DOI: 10.1002/anie.201205973

Drug Discovery

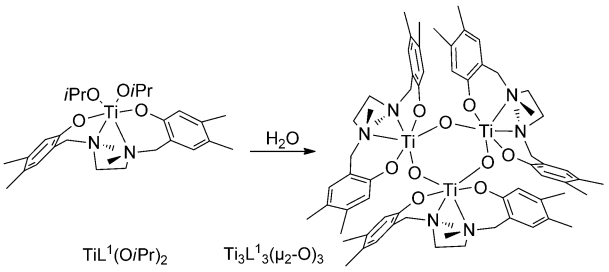
High Antitumor Activity of Highly Resistant Salan–Titanium(IV) Complexes in Nanoparticles: An Identified Active Species**

Sigalit Meker, Katrin Margulis-Goshen, Ester Weiss, Shlomo Magdassi, and Edit Y. Tshuva**

Titanium(IV)-based anticancer complexes were the first to enter clinical trials after platinum compounds.[1] In particular, budotitane ($[(bzac)_2Ti(OEt)_2]$; bzac = benzoylacetonate) and titanocene dichloride ($[Cp_2TiCl_2]$; Cp = cyclopentadienyl) demonstrated high antitumor activity with reduced toxicity toward a range of cancer cells; however, use of these complexes, which both bear two labile ligands, was limited by their instability in water.[2] Therefore, mechanistic aspects remained unresolved, including the nature of the active species and its identification from the multiple hydrolysis products that were formed. We have recently introduced cytotoxic salan–Ti^{IV} complexes,[3] which are: a) substantially more hydrolytically stable than known Ti^{IV} complexes, and b) markedly more active than $[(bzac)_2Ti(OiPr)_2]$, $[Cp_2TiCl_2]$, and *cis*-platin toward a variety of cancer-derived cell lines. Structure–activity-relationship studies based on both salan[3a,b,e–h,4a,b] and labile ligand[3g,4c,d] variations revealed that reduced steric bulk is favored for cytotoxicity. Additionally, all cytotoxic complexes slowly gave defined oxo-bridged polynuclear hydrolysis products.[3b,e–g] Particularly, N-methylated complexes produced well-defined trinuclear clusters,[3f,g] which were stable for weeks in water. Several observations indicated that the hydrolysis products play a meaningful role in the cytotoxicity mechanism;[3] nevertheless, direct measurements on the isolated clusters showed no activity.[3f,g] Herein we address the hypothesis that cellular penetration, which is size-dependent, and/or impaired solubility were limiting factors, and that labile ligands may actually not be required for cytotoxicity of Ti^{IV} complexes, unlike for *cis*-platin.[6] This paper presents the high activity of a hydrolysis product and other particularly resistant Ti^{IV} complexes, the solubility and cell-penetration of which are improved through the reduction of their particle size to the nanoscale dimension.

Reduction of the particle size to the nanometric region accelerates intercellular permeability and increases the solubility and dissolution rate.[7] Nanoparticles of salan–Ti^{IV} complexes were obtained by a rapid conversion of a volatile oil-in-water microemulsion into a dry powder composed of nanoparticles. Rapid evaporation of the volatile droplets that contain the complex gave a powder[8] that was easily dispersible in aqueous media to form a dispersion of stable nanoparticles. Notably, the surfactants used are approved by the Food and Drug Administration (FDA) for incorporation into pharmaceutical dosage forms.

$Ti_3L^1{}_3(\mu_2\text{-}O)_3$ is the crystallographically characterized trinuclear hydrolysis product of the cytotoxic $TiL^1(OiPr)_2$ (Scheme 1) and was previously reported as inactive.[3g] This



Scheme 1. Salan complex $TiL^1(OiPr)_2$ and its trinuclear hydrolysis product $Ti_3L^1{}_3(\mu_2\text{-}O)_3$.

trimer was incorporated into a microemulsion of *n*-butyl acetate in water, ultimately forming nanoparticles with a mean size of (17.6 ± 1.7) nm for 0.2 wt% of the powder in water. Cytotoxicity of the clear and stable aqueous dispersion was measured on colon HT-29 and ovarian OVCAR-1 cancer cell lines by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Figure 1, Table 1). The nanoformulated trimer exhibited high cytotoxicity, with IC_{50} values comparable to those of its monomeric precursor, as previously recorded without formulations.[3g,h] Control measurements showed no activity for empty nanoparticles. Thus, inactivity of the non-formulated trimer resulted from insufficient solubility and/or inhibited cellular penetration, rather than impaired interaction with the biological target. Apparently, any activity observed previously for partly hydrolyzed salan–Ti^{IV} complexes with some labile ligands does not necessarily identify the active species, but rather may be a consequence of additional hydrolysis steps.[9] A previous report on diketonato derivatives also presented cytotoxicity for a tetrameric hydrolysis product encapsulated in a liposome.[10] It is thus plausible that the hydrolysis product serves as a cellular active

[*] S. Meker, E. Weiss, Prof. E. Y. Tshuva
Institute of Chemistry, The Hebrew University of Jerusalem
Jerusalem, 91904 (Israel)
E-mail: edit.tshuva@mail.huji.ac.il
Homepage: http://chem.ch.huji.ac.il/~tshuva
K. Margulis-Goshen, Prof. S. Magdassi
Casali Institute of Applied Chemistry, Institute of Chemistry, The Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem
Jerusalem, 91904 (Israel)
E-mail: magdassi@mail.huji.ac.il

[**] We thank Dr. Shmuel Cohen for crystallography work. Funding was received from the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC Grant agreement (No.239603), and partially from the Israel Science Foundation (grant No.124/09) and Lower Saxony Ministry of Science.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205973.

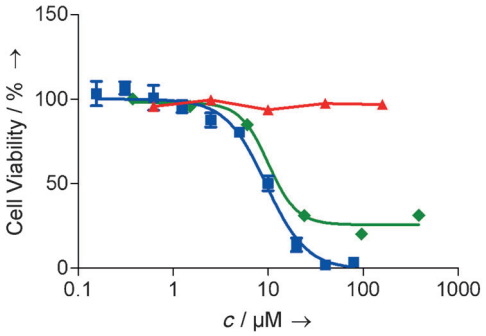


Figure 1. Dependence of OVCAR-1 cell viability on concentration of $TiL^1(OiPr)_2$ (previously reported without nanoformulation[3g,h]) and its hydrolysis product $Ti_3L^1{}_3(\mu_2\text{-}O)_3$ administered in a nanoformulated and non-nanoformulated[3g] manner following an incubation period of three days (concentration of polynuclear compounds was determined per entire cluster); green: $TiL^1(OiPr)_2$ non-nanoformulated; red: $Ti_3L^1{}_3(\mu_2\text{-}O)_3$ non-nanoformulated; blue: $Ti_3L^1{}_3(\mu_2\text{-}O)_3$ nanoformulated.

Table 1: Mean particle size measured for 0.2 wt% dispersion in water and IC_{50} (μM) values toward OVCAR-1 and HT-29 cancer cells for the nanoformulated complexes.

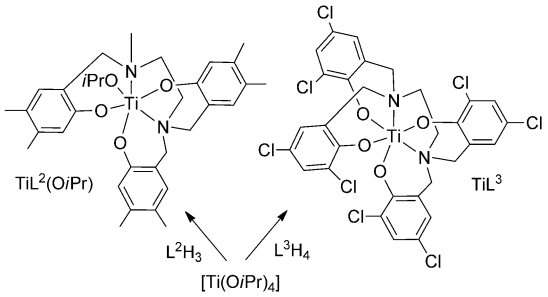
Complex[a]	Particle size [nm]	OVCAR-1 [μM]	HT-29 [μM]
$TiL^1(OiPr)_2$[b],[3h]	–	12±1	14±1
$Ti_3L^1{}_3(\mu_2\text{-}O)_3$	17.6±1.7	10±3	13±3
$TiL^2(OiPr)$	9.0±0.6	70±22	54±16
$Ti_2L^2{}_2(\mu_2\text{-}O)$	110±12.7	110±50	200±30
TiL^3	5.3±0.3	14±4	12±2

[a] $Ti_3L^1{}_3(\mu_2\text{-}O)_3$, $TiL^2(OiPr)$, and TiL^3 were all inactive when administered directly in a non-nanoformulated manner; [b] non-formulated reference.

species as a general mechanistic pathway for Ti^{IV} complexes, thus further questioning the requirement of labile ligands for the cytotoxicity of Ti^{IV} centers.[6]

Under the notion that labile ligands are not essential for cytotoxicity, pre-designed, inert, and hydrolytically stable complexes should be cytotoxic as are, thus eliminating the hydrolysis step and the accompanying undesired release of side products, such as free labile ligands. Thus, tris- and tetrakis(phenolato) ligands (L^2H_3 and L^3H_4, respectively) were prepared,[11] and afforded the monomeric octahedral complexes $TiL^2(OiPr)$ and TiL^3, respectively (Scheme 2). ^1H NMR spectroscopy confirmed that a single product of each complex had formed and the X-ray crystal structure of TiL^3 featured an octahedral C_2-symmetrical complex (Figure 2).

Comparative hydrolysis measurements by ^1H NMR spectroscopy were carried out as previously described[3f,g] by monitoring the integration of selected signals over time following addition of 10% D_2O to a solution of the complexes in [D_8]THF. The $t_{1/2}$ value for isopropoxo hydrolysis of $TiL^2(OiPr)$ was approximately 100 hours, markedly higher than the value previously reported for $TiL^1(OiPr)_2$ (ca. 5 h), which was obtained under similar conditions.[3g] TiL^3 demonstrated even higher stability, where no substantial hydrolysis was observed for over a week. As expected, the decrease in the number of labile ligands dramatically increased the hydrolytic stability of the complexes.



Scheme 2. Ti^{IV} complexes of reduced lability.

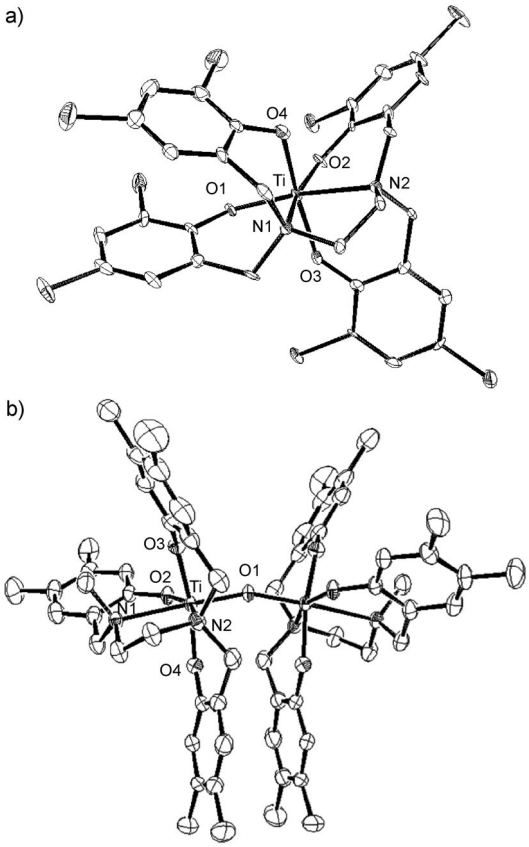


Figure 2. ORTEP drawings of a) TiL^3 and b) $Ti_2L^2{}_2(\mu_2\text{-}O)$ with thermal ellipsoids at 50% probability; hydrogen atoms and solvent molecule were omitted for clarity.

Although no hydrolysis products could be detected for TiL^3, $TiL^2(OiPr)$ slowly hydrolyzed to give a singly oxo-bridged dimeric product.[3e] The X-ray structure showed a C_i-symmetrical complex, in which each Ti^{IV} center was bound to the tris(phenolato) ligand (Figure 2).

$TiL^2(OiPr)$, TiL^3, and the hydrolysis product $Ti_2L^2{}_2(\mu_2\text{-}O)$ were all inactive when measured directly on HT-29 and OVCAR-1 cells. However, when formulated into nanoparticles, all three were cytotoxic (Figure 3, Table 1). Particularly, the most stable complex TiL^3 exhibited the highest cytotoxicity, with IC_{50} values that are comparable to those of the non-nanoformulated $TiL^1(OiPr)_2$[3g,h] and its nanoformulated hydrolysis product $Ti_3L^1{}_3(\mu_2\text{-}O)_3$ (Table 1).

To conclude, this work showed that defined hydrolysis products from salan–Ti^{IV} complexes serve as active species

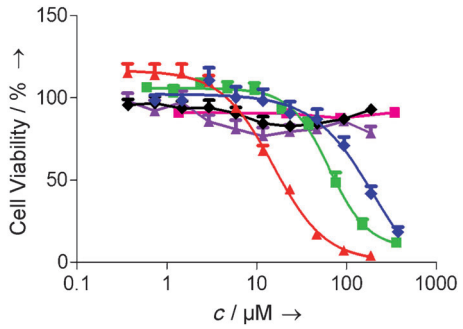


Figure 3. Dependence of OVCAR-1 cell viability on concentration TiL^2-(O*i*Pr), TiL^3, and the hydrolysis product $Ti_2L^2_2(\mu_2\text{-O})$ administered in a nanoformulated (green: TiL^2(O*i*Pr), red: TiL^3, blue: $Ti_2L^2_2(\mu_2\text{-O})$) and non-nanoformulated (pink: TiL^2(O*i*Pr), purple: TiL^3, black: $Ti_2L^2_2(\mu_2\text{-O})$) manner following an incubation period of three days (concentration of polynuclear compounds was determined per entire cluster).

inside the cell. This conclusion is enabled by the particular advantage of the salan–Ti^{IV} system, which, in addition to being highly cytotoxic, gives defined hydrolysis products over long periods that may be more easily monitored for mechanistic evaluation. Formulating the hydrolysis products into nanoparticles enabled overcoming limitations regarding solubility and cellular penetration of these bulky compounds, which apparently had inhibited their activity when administered directly. Furthermore, the results presented herein attest to the ability of biologically friendly Ti^{IV} to form complexes that are stable for weeks in an aqueous environment and are highly cytotoxic. It is now well-established that, unlike for platinum compounds, ligand lability is not a prerequisite for cytotoxicity of Ti^{IV} complexes,[6] which serves as a particular advantage in this case because of the rich aquatic chemistry of Ti^{IV} compounds. Thus, stable Ti^{IV} complexes are certainly attainable and may lead to high activity in a controlled manner without the accompanying release of undesired products. For this reason, administering the stable active species directly is advantageous over receiving it through a hydrolysis step. Future design of Ti^{IV}-based antitumor drugs should probably focus on the isolation of small, water-soluble, coordinatively saturated complexes, with the fewest polydentate ligands possible, with an advantage for a single hexadentate strongly bound ligand. The development of such systems is underway in our group, as well as the mechanistic investigation of possible biological targets for these bulky and inert compounds.

Received: July 26, 2012
Published online: September 7, 2012

Keywords: antitumor agents · *cis*-platin · ligand design · nanoformulation · titanium

[1] K. M. Buettner, A. M. Valentine, *Chem. Rev.* **2012**, *112*, 1863–1881.
[2] a) P. M. Abeysinghe, M. M. Harding, *Dalton Trans.* **2007**, 3474–3482; b) F. Caruso, L. Massa, A. Gindulyte, C. Pettinari, F. Marchetti, R. Pettinari, M. Ricciutelli, J. Costamagna, J. C. Canales, J. Tanski, M. Rossi, *Eur. J. Inorg. Chem.* **2003**, 3221–3232; c) F. Caruso, M. Rossi, *Mini-Rev. Med. Chem.* **2004**, *4*, 49–60; d) F. Caruso, M. Rossi, C. Pettinari, *Expert Opin. Ther. Pat.* **2001**, *11*, 969–979; e) C. V. Christodoulou, A. G. Eliopoulos, L. S. Young, L. Hodgkins, D. R. Ferry, D. J. Kerr, *Br. J. Cancer* **1998**, *77*, 2088–2097; f) G. Kelter, N. J. Sweeney, K. Strohfeldt, H.-H. Fiebig, M. Tacke, *Anti-Cancer Drugs* **2005**, *16*, 1091–1098; g) B. K. Keppler, C. Friesen, H. G. Moritz, H. Vongerichten, E. Vogel, *Struct. Bonding (Berlin)* **1991**, *78*, 97–127; h) P. Köpf-Maier, H. Köpf, *Chem. Rev.* **1987**, *87*, 1137–1152; i) P. Köpf-Maier, H. Köpf, *Struct. Bonding (Berlin)* **1988**, *70*, 103–185; j) E. Meléndez, *Crit. Rev. Oncol. Hematol.* **2002**, *42*, 309–315; k) K. Strohfeldt, M. Tacke, *Chem. Soc. Rev.* **2008**, *37*, 1174–1187; l) J. H. Toney, T. J. Marks, *J. Am. Chem. Soc.* **1985**, *107*, 947–953.
[3] a) C. M. Manna, G. Armony, E. Y. Tshuva, *Chem. Eur. J.* **2011**, *17*, 14094–14103; b) C. M. Manna, G. Armony, E. Y. Tshuva, *Inorg. Chem.* **2011**, *50*, 10284–10291; c) C. M. Manna, O. Braitbard, E. Weiss, J. Hochman, E. Y. Tshuva, *ChemMedChem* **2012**, *7*, 703–708; d) C. M. Manna, E. Y. Tshuva, *Dalton Trans.* **2010**, *39*, 1182–1184; e) S. Meker, C. M. Manna, D. Peri, E. Y. Tshuva, *Dalton Trans.* **2011**, *40*, 9802–9809; f) D. Peri, S. Meker, C. M. Manna, E. Y. Tshuva, *Inorg. Chem.* **2011**, *50*, 1030–1038; g) D. Peri, S. Meker, M. Shavit, E. Y. Tshuva, *Chem. Eur. J.* **2009**, *15*, 2403–2415; h) M. Shavit, D. Peri, C. M. Manna, J. S. Alexander, E. Y. Tshuva, *J. Am. Chem. Soc.* **2007**, *129*, 12098–12099; i) E. Y. Tshuva, J. A. Ashenhurst, *Eur. J. Inorg. Chem.* **2009**, 2203–2218; j) E. Y. Tshuva, D. Peri, *Coord. Chem. Rev.* **2009**, *253*, 2098–2115; k) H. Glasner, E. Y. Tshuva, *J. Am. Chem. Soc.* **2011**, *133*, 16812–16814.
[4] a) A. D. Tinoco, H. R. Thomas, C. D. Incarvito, A. Saghatelian, A. M. Valentine, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 5016–5021; b) T. A. Immel, M. Debiak, U. Groth, A. Burkle, T. Huhn, *ChemMedChem* **2009**, *4*, 738–741; c) T. A. Immel, U. Groth, T. Huhn, *Chem. Eur. J.* **2010**, *16*, 2775–2789; d) T. A. Immel, M. Grutzke, A.-K. Spate, U. Groth, P. Ohlschlager, T. Huhn, *Chem. Commun.* **2012**, *48*, 5790–5792.
[5] D. Peri, PhD Thesis, The Hebrew University of Jerusalem (Israel), **2011**.
[6] a) M. Shavit, D. Peri, A. Melman, E. Y. Tshuva, *J. Biol. Inorg. Chem.* **2007**, *12*, 825–830; b) F. Caruso, M. Rossi, C. Opazo, C. Pettinari, *Bioinorg. Chem. Appl.* **2005**, *3*, 317–329; c) M. Ravera, C. Cassino, E. Monti, M. Gariboldi, D. Osella, *J. Inorg. Biochem.* **2005**, *99*, 2264–2269.
[7] a) A. A. Noyes, W. R. Whitney, *J. Am. Chem. Soc.* **1987**, *109*, 930–934; b) L. F. Knapp, *Trans. Faraday Soc.* **1922**, *17*, 457–465; c) A. P. Simonelli, S. C. Mehta, W. I. Higuchi, *J. Pharm. Sci.* **1970**, *59*, 633–638.
[8] a) K. Margulis-Goshen, H. D. Netivi, D. T. Major, M. Gradzielski, U. Raviv, S. Magdassi, *J. Colloid Interface Sci.* **2010**, *342*, 283–292; b) K. Margulis-Goshen, B. F. B. Silva, E. F. Marques, S. Magdassi, *Soft Matter* **2011**, *7*, 9359–9365; c) K. Margulis-Goshen, E. Kesselman, D. Danino, S. Magdassi, *Int. J. Pharm.* **2010**, *393*, 231–238; d) K. Margulis-Goshen, S. Magdassi, *Nanomedicine* **2009**, *5*, 274–281.
[9] T. A. Immel, M. Grutzke, E. Batrogg, U. Groth, T. Huhn, *J. Inorg. Biochem.* **2012**, *106*, 68–75.
[10] F. Caruso, M. Rossi, J. Tanski, R. Sartori, R. Sariego, S. Moya, S. Diez, E. Navarrete, A. Cingolani, F. Marchetti, C. Pettinari, *J. Med. Chem.* **2000**, *43*, 3665–3670.
[11] C. S. Higham, D. P. Dowling, J. L. Shaw, A. Cetin, C. J. Ziegler, J. A. Farrell, *Tetrahedron Lett.* **2006**, *47*, 4419–4423.
[12] CCDC 893691 (TiL^3) and 893692 ($Ti_2L^2_2(\mu_2\text{-O})$) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

