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Conjugation of Vitamin E Analog α -TOS to Pt(IV) Complexes for Dual-Targeting Anticancer Therapy

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Abstract

We report two platinum(IV) complexes conjugated with a vitamin E analog, α -tocopherol succinate (α -TOS). One of the conjugates displays the activity of both cisplatin and α -TOS in cancer cells, causing damage to DNA and mitochondria simultaneously. Accordingly, it serves as promising dual-targeting anticancer agent.

Platinum-based anticancer agents induce apoptosis by damaging nuclear DNA in cancer cells.^{1,2} Following the success of cisplatin over the past few decades, platinum(II) analogues, including carboplatin, oxaliplatin, nedaplatin, and lobaplatin, have been introduced into the clinic to treat various cancers. Despite their efficacy, the inherent side effects and limitations of platinum(II) compounds remain problematic.^{2,3} Platinum(IV) prodrugs offer a viable alternative to platinum(II) therapy.⁴

Surrounded by six ligands in an octahedral coordination environment, the platinum(IV) metal centre is more inert toward substitution reactions than platinum(II). Upon reduction, two axial ligands are released and the corresponding divalent species is generated. Chemical modification of the axial ligands can significantly influence the efficacy and pharmacokinetics of platinum(IV) agents. The axial ligands provide synthetic chemists a means by which to impart and fine-tune desirable properties such as lipophilicity, redox stability, specific targeting, and improved cellular uptake.⁵

Single agents with dual-targeting capabilities provide a powerful approach to attack cancer cells. We previously reported the preparation and activity of mitaplatin, a platinum(IV) derivative of cisplatin bearing two dichloroacetate (DCA) ligands in the axial positions.⁶ Upon intracellular reduction, the DCA molecules target mitochondria and compromise the metabolism of cancer cells, while the released platinum(II) complex, cisplatin, forms DNA lesions. The widely differing effective doses of its individual components, DCA and cisplatin, cannot be recapitulated in mitaplatin, however, where the stoichiometry of these species is fixed at 2:1.

In the present study, we introduced a vitamin E analog, α -tocopherol succinate (α -TOS), as the axial ligand(s) in order to achieve a better match in activity between the intracellular reduction products. Two platinum(IV) complexes were constructed for simultaneously

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targeting of genomic DNA and mitochondria. α -TOS, (Fig. 1), displays potent in vitro cytotoxicity in a variety of cancer cell types including prostate, breast, lung, and colon cells.^{7–10} α -TOS inhibits anti-apoptotic proteins, Bcl-2 and Bcl-xL, thereby inducing mitochondria-mediated apoptotic cell death.¹¹ A synergistic effect has been reported between an analog of α -TOS and cisplatin in cisplatin-resistant ovarian cancer cells.¹²

The Pt(IV) design adopted here is based on the following premises: (1) α -TOS is highly lipophilic and will facilitate cellular uptake of Pt(IV); (2) α -TOS has an IC₅₀ value in the micromolar range, comparable to that of platinum(II) complexes;^{13,14} (3) Intracellular reduction or aquation of the Pt(IV) complexes is likely to release α -TOS, which will inhibit Bcl-xL and disrupt mitochondrial function, and cisplatin, which will target nuclear DNA. Together these effects were expected to induce apoptosis in a “dual-threat” manner. The results of our studies are reported below.

Pt(IV)(α -TOS)₂ and Pt(IV)(α -TOS)(OEt) (Fig 1) were prepared in a single step in reactions of *c,c,t*-[Pt(NH₃)₂Cl₂(OH)₂] and *c,c,t*-[Pt(NH₃)₂Cl₂(OH)(OEt)], respectively, with the acid anhydride derivative of α -TOS (Scheme S1, ESI). The high lipophilicity of the final compounds facilitated isolation through precipitation from methanol or by silica column chromatography. Both Pt(IV)(α -TOS)₂ and Pt(IV)(α -TOS)(OEt) are fairly insoluble in water, but readily dissolve in organic solvents such as dimethyl formaldehyde, dimethyl sulfoxide, and chloroform. The final compounds were characterized by ¹H, ¹³C, and ¹⁹⁵Pt NMR spectroscopy, ESI-MS, and elemental analysis (Fig. S1–10).

The anti-proliferative properties of Pt(IV)(α -TOS)(OEt), Pt(IV)(α -TOS)₂, α -TOS, cisplatin, mixtures of α -TOS and cisplatin were evaluated by the MTT assay and the results are summarised in Table 1. Pt(IV)(α -TOS)₂ displayed moderate to low toxicity against all cell lines. Conversely, Pt(IV)(α -TOS)(OEt) exhibited impressive potency, 7–220 times greater than that of cisplatin or combinations of cisplatin and α -TOS, across several tumour cell lines. Encouragingly Pt(IV)(α -TOS)(OEt) exhibited lower toxicity against healthy cells. Notably, a 265-fold reduction in toxicity was observed for non-tumorigenic lung fibroblast cells (MRC-5) compared to ovarian carcinoma cells (A2780). The difference in toxicities of Pt(IV)(α -TOS)₂ and Pt(IV)(α -TOS)(OEt) can be attributed to their differing lipophilities and, consequently, bioavailability. Pt(IV)(α -TOS)₂ is highly lipophilic and thus susceptible to detoxification through sequestration by extra- or intra-cellular proteins and entrapment inside the cell membrane. Pt(IV)(OAc)₂, bearing biologically inactive acetate ligands, exhibited low toxicity (IC₅₀ > 100 μ M) against all cell lines (Fig. S11). This emphasises the important contribution of α -TOS towards the potency of Pt(IV)(α -TOS)(OEt).

Cellular uptake studies were carried out to measure the distribution of platinum species in different subcellular compartments. A549 cells were treated with 10 μ M Pt(IV)(α -TOS)₂, Pt(IV)(α -TOS)(OEt), or cisplatin for 3 h and the cells were fractionated into three pools: membrane, nucleus, and cytosol. The platinum levels in each pool were measured by graphite-furnace atomic absorption spectroscopy (GF-AAS). The results are presented in Fig. 2. Both Pt(IV) agents showed 15–20 times greater cellular uptake than cisplatin. This property could be due to their higher intrinsic lipophilicity.

Nuclear DNA from A549 cells treated with cisplatin, mixtures of α -TOS and cisplatin, Pt(IV)(α -TOS)₂, Pt(IV)(α -TOS)(OEt), and Pt(IV)(OAc)₂ (10 μ M for 3 h) were extracted and the platinum content was determined by GF-AAS (Fig. S12). Genomic DNA extracted from cells dosed with Pt(IV)(α -TOS)₂ and Pt(IV)(α -TOS)(OEt) displayed significantly (*t* test, *p* < 0.005) higher levels of platinum (7–20 fold) than those exposed to cisplatin, mixtures of α -TOS and cisplatin, or Pt(IV)(OAc)₂. Therefore the Pt(IV)- α -TOS conjugates

are more adept at targeting nuclear DNA than cisplatin or Pt(IV) agents with biological inactive ligands.

α -TOS disrupts Bcl-xL-Bax interactions, activates Bax, and thus mediates mitochondrial centred apoptotic cell death.¹¹ Co-immunoprecipitation studies were carried out to determine whether the Pt(IV)- α -TOS conjugates could behave in a similar manner. To probe the Bcl-xL-Bax interaction dynamics, A549 cells were incubated with α -TOS, cisplatin, Pt(IV)(α -TOS)₂, Pt(IV)(α -TOS)(OEt), and Pt(IV)(OAc)₂ at the respective IC₅₀ values for 48 h, the cell lysates were then subjected to immunoprecipitation with Bcl-xL antibody, and, finally, the immunoprecipitates were probed for Bax content using immunoblotting. As expected, upon α -TOS treatment, the level of Bax associated to the Bcl-xL immunoprecipitate was markedly lower than the untreated control (Fig. S13). Pt(IV)(α -TOS)(OEt) treatment also led to reduced Bcl-xL-Bax interactions; moreover, the inhibitory effect was comparable to that of α -TOS treatment (Fig. S13). Bcl-xL-Bax interactions were less affected by cisplatin, Pt(IV)(α -TOS)₂, or Pt(IV)(OAc)₂ incubation. Additional immunoblotting studies were carried to determine the effect of Pt(IV)(α -TOS)(OEt) treatment on transcription. Remarkably, Pt(IV)(α -TOS)(OEt) induced a reduction in Bcl-xL expression. A similar trend was observed for cisplatin, Pt(IV)(α -TOS)₂, and Pt(IV)(OAc)₂ but not α -TOS (Fig. S14). Overall Pt(IV)(α -TOS)(OEt) is able to (1) downregulate Bcl-xL expression and (2) inhibit the interaction of Bcl-xL with Bax, a prerequisite for mitochondrial dysfunction. The former is inherent to cisplatin but not α -TOS, and the latter is inherent to α -TOS but not cisplatin. Pt(IV)(α -TOS)(OEt) is able to combine properties intrinsic to cisplatin and α -TOS, and thereby display transcriptional and post-transcriptional effects superior to α -TOS or cisplatin alone.

To gain further insight into the cellular response evoked by Pt(IV)(α -TOS)(OEt) treatment, biomarkers related to DNA damage and apoptosis were monitored. In the event of DNA damage, γ H2AX, the phosphorylated form of histone H2AX, is upregulated upon activation by the apical kinases, ATM and ATR.^{15,16} After treatment with increasing concentrations (0.75 – 6 μ M) of Pt(IV)(α -TOS)(OEt) for 48 h, A549 cells showed an increase in γ H2AX expression (Fig. 3A), indicative of genomic DNA damage. The expression of p53, a downstream effector of DNA damage,¹⁷ also increased in a dose-dependent manner. In addition, expression levels of apoptosis-related proteins, cleaved caspase-9 and PARP-1, increased with Pt(IV)(α -TOS)(OEt) treatment, revealing that cells undergo apoptosis. We therefore conclude that Pt(IV)(α -TOS)(OEt) induces DNA damage that results in apoptosis, like cisplatin and other Pt(IV)-based agents.

To investigate the effect of Pt(IV)(α -TOS)(OEt) on mitochondria, changes in the mitochondrial membrane potential were assessed by the JC-1 assay. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) is a positively charged lipophilic dye, which accumulates in the mitochondria of healthy cells as red-emitting aggregates.^{18,19} When the mitochondrial membrane is compromised, JC-1 de-aggregates into monomeric forms that emit green fluorescence. Untreated A549 cells showed both red and green fluorescence with slightly greater red fluorescence. Cells treated with mitochondrial membrane depolarizer, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), had increased green fluorescence and diminished red fluorescence (Fig. S15, ESI), indicative of mitochondrial membrane disruption. A similar phenomenon was observed in Pt(IV)(α -TOS)(OEt)- but not in cisplatin-treated cells (Fig. 3B). We further confirmed our findings using flow cytometry. A large population of untreated and Pt(IV)(α -TOS)(OEt)-treated A549 cells were examined. Upon Pt(IV)(α -TOS)(OEt) treatment, a marked increase in cells expressing green fluorescence was observed, indicative of unhealthy mitochondria (Fig. 3C & S16). This observation leads us to believe that Pt(IV)(α -TOS)(OEt) can indeed

cause disruption of mitochondrial function and promote apoptosis in cancer cells in an orthogonal manner to genomic DNA damage.

We performed flow-cytometric measurements to determine the effect of Pt(IV)(α -TOS)(OEt) exposure on cell cycle progression. A549 cells were treated with Pt(IV)(α -TOS)(OEt), 3 μ M, for 24, and 48 h. The cell cycle distribution was compared with untreated cells (Fig. S17). Cells treated with Pt(IV)(α -TOS)(OEt) displayed large S-phase populations after 24 and 48 h, indicative of S-phase arrest. It is therefore clear that Pt(IV)(α -TOS)(OEt) can disturb DNA synthesis and arrest at S-phase.

Finally, we carried out the PI-annexin-V dual staining assay to quantify apoptosis induced by Pt(IV)(α -TOS)(OEt) in A2780 ovarian cancer cells. After cells were exposed to Pt(IV)(α -TOS)(OEt) (4 μ M) for 48 h, 28% of the cell population displayed characteristics associated with early apoptosis. Only 10% of cisplatin-treated cells displayed apoptotic properties; therefore, Pt(IV)(α -TOS)(OEt) shows a superior ability to induce apoptosis. The data are shown in Fig. S18 (ESI).

In summary, the lead vitamin E analog α -TOS conjugated Pt(IV) compound, Pt(IV)(α -TOS)(OEt), displays dual-targeting effects in killing cancer cells. The platinum moiety causes nuclear DNA damage and α -TOS disrupts Bcl-xL-Bax interactions leading to mitochondrial dysfunction. Pt(IV)(α -TOS)(OEt) displays superior efficacy in cell killing than cisplatin. More generally, these results demonstrate the dual-targeting strategy to be a valuable route to pursue in the design of platinum agents with higher efficacy and improved therapeutic index.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structures of vitamin E analogue, α -TOS, and α -TOS conjugated Pt(IV) complexes.

Fig. 2.

Cell uptake of Pt(IV)(α-TOS)₂, Pt(IV)(α-TOS)(OEt), or cisplatin in A549 cells. Cells were exposed to 10 μM of platinum compounds for 3 h.

Fig. 3.

(A) Analysis of protein expression in A549 cells following treatment with Pt(IV)(OEt)(α-TOS) at 0, 0.75, 1.25, 3, 6 μM for 48 h. Whole cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting against γH2AX, p53, and cleaved caspase-7, cleaved caspase-9, cleaved PARP-1, and β-actin as control. (B) A JC-1 assay to observe the disruption of mitochondrial function in A549 cells by Pt(IV)(α-TOS)(OEt). Fluorescence microscopy of A549 cells treated with cisplatin or Pt(IV)(α-TOS)(OEt) at 20 μM, for 4 h. (C) Flow cytometry analysis of A549 cells treated with cisplatin or Pt(IV)(α-TOS)(OEt) at 25 μM, for 4 h. (Histogram representing the change in FL1-H for cells stained with JC-1 in absence (red) and presence (blue) of cisplatin (top) and Pt(IV)(α-TOS)(OEt) (bottom)).

Table 1

The cytotoxicity of Pt(IV)(α -TOS)₂ in comparison to those of cisplatin and α -TOS in cancer cell lines by IC50 (μ M).

Cell name	Type	CisPt	α -TOS	CisPt + α -TOS	Pt(IV)(α -TOS)(OEt)	Pt(IV)(α -TOS) ₂
A549	Lung	2.5(0.8)	26.9(1.4)	6.4(0.2)	1.3(0.1)	13.9(0.1)
HeLa	Cervical	1.4(0.3)	25.1(1.0)	3.4(0.5)	1.9(0.3)	24.7(0.6)
A2780	Ovarian	0.56*	13.8(1.08)	4.4(1.2)	0.02(0.01)	56.4(4.6)
A2780/CP70	Ovarian	6.0*	21.7(1.7)	7.3(0.6)	1.1(0.1)	>100
PC-3	Prostate	15.1(0.9)	37.0(0.1)	19.2(0.2)	2.5(0.2)	>100
HCT116	Colon	6.6(0.4)	31.2(1.8)	17.3(0.9)	1.24(0.01)	>100
MCF-7	Breast	18.2(0.5)	40.5(5.0)	18.3(0.5)	5.9(0.1)	>100
MRC-5	Lung (Normal)	6.3(0.4)	28.5(4.2)	18.8(0.7)	5.3(0.2)	80.5(0.6)

Note: average of three independent experiments.

* Data taken from reference 6.