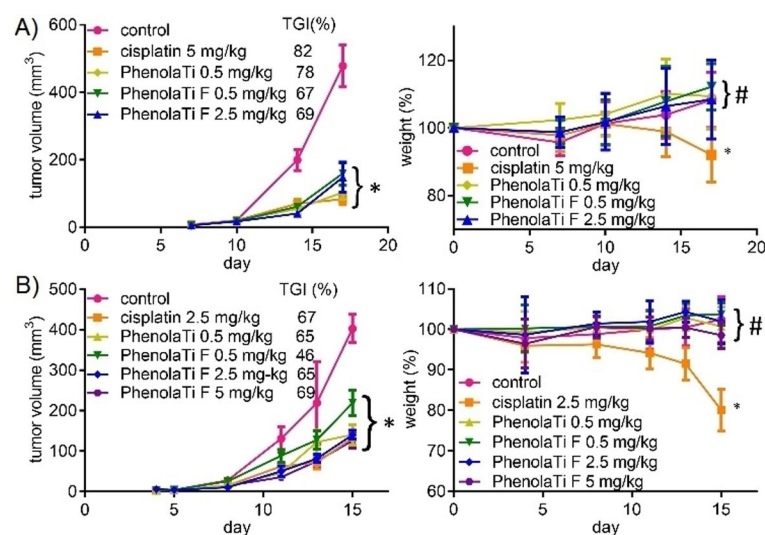
In an additional set of experiments, using the above-mentioned mice strains and cancer cell lines, the effect of a combination of phenolaTi and cisplatin was addressed. PhenolaTi and cisplatin were compared with regards to tumor growth inhibition (tumor volume and weight) as well as toxicity (body and spleen weight) (Figure 6). In both models, the combinations showed enhanced efficacy. Notably, the spleen weights of mice treated with cisplatin were lower than those of the control group, whereas the spleens of those treated with phenolaTi and phenolaTi F remained similar to those of the control group. Furthermore, within the timeframes used (up to 17 and 15 days post-inoculation for Figures 6A,B, respectively), phenolaTi did not enhance the toxic outcomes of cisplatin treatment, while again demonstrating antitumor efficacy.

The studies were expanded to include a human cancer model. Findings similar to the above were observed with immune-deficient Nude mice, inoculated s.c. with human HT-29 colon adenocarcinoma cells that were subjected to phenolaTi, phenolaTi F, cisplatin, and oxaliplatin commonly used in the clinic for colon cancer. Mice were subjected to i.p. injections of the drugs every day/other day. All drugs (or combinations thereof) were antitumorigenic with the combination of phenolaTi with cisplatin showing increased efficacy. In one experiment (Figure 7A), the side effects of cisplatin were even somewhat diminished when combined with phenolaTi, whereby the body weights remained similar to that in the control group. Thus, the phenolaTi consistently showed no side effects relative to the marked toxicity demonstrated by cisplatin alone, although decreased effects were developed by oxaliplatin as well. Nevertheless, somewhat reduced efficacy was observed for oxaliplatin and its combinations.

Nephrotoxicity test

The Nude mice inoculated with HT-29 cells and treated with phenolaTi or cisplatin (Figure 7B) underwent evaluation for chronic renal dysfunction by morphological damage to the kidney. Histological examination revealed necrosis, protein casts, vacuolization and desquamation of renal tubular epithelial cells in the cisplatin-treated mice. PhenolaTi at 5 mg kg^{-1} did not cause tubular damage as determined by PAS staining of the kidney (Figure 8), indicating no nephrotoxicity induced by the novel drug.

Discussion

This is the first study to demonstrate that both phenolaTi and its formulated version phenolaTi F effectively impair solid tumor development in both immune-competent and immune-deficient mice injected with murine and human cancer cell lines, respectively, including cisplatin-resistant ovarian carcinoma cells. Of added significance is the lack of apparent toxicity that distinguishes the Ti drug from the commonly used Pt-based chemotherapeutics. No body weight loss or spleen weight changes were de-

Figure 5. Effect of phenolaTi (formulated or non-formulated) and cisplatin (injected i.p.), on tumor volume (left) and body weight (right) of (A) Balb/c mice injected with CT-26 colon cancer cells (drug applied three times per week), and (B) C57BL/6 mice injected with LLC-1 lung cancer cells (drug applied five times per week). Data are the mean ± SEM in 8–10 animals per group. *$P < 0.0001$ relative to control; #$P < 0.0001$ relative to cisplatin-treated group.
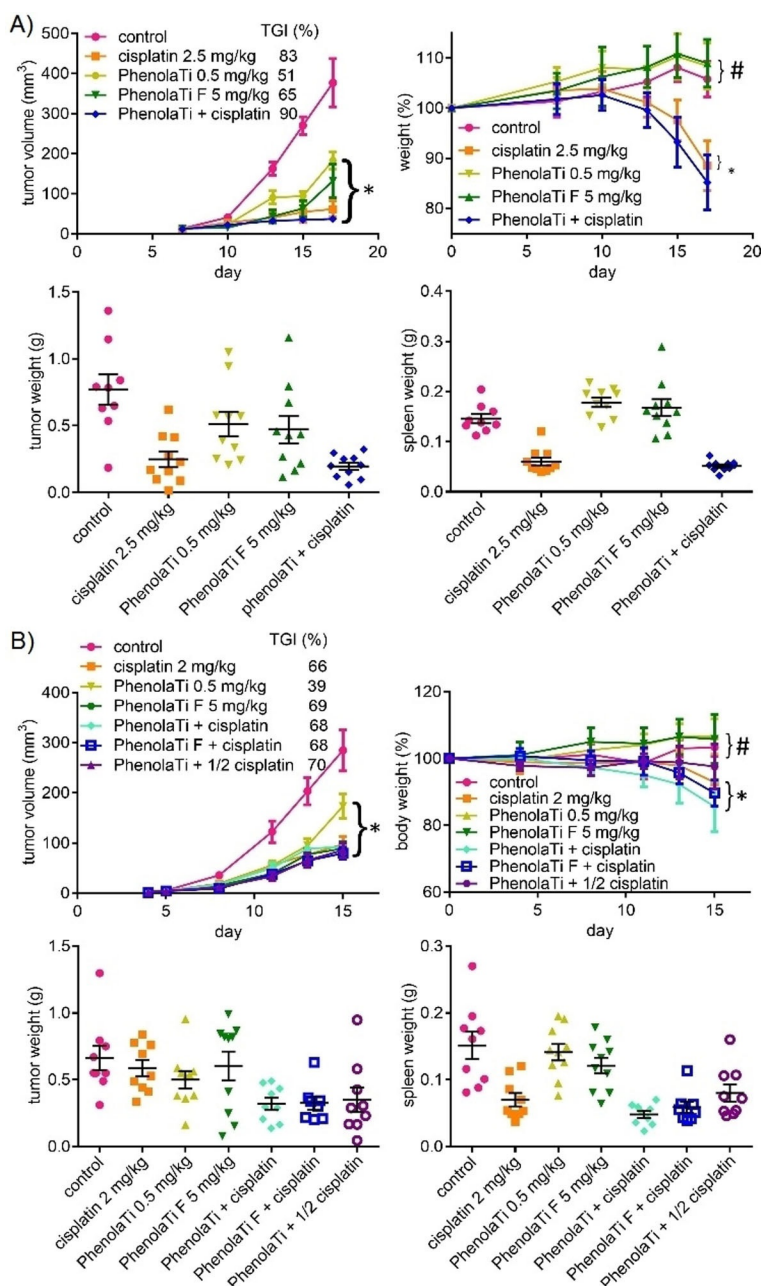
Figure 6. Effect of phenolaTi and cisplatin, alone and combined, applied five times per week, on tumor growth (left): overtime volume (top) and final weight (bottom) and toxicity parameters (right): overtime body (top) and final spleen weights (bottom). (A) Balb/c mice inoculated with CT-26 colon cancer cells; (B) C57BL/6 mice inoculated with LLC-1 lung cancer cells. For combinations: the added drugs were applied at the same concentration each as when applied alone; "1/2 cisplatin" refers to half the concentration of cisplatin as applied alone. Data are the mean ± SEM in 9–10 animals per group. *$P < 0.0001$ relative to control; #$P < 0.0001$ relative to cisplatin-treated group.

tected in phenolaTi-treated animals, as well as no hair loss, grooming or any behavioral changes. Moreover, because cisplatin is known to be nephrotoxic, various parameters relating to kidney function were evaluated and none were impaired by the phenolaTi complex. Therefore, the phenolaTi titanium complex is an attractive candidate for anticancer chemotherapy.

Combination therapy is a common methodology, as combining drugs may achieve a desired effect with reduced doses of each drug, thereby reducing side effects. In addition, multiple mechanisms of action can overcome drug resistance.[45–48] In all combinations studied herein, no antagonistic behaviors were detected, implying unrelated mechanisms, as also supported by previous NCI-60 results.[43] Moreover, in some experiments the combined drugs achieved better efficacy than each drug alone, whereas the side effects of cisplatin remained similar as when the drug was administered alone; therefore, combining phenolaTi with a decreased concentration of cisplatin gave similar efficacy, but with reduced Pt-generated side effects.

In the present study, in order to find an optimal dose for treatment, especially considering the lack of toxicity of phenolaTi, various concentrations of phenolaTi were examined. Interestingly, a clear dose–response was not detected, which may imply that alternative formulations should be evaluated. As the formulation degradation in the animal is presently unknown, it is possible that the active material is released before arriving at its biological target, and due to the limited solubility, is only partially effective. Because the efficacy of phenolaTi in all concentrations used was high (mostly TGI > 50 %), and also similar to that of cisplatin, it is also possible that the efficacy recorded is the highest achievable under the experimental conditions.

Conclusions

The phenolaTi complex is an effective anticancer drug as established on several murine and human solid tumor models and is nontoxic at a range of highly effective doses. Taken together with the ability to circumvent drug resistance, this complex is an attractive novel anticancer drug. Further preclinical studies with alternative formulations should specifically establish the therapeutic window and pharmacokinetics of the drug, to enable its subsequent evaluation in clinical settings.

Experimental Section

Drugs and chemicals: PhenolaTi was synthesized as previously described.[43] Cis-dichlorodiammine platinum(II) (cisplatin) 99 % was purchased from Acros (cat. 193760010), and oxaliplatin was purchased from Glentham Life Scientific Ltd. (cat. GP0792). Nanoparticles powder was prepared as previously described, increasing solubility of phenolaTi in water from 0.4 to 25 mg mL^{-1}.[36,43]

Cells: HT-29 (HTB-38TM) human colon cancer cells, as well as CT-26 (CRL-2638TM) and LLC-1 (CRL-1642TM) murine colon and lung cancer cells, respectively, were obtained from ATCC Inc. (VA, USA). HT-29 and CT-26 cells were maintained as attached culture in a medium containing: 1 % penicillin/streptomycin antibiotics, 1 % L-glutamine, 10 % fetal bovine serum (FBS), and 88 % RPMI-1640 medium, (all purchased from Biological Industries Inc., Bet-
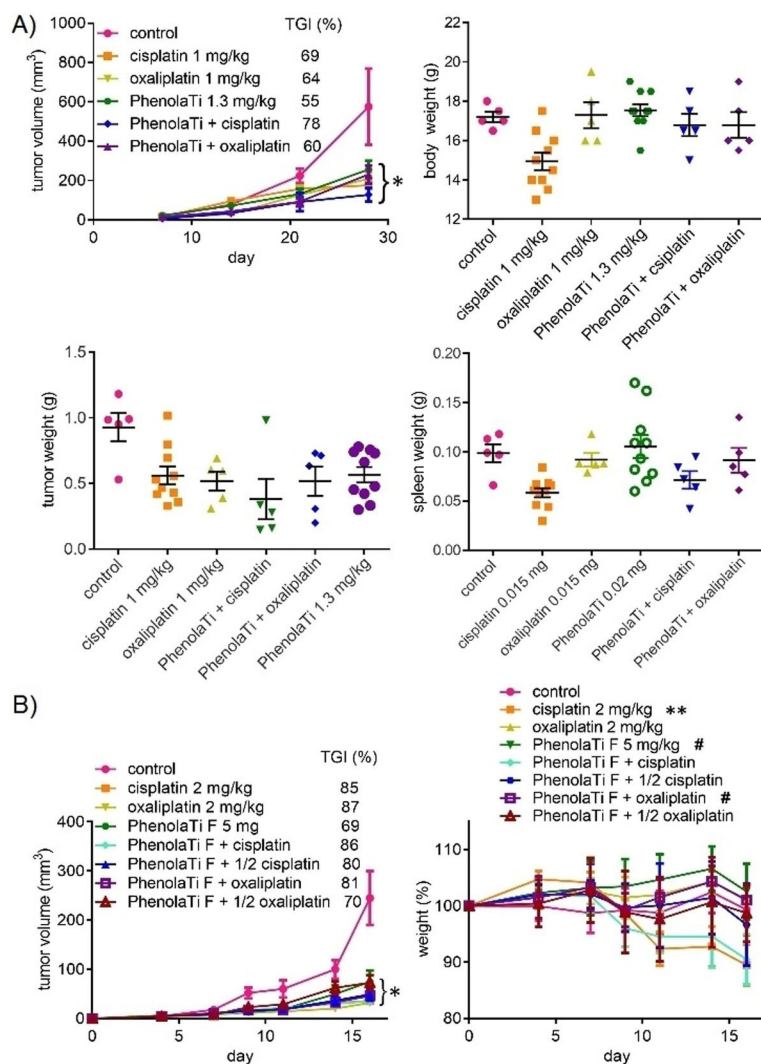
Figure 7. (A) Effect of non-formulated phenolaTi, cisplatin, and oxaliplatin, alone or combined, applied three times per week, on tumor growth (left): overtime volume (top) and final weight (bottom) and toxicity parameters (right): final body (top) and final spleen (bottom) weights in Nude mice inoculated with HT-29 human colon cancer cells. Here, treatment started two days post-cancer cell inoculation. (B) Effect of formulated phenolaTi, cisplatin, and oxaliplatin, applied five times per week, on overtime tumor volume (left) and body weight (right). For combinations: the added drugs were applied at the same concentration each as when applied alone; "1/2 cisplatin" or "1/2 oxaliplatin" refers to half the concentration of the Pt drug as applied alone. Data are the mean±SEM in 5–10 animals per group. *$P < 0.0001$ relative to control; **$P < 0.01$ relative to control; #$P < 0.0001$ relative to cisplatin-treated group.

consequently treated with the reagent tested at 10 different concentrations. Doses for control Pt-based drugs were selected based on literature and toxicity limitations.[50–53] After a standard of 3 days incubation, MTT (0.1 mg in 20 μL RPMI) was added and the cells were incubated for additional 3 h. After the incubation period, the MTT solution was removed, and the cells were dissolved in 200 μL isopropanol. The absorbance at 550 nm was measured by a Spark 10M Multimode Microplate Reader spectrophotometer (Tecan Group Ltd. Mannedorf, Switzerland). Relative IC_{50} values were determined by a nonlinear regression of a variable slope (four parameters) model by GraphPad Prism 5.04 software, with error values based on the STD of at least 3×3 repetitions (three separate measurements conducted on three different days to give nine repeats altogether). Cytotoxicity measurements on HT-29 human colon cancer cells were published previously.[44]

In vivo studies: For tumor growth inhibition experiments, 5–6-week-old Balb/c mice, C57BL/6, or immune-deficient (Nude) mice were inoculated subcutaneously (s.c.) with 1×10^6 CT-26 colon cancer cells, 5×10^5 LLC-1 lung cancer cells, or 5×10^6 HT-29 human colon adenocarcinoma cells, respectively. Tumors manifested within 4–10 days post-inoculation when mice were randomized into groups with similar average tumor dimensions. The mice were then treated 3 to 5 times weekly with the tested drug by intraperitoneal (i.p.) injections. Control groups were subjected to phosphate-buffered saline (PBS) or microemulsion solution in PBS devoid of the active drug. For all models, tumor volume ($length \times width^2 \times 0.52$) was assessed by caliper measurements every 2 to 4 days. Mice were euthanized once the tumors reached ethical limit of 15 mm length or if the animals displayed health indicators that met the ethical criteria for sacrifice. Tumor growth inhibition (TGI) was defined as the difference in size between mean control group and mean treated group, expressed as a percentage of mean control group: %TGI = [1 − ($mean_{drug-treated}$/$mean_{control}$)] × 100. A regimen of an agent that produces at least 50% TGI is generally classified as potentially therapeutically active.

Histological examination for tubular damage: Following euthanasia, kidneys were removed and fixed with 10% formalin, renal tissues were sectioned (3 μm) and stained with periodic acid–Schiff (PAS) reagents for histological examination. Tubular damage in PAS-stained sections was examined by microscopy (200× magnification) as described earlier.[54]

Nephrotoxicity test: Eight- to 10-week-old male C57BL/6 mice were euthanized 72 h after a single i.p. injection (20 mg kg^{-1}) of *cis*-diammineplatinum(II) dichloride (cisplatin), phenolaTi, or PBS as a vehicle control. Urine was collected before euthanasia using mouse metabolic cages (CCS2000 Chiller System, Hatteras Instruments, NC, USA). Blood was collected under deep anesthesia by retro-orbital bleeding, and serum and urine levels of creatinine as well as serum urea levels were measured using the Cobas C-111 chemistry analyzer (Roche, Switzerland). Blood urea nitrogen (BUN) was calculated by serum urea levels (BUN mg dL^{-1} = [urea] mM × 2.801). Creatinine clearance (CCr) was calculated using urine and serum creatinine levels (CCr mL h^{-1} = [urine creatinine] mg dL^{-1} × (urine volume)/[serum creatinine] mg dL^{-1} × 24 h). Urine levels of al-

Haemek, Israel) in a humidified atmosphere of 5% CO_2 at 37 °C. LLC-1 cells were maintained under similar conditions but with DMEM instead of RPMI-1640.

Mice: Balb/c, C57BL/6, and immune-deficient (Nude) female mice (5–6 weeks old) were obtained from Harlan (Israel) and held in an SPF facility (AAALAC accreditation #1285). Mice were treated in accordance with NIH guidelines and approval by the institutional committee for ethics in animal experimentation.

Growth inhibition assay: Cytotoxicity was measured on CT-26 colon cells and LLC-1 lung cells using the MTT assay as previously described.[49] Approximately 0.6×10^6 cells in medium were seeded into a 96-well plate and allowed to attach for a day. The cells were
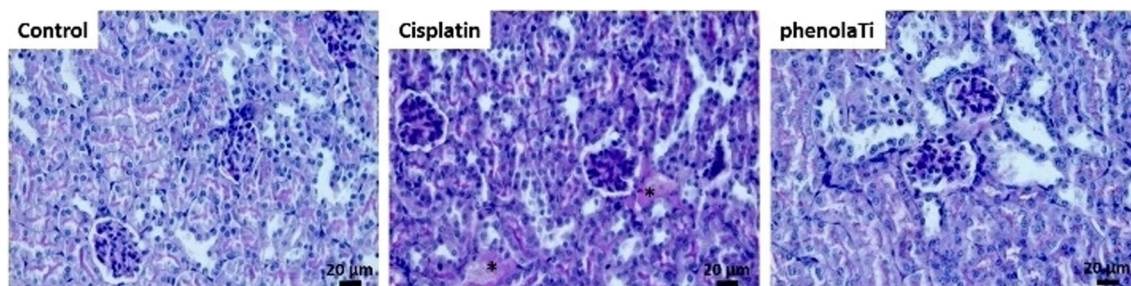
Figure 8. Chronic phenolaTi treatment does not induce nephrotoxicity in Nude mice inoculated with HT-29 human colon cancer cells. Representative images of PAS staining show that only cisplatin induced profound tubular injury after chronic administration to mice, as evidenced by protein cast, vacuolation and desquamation of the renal tubular epithelial cells. Note the normal histopathological appearance of the kidney of mice treated with phenolaTi.

bumin and kidney injury marker (KIM-1) were measured by ELISA kits (Albumin, Bethyl Laboratories, TX, USA; KIM-1, R&D Systems, MN, USA).

Statistical analysis: Two-way ANOVA with Bonferroni multiple comparisons test was performed for the tumor volume and body weight changes over time using GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego, CA, USA: www.graphpad.com). Statistical significance was determined at the level of $P < 0.05$. One-way ANOVA with Bonferroni multiple comparisons test was performed for the tumor and spleen final weight, available in the Supporting Information (Table S1).

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Keywords: cisplatin · drug combinations · metallodrugs · nephrotoxicity · titanium

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