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Cytotoxic activity of the titanium alkoxide (OPy)₂Ti(4AP)₂ against cancer colony forming cells

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Summary

A novel family of titanium alkoxides with two stable pyridinemethoxide moieties bound to a titanium metal center were synthesized and tested for cytotoxic activity on a variety of cancer cell lines using colony formation assays. One compound, (OPy)₂Ti(4AP)₂, where OPy is NC₅H₅CH₂O, and 4AP is 4-aminophenoxide (⁻OC₆H₅(NH₂)-4), demonstrated increased cytotoxicity in breast, colon, and pancreatic cancer cell lines at 100 nanomolar levels with only short exposures. Further, (OPy)₂Ti(4AP)₂ had activity in colon and pancreatic cancer cell lines that are usually resistant to chemotherapy. This demonstrates that these titanium compounds may have a role in anti-cancer therapy, similar to platinum-based compounds, and the (OPy)₂Ti(4AP)₂ compound specifically deserves further investigation as an anti-cancer agent in chemo-resistant solid tumors.

Keywords

Titanium alkoxides; Colony forming cell; Pancreatic cancer; Lung cancer; Colon cancer

Introduction

Several metalorganic compounds have demonstrated exceptional anticancer activity. Classic examples of such compounds are the derivatives of cisplatin, including carboplatin and oxaloplatin, where a platinum molecule is linked to an organic structure. These have found a wide variety of clinical applications in treating human neoplasia [1–3]. Recently, organotitanium molecules have generated interest, in hopes that they might function similarly to the platinum cancer drugs. Of these compounds, titanium alkoxides [(η^5 -Cp)₂Ti(OR)₂ and (η^5 -Cp)Ti(Cl)(OR)₂, where Cp is C₅H₅ and OR is an alkoxide] as well as titanocene dichloride [(Cp)₂TiCl₂] were found to exhibit sufficient activity in cancer cell line studies and in murine xenograft models to merit Phase I and Phase II trial studies [4–8]. Renal insufficiency was the major dose-limiting toxicity. However, the few responses noted in these trials has limited further clinical development. Thus far, investigation with titanium compounds has focused on the use of Cp derivatives coupled with either halide or alkoxide

ligands [4–7]. The active moiety of the organometallic species has been postulated to participate in metal alkoxide-DNA interactions, not unlike cisplatin. However, while there is direct molecular interaction between these titanium compounds and DNA, the molecular mechanism of cytotoxicity for these compounds has yet to be elucidated [8, 9].

Recently, a family of novel metalorganic species with structural characteristics similar to those noted for the Cp compounds has been synthesized in our laboratory [10]. This novel family of titanium alkoxide compound is stabilized by two pyridine methoxide ($(\text{NC}_5\text{H}_5)\text{CH}_2\text{O}$, termed OPy) ligands, which were chemically stable enough to introduce a variety of alkoxide substitutions without inducing structural rearrangements. Since the ligands could be easily manipulated and the general structure was stable and similar to those noted for anticancer agents, determining the cancer cell cytotoxicity activity of these species became of interest. While several members of this family had some cytotoxic activity in selected cancer cell types, one compound $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ (where 4AP is 4-aminophenoxide or $-\text{OC}_6\text{H}_5(\text{NH}_2)-4$) showed cytotoxic effects at 100 nanomolar levels on the colony forming cells of multiple cancer cell lines, many of which are characteristically resistant to most chemotherapeutic agents.

A variety of cancer cell lines were used to study the cytotoxic effects of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$, including the pancreatic cancer cell lines Panc1, BxPC3, and McCain's nut; the breast cancer cell lines T47D, MFC7, MCF10a, and MDA-MB-231; and the colon cancer cell lines SW48, CaCo2, HT29 and T84. Cytotoxic activity at 100 nanomolar levels of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was observed on all of these lines except T84 and MCF-10a, where it had activity in the micromolar range. Since there are few agents with significant activity against many colon and pancreatic cancers, and $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ is particularly active against these types of cancers. Therefore, these results may be of significance for the development of future therapy in these cancer types.

Materials and methods

Titanium compound synthesis

A series of novel organotitanium compounds were synthesized as previously described [10, 11]. These compounds were tested for cytotoxic activity in cancer cell lines, wherein $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was found to possess significant activity, which is reported here. The synthesis of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was previously published [10], but a brief description follows. To a stirring clear solution of $\text{Ti}(\text{OCHMe}_2)_4$ in toluene, two equivalents of H-OPy was added via pipette and left to stir. After 12 h, the reactions were set aside to slowly evaporate the volatile component of the reaction until crystals of $(\text{OPy})_2\text{Ti}(\text{OCHMe}_2)_2$ formed. After isolating this powder, it was redissolved in toluene, two equivalents of H-4AP were added and the reaction was left to stir overnight, whereupon a precipitate formed. Pyridine was added and the reaction heated until clear. Upon cooling, X-ray quality crystals were isolated and tested for assessment of structure as reported [10, Fig. 1]. After purification using liquid chromatography, $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was dissolved in aliquots of DMSO for cancer cell cytotoxicity studies, and stored at 4°C. There was little diminishment of its activity observed over time. (Figs. 2, 3, and 4)

Colony formation assays

The cell lines A549, Panc1, BxPC3, McCain's nut, T47D, MFC7, MCF10a, MDA-MB-231, SW48, CaCo2, HT29 and T84 were all obtained from ATCC (Manassas, VA). These cell lines were chosen because they represented common solid tumors of multiple types, and several of these types had few effective chemotherapeutic agents available. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Colony

formation assays were performed as previously described [12, 13]. One million cells were plated in 100 mm cell culture dishes, allowed to adhere overnight. After overnight proliferation, plated cells were exposed to the titanium compounds at various concentrations (0–1,000 nM). The cells were exposed to the compounds for 4 h at the various concentrations, trypsinized, and then re-plated at 500–5,000 cells per 100 mm plate in triplicate. Treated cells were then incubated for 10–14 days, until visible colonies formed. The plates were then washed in phosphate-buffered normal saline, and colonies stained with 1% methylene blue in methanol for 15 min. Plates were then washed twice in tap water, and colonies containing greater than 50 cells were counted on an inverted microscope. Survival was compared to the plating efficiency of vehicle-treated control cells. Statistical significance was assessed using Student's *t* test.

Results

A series of sterically encumbered heterocyclic organotitanium compounds were synthesized according to our previously published methods [10]. In brief, titanium iso-propoxide [$\text{Ti}(\text{OPr}^i)_4$] was mixed with pyridinecarbinol (H-OPy) to form bis-pyridinecarbinol titanium iso-propoxide [$(\text{OPy})_2\text{Ti}(\text{OCHMe}_2)_2$]. This compound was further reacted with four aminophenol (H-4AP) to form bis-pyridine titanium bis 4-aminophenoxide, $(\text{OPy})_2\text{Ti}(\text{4AP})_2$. This compound had two terminal amine groups that were thought to provide reactivity with cellular biomolecules and was also found to be soluble in DMSO (Fig. 1). Because of the interest in organotitanium compounds as novel cancer therapeutics, this DMSO-soluble titanium compound was tested for cytotoxic activity in colony forming cells from cancer lines. In these assays equivalent concentrations of DMSO served as the intrinsic colony forming cell capacity control. However, we noted no difference in colony forming cell survival with or without DMSO.

Activity in pancreatic cancer cells

Three pancreatic cell lines were examined for sensitivity to $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ using colony formation assays, Panc1, BxPC3, and McCain's nut. $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was incubated at 0–1,000 nM concentrations for 4 h, and then washed off, and surviving cell colonies counted after 14 days. There was no effect of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ in Panc1 cells at 100 nM, but at 600 nM and 1,000 nM essentially all cells were killed. Likewise, $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ essentially killed all BxPC3 pancreatic cancer cells at concentrations equal to or exceeding 600 nM. Similarly, at concentrations above 600 nM essentially all McCain's nut pancreatic cancer cells were killed.

Activity in breast cancer cells

Four breast cancer cell lines were examined for sensitivity to $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ using colony formation assays, T47D, MCF7, MCF10a, and MDA-MB-231. $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ was incubated at 0–1,000 nM concentrations for 4 h, and then washed off, and surviving cell colonies counted after 10 days. Exposure of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ to T47D breast cancer cells resulted in an 8-fold decrease in colony survival at 100 nM, and essentially no cells survived exposure to concentrations equal to or greater than 300 nM. MCF7 breast cancer cells had a 12-fold decrease in colony survival after exposure to 300 nM $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ and little colony forming cell survival at any concentration exceeding that. MCF10a cells were more resistant to $(\text{OPy})_2\text{Ti}(\text{4AP})_2$, with no survival decrease until 1,000 nM. MDA-MB-231 breast cancer cells, which are estrogen receptor negative, are more sensitive to $(\text{OPy})_2\text{Ti}(\text{4AP})_2$. Little colony survival was noted at 300 nM or greater.

Activity in colon cancer cells

Four colon cancer cell lines were also examined for sensitivity to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ using colony formation assays, SW48, CaCo2, HT29, and T84. $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ was incubated at 0 nM, 10 nM, 100 nM, and 1,000 nM concentrations for 4 h, and then washed off, and surviving cell colonies counted after 14 days. SW48 colon cancer cells had a 2-fold decrease in colony forming cell survival at 100 nM of $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, a 2.5-fold decrease in colony survival at 300 nM $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, and no colony survival at concentrations exceeding 300 nM. CaCo2 cells had a 9-fold decrease in survival at 300 nM $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, and essentially no colony forming cell survival at concentrations exceeding that. HT29 colon cancer cells were somewhat more resistant to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, with no cytotoxic effect seen until 600 nM, but at 1,000 nM of $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ essentially all HT29 cells are killed. T84 colon cancer cells were also relatively more resistant to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, as there was little cytotoxicity until 1,000 nM, where there was a 13-fold decrease in colony forming cell survival.

Discussion

The capacity of $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ to kill colony forming cells from a variety of solid tumor cancer cell lines, chosen mainly for their resistance to conventional chemotherapy, was examined. In this study we found that exposure to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ resulted in significant killing of these colony forming cells at 100 nanomolar levels. Colony formation assays are more appropriate *in vitro* measures of investigational new drug activity than proliferation assays or expression of apoptosis markers for several reasons. First, colony formation assays measure not just stasis of proliferation, but actual cell killing. A non-proliferative cell may still be able to repair an insult and resume proliferation at some point after drug exposure, and this would be assessed in a colony formation assay. Second, only a fraction of cells in a cancer cell line culture population can form colonies. Only the most proliferative and most hardy cells form colonies, which can serve as a distant approximation of a cancer stem cell [14]. These colony forming cells may get lost within the overall population when growth of a population is measured, yet it is these cells that will ultimately be selected for and overgrow the population. In a similar manner, overall expression of apoptosis markers does not always correlate with mortality of these colony forming cells, as these cells can be overwhelmed within the overall population.

Thus, colony formation more accurately represents the opportunity for a clinical response, given that cancer cells can regain proliferation after a cytostatic agent, whereas they cannot after a cytotoxic agent. These assays identify a cell that not only repairs and survives the insult of exposure to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$, but is able to replicate to form colonies. Thus, colony forming cells are perhaps a more accurate assessment of cytotoxicity of this cancer drug than proliferation assays, since a small resistant population could overgrow a dead population rapidly, but the overall population masks the survival of a resistant clone.

The cytotoxic effect that $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ had on pancreatic cell carcinoma lines (Panc1, BxPC3 and McCain's Nut) was one of the most significant findings of this study, since this cancer type is highly resistant to most forms of chemotherapy [15]. The only drug approved specifically for the treatment of pancreatic cancer is gemcitabine, and it is only marginally effective [15]. Panc1 cells are resistant to gemcitabine, while BxPC3 cells are sensitive to gemcitabine [16], yet both are equally sensitive to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$. This indicates that $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ is able to overcome resistance of at least one pancreatic cancer cell line to the best current pancreatic cancer chemotherapeutic agent. In like manner, SW48 colon cancer cells are resistant to the most common colon cancer drug 5-fluorouracil [17], but were found to be sensitive to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$. Interestingly, $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ was able to overcome p-glycoprotein resistance in at least one cell line. Caco2 colon cancer cells are

intrinsically resistant to many chemotherapeutic agents because of high p-glycoprotein expression [18], yet they are quite sensitive to $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ in studies here.

$(\text{OPy})_2\text{Ti}(4\text{AP})_2$ showed cytotoxic effects in all cancer cell lines tested with the exceptions of the T84 colon cancer and the MCF10a breast cancer cell lines. There is little these cell lines have in common that can be found. MCF10a cells are thought to be closer to normal tissue and less transformed than other breast cancer cell lines. It has served as a model for investigating steps of transformation to frank malignancy in some studies [19]. However, it is doubtful that this relatively decreased sensitivity of MCF10a represents a lack of toxicity of $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ to normal tissue, but rather its lack of cytotoxicity in this cell line more likely indicates activity is specific for individual cancers.

The other structurally similar titanium alkoxide species lacking the 4AP ligand described by us previously [10] did not show cytotoxicity in these colony formation assays (not shown). This implies the necessity of the 4AP ligand for cancer colony forming cell cytotoxicity. Therefore, functional groups on the phenoxide ring with acidic protons or with the ability to hydrogen bond are being studied. Recent reports by Harding and colleagues and Gao and Melendez have suggested a connection between stable amino-group functionalization and increased anticancer activity and aqueous solubility [20, 21]. By modifying titanocene dichloride and replacing the chloride ligands with varying amino acids and ligands with amino functional groups, they obtained a family of compounds with anticancer activity. This has lead into continued investigation on the generation of a ligand set similar to 4AP with varying functional groups in order to enhance aqueous solubility and increase anti-cancer activity.

How titanium compounds actually kill cancer cells is not completely understood. Titanocene dichloride induced a block in late S/early G2 phase of the cell cycle, similar to that seen in DNA damage. However, apoptotic cell death occurred in any phase of cycle. Titanium-DNA adducts were detected in A2780 cells treated with titanocene dichloride using atomic absorption spectrometry, suggesting that DNA may be a target for this drug [8]. In agreement with this finding, p53 accumulated rapidly in treated A2780 cells, indicative of a role for titanocene dichloride as a DNA-damaging agent. However, whether titanocene dichloride actually causes DNA breaks is not clear [9]. It may function less like cisplatin, which cross-links DNA, causing replication fork collapse, and resultant DNA double strand breaks, and more like a single strand alkylating agent, which does not function in that manner. In recent studies on the anticancer activity of $(\text{Cp})_2\text{TiCl}_2$, the action of titanium entry into tumor cells has been attributed to an interaction between the surface protein transferrin and titanium ions (20). Once in the cell, it has been postulated that titanium has a variety of effects on transcription factors and polymerase proteins, effectively halting protein synthesis, or causing cytotoxicity [8]. However, little research has been conducted on the direct intracellular actions of titanium-based chemotherapy due to the propensity of such compounds to hydrolyze and form insoluble polymers after exposure to aqueous solvent systems, especially if such solvent systems contained serum proteins. This also may be a reason for the lack of clinical activity in the Phase I/II trials with titanocene dichloride [6–8]. Previous experiments which attempted encapsulation with phosphatidylserine found no cytotoxic activity of these encapsulated in cell culture [21]. It has been postulated that micelle encapsulation may interrupt the transferrin-Ti interaction or may inhibit hydrolysis and ligand-Ti dissociation so as to hinder the anti-cancer activity of the compound [21, 22]. Thus, there remain barriers to effective use of titanium compounds for clinical cancer care. Perhaps the sterically encumbered $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ may overcome some of these barriers.

The activity of $(\text{OPy})_2\text{Ti}(4\text{AP})_2$ compares favorably with several recently generated titanocene dichloride derivatives, titanocene X, Y, and Z [23]. For the two cell lines tested in

common for in vitro colony formation, Panc1 and MCF7, titanocene X, Y, and Z had IC₅₀ values near 100 micromolar, whereas (OPy)₂Ti(4AP)₂ had IC₅₀ values below 1 micromolar for Panc1 and MCF-7. Thus, (OPy)₂Ti(4AP)₂ appears to have greater anti-cancer cytotoxicity than the recent titanocene dichloride derivatives, at least *in vitro*. Animal testing for (OPy)₂Ti(4AP)₂ *in vivo* anti-neoplastic activity remains to be performed. In summary, we have found that (OPy)₂Ti(4AP)₂ has significant cytotoxic activity against cancer colony forming cells, even in cell lines that are normally resistant to current chemotherapeutic agents. Importantly, (OPy)₂Ti(4AP)₂ can overcome p-glycoprotein resistance in at least some cancer cell lines. In addition, this activity occurs at the 100 nanomolar level, which is likely to be achievable *in vivo*. Further investigation will include study of the action of this compound within cancer cells, as well as varying the functional ligands of the compound to increase solubility in plasma.

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Fig. 1. The structure of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$ (10). **a** Unit cell view of the 3-dimensional structure of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$. The titanium is represented by *green*, oxygen by *red*, nitrogen by *blue*, and carbon by *grey*. Hydrogen atoms have been removed for clarity. **b** The Schematic structure of $(\text{OPy})_2\text{Ti}(\text{4AP})_2$

Fig. 2.

The effect of (OPy)₂Ti(4AP)₂ on pancreatic cancer cell lines. **a** Panc1, **b** BxPC3, and **c** McCain's nut. Indicated cell numbers were adhered to culture dishes, exposed to varying nM concentrations of compound for 4 h, then washed, and incubated for 10–14 days for colony formation. Each experiment was performed twice in triplicate, with standard error of the mean indicated. Colony survival was compared to the plating efficiency of cells treated with vehicle alone as a control. Statistical differences at the $p < 0.05$ level using Student's t test between test and control cells is indicated by a *star*

Fig. 3.

The effect of (OPy)₂Ti(4AP)₂ on breast cancer cell lines. **a** T47D, **b** MFC7, **c** MCF10a, and **d** MDA-MB-231. Indicated cell numbers were adhered to culture dishes, exposed to varying nM concentrations of compound for 4 h, then washed, and incubated for 10–14 days for colony formation. Each experiment was performed twice in triplicate, with standard error of the mean indicated. Colony survival was compared to the plating efficiency of cells treated with vehicle alone as a control. Statistical differences at the $p < 0.05$ level using Student's *t* test between test and control cells is indicated by a *star*

Fig. 4.

The effect of (OPy)₂Ti(4AP)₂ on colon cancer cell lines. **a** SW48, **b** CaCo2, **c** HT29 and **d** T84. Indicated cell numbers were adhered to culture dishes, exposed to varying nM concentrations of compound for 4 h, then washed, and incubated for 10–14 days for colony formation. Each experiment was performed twice in triplicate, with standard error of the mean indicated. Colony survival was compared to the plating efficiency of cells treated with vehicle alone as a control. Statistical differences at the $p < 0.05$ level using Student's *t* test between test and control cells is indicated by a *star*