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Synthesis, Structure, and Antitumor Activity of a Novel Tetranuclear Titanium Complex

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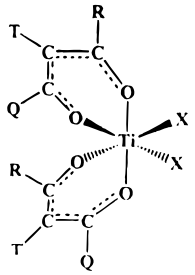
The coordination complex *cyclo*-tetrakis[bis(1-phenyl-3-methyl-4-benzoylpyrazolon-5-ato)μ-oxotitanium(IV)] has been synthesized and characterized with IR and NMR spectroscopies and X-ray diffraction. The core of this species consists of an eight-membered Ti-μ-oxo ring with alternate short−long Ti−O bond lengths. Besides these two O ligands, each metal is bound octahedrally to four O atoms from two chelating 1-phenyl-3-methyl-4-benzoylpyrazolon-5-ato anions. Several sets of Ti−O bond lengths are present: the shortest are the two Ti−O(oxo) (which are *cis* to each other), the longest are the two Ti−O(acyl) (*cis* to each other), and the two Ti−O(pyrazolonato) (*trans* to each other) are intermediate. The β-diketonate ligand asymmetry, a feature considered essential in other antitumor Ti compounds, induces the short−long Ti−O(oxo) sequence of bond lengths. The antitumor activity of this compound, encapsulated in a dipalmitoylphosphatidylcholine liposome, has been studied in vitro using TA-3 (mouse mammary adenocarcinoma), HEP-2 (human epithelial larynx carcinoma), and VERO (African green monkey kidney) cell lines and in vivo in CF-1 and AJ female mice ip inoculated with TA-3. In vitro cytotoxicity is greater for TA-3 than for HEP-2 and null for VERO cell lines. In vivo results show a marked increase in survival time (T/C = 293% for AJ and 208% for CF-1), whereas tumor weight decrease was observed for CF-1-treated mice. These results suggest the Ti complex−liposome system may be promising as an antitumor drug.

Introduction

After the serendipitous discovery of the antitumor properties of *cis*-diaminedichloroplatinum(II), cisplatin,[1] and the leading role of this drug in cancer chemotherapy, research in this field produced other useful Pt drugs such as carboplatin.[2] A growing interest in other metal complexes was then developed, with those of Ti, Au, Cu, Sn, Rh etc., being tested for activity.[3] Keppler et al. succeeded in finding the first inorganic (non-Pt) compound that reached clinical trials, budotitane[4] = $(bzac)_2Ti(OC_2H_5)_2$, where bzac = benzoylacetonate. It is the most active of a family of $(a\text{-}dik)_2TiX_2$ species, where a-dik = an asymmetric classical β-diketonate (an acetylacetonate derivative) anion and X = a leaving group such as halide or alkoxide. The ligand asymmetry is considered an essential condition for antitumor activity.

The two *cis* leaving groups resemble a structural feature similar to that found in active Pt species; however, the biological application of budotitane and related compounds (colon−rectal tumor) is different from that of Pt derivatives (testes, ovarian, neck, and lung tumors). Another Ti species currently undergoing clinical trials is $[Ti(Cp)_2Cl_2]$, where Cp = cyclopentadienyl,[3] which shares the *cis* leaving group feature with budotitane as well.



X = OMe, OEt, O^{n}Bu, O^{i}Pr, Cl, Br or I
T = Me or H
R,Q= Me, Ph, ^{t}Bu or H

Recently the first specific Ti^{IV}−protein complex (a transferrin derivative) was considered relevant to understanding the mechanism of action of anticancer Ti−Cp species.[5] However, that of $(a\text{-}dik)_2TiX_2$ complexes is not clear, and in an effort to clarify this, we are investigating Ti complexes of some alternative β-diketonate ligands.

We have previously characterized several Sn potential antitumor complexes of such ligands and found a large variety of coordination properties permitting structural variations.[6] These ligands allow metal coordination through a large range of bite angles. In addition, the

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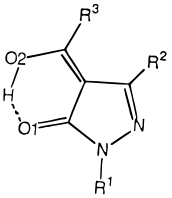

six-membered ring chelate framework containing the metal also shows variable electron delocalization. These characteristics make them useful to establish a possible structure−activity relationship (SAR).

Keppler reports that anhydrous media are needed in $(bzac)_2TiX_2$ synthesis.[4] One of the aims of our program is to find less severe conditions of synthesis, so that a more manageable drug formulation would be allowed. During our work we synthesized and characterized the title compound, which is the first $(a\text{-}dik)_2TiX_2$ species to show a tetrameric structure. Such an oligomer was not detected by Keppler in his studies, because when they performed vapor pressure osmometry in benzene, only a mixture of dimers and trimers was found.[4] In those experiments, the crystalline quality was not good enough to allow diffraction studies and confirm the nature of the polymerization. The title compound is then representative of this hydrolytic reaction process, leading to polymeric species.

Many potential antitumor compounds have low solubility in a physiological medium, reducing their utility. Nevertheless, this problem has been solved in many cases by associating drugs with soluble polymers[7] or by encapsulating them into liposomes,[8] which are a promising, broadly applicable, and highly researched drug delivery system. Liposomes are microscopic and submicroscopic vesicles ranging in size from 20 μm to 10 nm. They are composed of one or several lipid bilayers enclosing aqueous compartments. When phospholipids are hydrated, they spontaneously form lipid spheres enclosing the aqueous mediun and the solute.[9] The particular nature of liposomes causes them to be distributed within the body in a pattern significantly different from that of free drugs.[10] Temperature-sensitive release can be engineered by the selection of pure lipids that undergo sharp transition temperatures to adjust the transition temperature of the liposome−complex adduct to the desired point.[11] Since [bis(1-phenyl-3-methyl-4-benzoylpyrazolon-5-ato)Ti-μ-O]$_4$ is insoluble in physiological medium, we tested its antitumor activity in the dipalmitoylphosphatidylcholine (DPPC) liposome (T_m = 41 °C).

Results and Discussion

The oligomer complex *cyclo*-tetrakis[bis(1-phenyl-3-methyl-4-benzoylpyrazolon-5-ato)μ-oxotitanium(IV)] was obtained after fast hydrolysis of its nonisolable mononuclear species, [bis(1-phenyl-3-methyl-4-benzoylpyrazolon-5-ato)dichlorotitanium(IV)].

The IR spectrum of the tetranuclear compound in Nujol mull (range 4000−100 cm^{-1}) contains bands (in the chelating carbonyl stretching region of 1600−1300 cm^{-1}) of frequencies similar to those of other metal−pyrazolonate derivatives.[6b] In addition, no bands were observed in the 1600−1750 cm^{-1} region where ketonic carbonyl modes are expected for the pyrazolone keto form.[12] Therefore both carbonyls are coordinated to the Ti atom. Broad bands in the 500−400 cm^{-1} region and some strong absorptions in the 400−300 cm^{-1} region are associated with Ti−O bonds. IR spectra in Nujol mull and chloroform are similar suggesting that the tetranuclear species is stable in chlorohydrocarbon solution. NMR data in solution agree with the molecular structure described below.

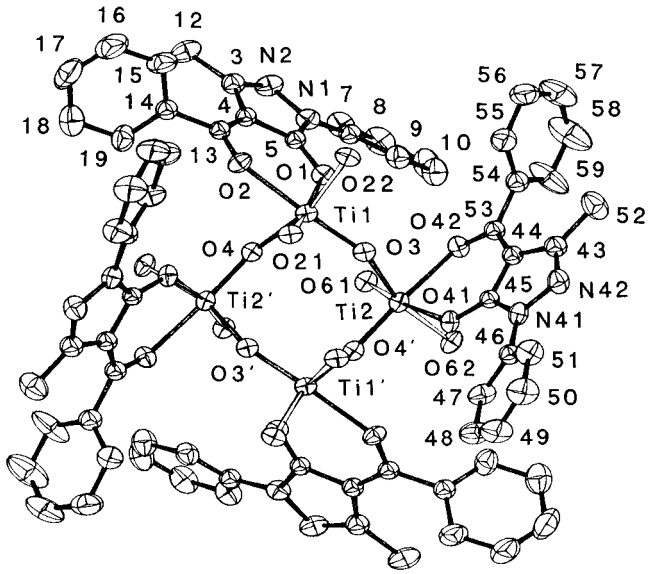

Figure 1. Molecular structure of the Ti complex obtained with X-ray diffraction. Each Ti atom is bound to two pyrazolonato ligands, although only one is shown completely for clarity. The second pyrazolonato is shown bound with two oxygens with unfilled bonds.

Table 1. Selected Structural Data

Bond Lengths (Å, with SDs in parentheses)			
Ti1−O1	1.979(4)	Ti2−O3	1.859(6)
Ti1−O2	2.146(7)	Ti2−O41	1.983(5)
Ti1−O3	1.767(6)	Ti2−O42	2.163(5)
Ti1−O4	1.868(4)	Ti2−O61	1.978(5)
Ti1−O21	1.980(4)	Ti2−O62	2.076(7)
Ti1−O22	2.056(5)	Ti2−O4′	1.758(5)
Bond Angles (deg, with SDs in parentheses)			
O22−Ti1−O4	163.0(3)	O42−Ti2−O4′	176.1(3)
O3−Ti1−O2	176.5(2)	Ti1−O3−Ti2	150.4(3)
O21−Ti1−O1	164.8(2)	Ti1′−O3′−Ti2′	150.4(3)
O61−Ti2−O41	164.7(2)	Ti1−O4−Ti2′	153.8(3)
O62−Ti2−O3	163.3(2)	Ti1′−O4′−Ti2	153.8(3)

Diffraction Study. The title compound has a crystal structure made up of discrete and well-separated molecules containing crystallographic symmetry. The asymmetric crystallographic unit is half a molecule; the other half is related by a 2-fold axis. The tetranuclear species contains an eight-membered ring formed by an alternating sequence of Ti−O atoms. Each metal is surrounded by six O atoms in an octahedral arrangement: two O are oxo atoms that bridge two other Ti neighbors, and the remaining four atoms belong to two pyrazolonates. The molecular structure is depicted in Figure 1 and relevant geometrical parameters are listed in Table 1.

Trans angles differ markedly from an ideal value of 180° and show Ti octahedron distortion. This is probably due to low values of O(pyrazolonato)−Ti−O(acyl) bite angles.

Recently, the structure of a tetranuclear Ti complex similar to the title compound was determined by dif-

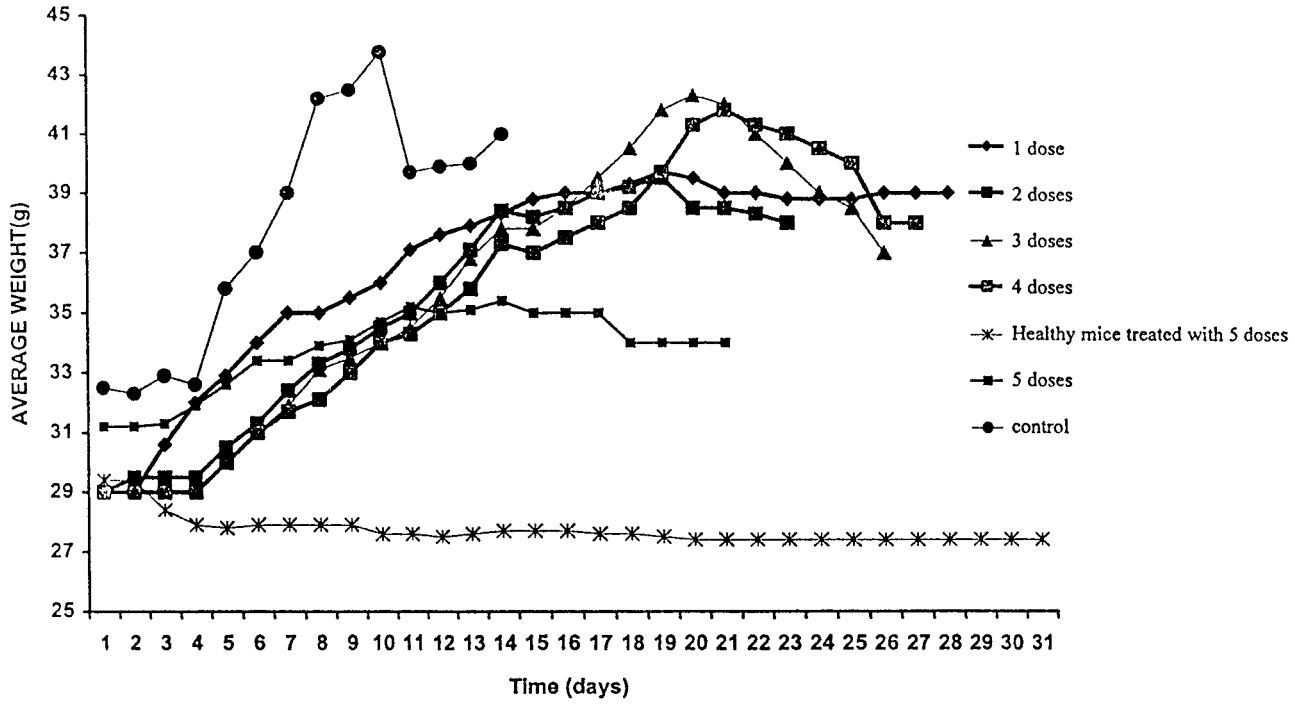

Figure 2. Mean weight of CF-1 mice treated with Ti complex−liposome. Errors are about 1% and not included. One ip inoculated dose equals 4.1 mg of Ti complex/kg of animal body weight. The last point on each curve, for mice with tumors, indicates survival time of the last mouse.

fraction methods. This complex,[13] *cyclo*-tetrakis[μ-oxo-bis(2,2,6,6-tetramethylheptane-3,5-dionate)titanium-(IV)], contains a symmetric β-diketonate anion and shows three sets of Ti−O bonds:

(a) the shortest bonds belong to the tetranuclear cyclic unit (Ti−μ-O bonds), about 1.81 Å; (b) the longest bonds (about 2.12 Å) are *trans* to the shortest bonds forming set a; and (c) the intermediate bonds are *cis* to the bonds forming sets a and b and are themselves *trans* to each other (about 1.97 Å).

The main structural difference between this species and our title compound resides in the further splitting of sets a and b in our species. Thus, in our set a (the Ti−μ-O bonds) an alternate sequence of short−long bonds is found: Ti1−O3 = 1.767(6) Å; O3−Ti2 = 1.859-(6) Å; Ti2−O4′ = 1.758(5) Å; O4′−Ti1′ = 1.868(4) Å. Table 1 shows that *trans* to each Ti−μ-O bond appears an O(acyl) ligand forming a weak (or secondary) Ti−O bond. These Ti−O(acyl) bonds are also longer than the Ti−O(pyrazolonato) bonds. Since Sn−O(pyrazolonato) bonds are covalent and Sn−O(acyl) bonds are weak in (4-acyl-5-pyrazolonate)$_2$SnR$_2$ complexes,[6] an O(acyl) *trans* to a strong (short) bond, as is the Ti−μ-O, can be expected. The sequence of short−long bonds in the tetranuclear moiety is associated with a long−short sequence opposite Ti−O(acyl) secondary bonds due to a dominant *trans* effect. In the title compound, Ti−O(pyrazolonato) bonds comprise set c. Since these Ti−O bonds are *trans* to each other there is no dominant *trans* effect and their bond lengths are the same as those of set c in the above-mentioned symmetric classical β-diketonate species.[13]

Keppler has shown that the mononuclear Ti species, after hydrolysis, loses the *cis* leaving groups (alkoxide or halide), followed by loss of the diketonates and leaving, in the final stage, the nontoxic TiO_2.[4] Instead, our tetranuclear compound is very stable, and its molecular structure shows that after the monomer undergoes hydrolysis and loss of the Cl *cis* leaving groups; such *cis* positions are then occupied by the O (oxo) atoms, therefore retaining the Ti-β-diketonate moiety configuration in the tetramer. SAR studies by Keppler[4] determined that an asymmetric β-diketonate is essential for antitumor activity. 4-Acyl-5-pyrazolones are intrinsically asymmetric, and this study describes the antitumor activity of the title Ti derivative.

Biological Analysis. Both DPPC and the titanium complex were dissolved in chloroform and dried under nitrogen to obtain the liposome film. The encapsulated complex shows a T_m around 20 °C. This temperature is lower than that of the pure DPPC liposome (41 °C). At temperatures higher than 20 °C the Ti complex is more strongly bound inside the liposome. The absorbance measured at different temperatures was lower than that of the free complex. This drug release pattern therefore indicates that it is almost completely cross-linked. Liposomes take advantage of the fact that the vasculature in tumors is such that the permeability of macromolecules circulating in blood vessels is vastly increased.[14] Therefore, the primary effect of the liposome is not the liberation of the Ti complex inside the animal body but simply to increase the complex permeability to the tumor cell.

Figures 2 and 3 show that mice weight of healthy animals was not affected by the liposome−Ti complex adduct. After treatment, the weight increase normally observed in mice with tumors (see Experimental Section for tumor cell line description) was drastically reduced in CF-1 mice and their life span was increased. AJ mice increased their survival time as well, but tumor size was not decreased. Table 2 and Figure 4 show that CF-1 has almost equivalent T/C values (about 200%) for 1−4

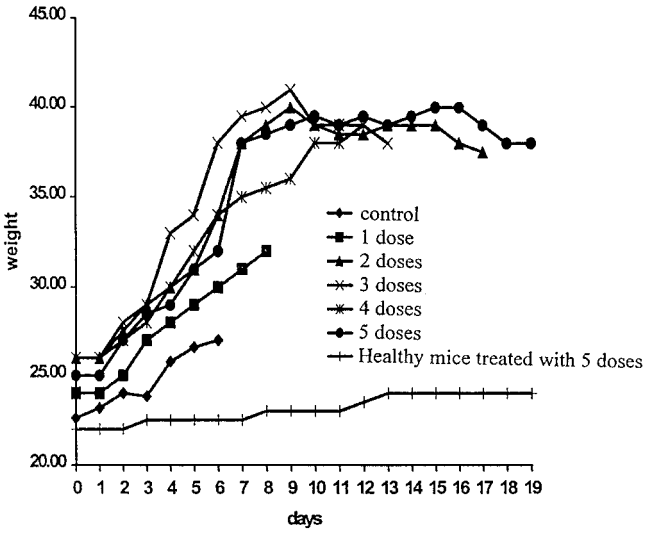

Figure 3. Mean weight of AJ mice treated with Ti complex−liposome. Errors are about 1% and not included. One ip inoculated dose equals 6.2 mg of Ti complex/kg of animal body weight. The end of each curve, for mice with tumors, indicates survival time of the last mouse.

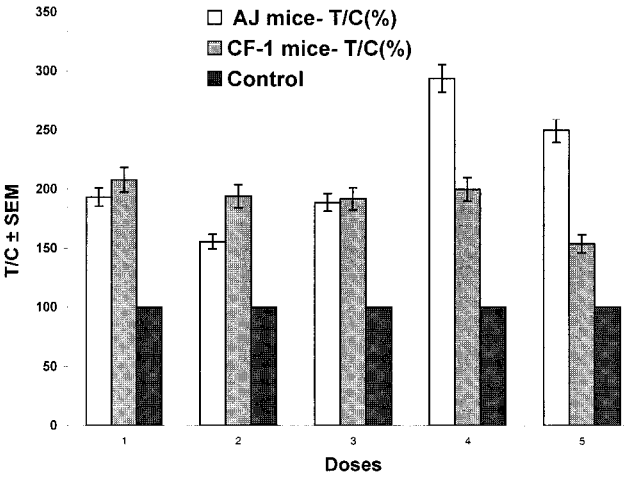

Figure 4. In vivo biological activity of Ti complex−liposome.

doses of treatment (each dose of 0.1 mL of Ti complex/liposome/0.9% NaCl corresponds to 4.1 mg of Ti complex/kg of animal body weight). In contrast, in AJ mice 4 doses of treatment is clearly better. Since 5 doses of treatment reduce antitumor activity for both types of mice, the recommended dosage appears to be 4 (each dose of 0.1 mL of Ti complex/liposome/0.9% NaCl corresponds to 6.2 mg of Ti complex/kg of animal body weight).

Figure 5 shows in vitro results of the Ti complex−DPPC adduct. Cytotoxicity increases with increasing adduct concentration more dramatically for TA-3 tumor cells than for HEP-2 cells. At a Ti complex concentration of 500 μM, all TA-3 cells were killed and its IC_{50} is 90 μM. No cytotoxic effect was observed against the VERO line (HEP-2 and VERO cell lines are described in the Experimental Section).

Conclusions

This work reports the synthesis of a tetranuclear Ti-μ-O-bis(4-acyl-5-pyrazolonate) compound obtained after formation of the corresponding mononuclear species, which could not be isolated because of its fast hydrolysis in the reaction mixture. During this process the halide *cis* leaving groups are replaced by O(oxo) with formation of an eight-membered ring. This tetranuclear compound retains the Ti-bis(β-diketonate) moiety configuration. In this intrinsically asymmetric β-diketonate species, the Ti−O(oxo) bonds are the strongest (shortest) and are arranged as alternate short−long lengths. These Ti−O(oxo) bonds form the core of the complex and are *trans* to the Ti−O(acyl) bonds, which are the weakest (longest) in the molecule. *Trans* effects are responsible for the several sets of Ti−O bond lengths.

This very stable tetranuclear species is insoluble in physiological medium. Therefore, its antitumor activity was analyzed as a DPPC liposome adduct. This study was performed in vitro against three tumor cell lines (TA-3, HEP-2, and VERO), with the complex more cytotoxic against TA-3 than HEP-2 and inactive against VERO cell lines, and in vivo in AJ and CF-1 mice inoculated with TA-3 cells that resulted in increased survival times between 200% and 300%. The asymmetric nature of the 4-acyl-5-pyrazolonato ligand confirms previous trends of cytotoxic activity shown by budotitane (a drug currently in clinical trials):[15] ligand asymmetry is essential for antitumor activity.[4] The novel Ti−μ-O bond splitting is induced by the ligand asymmetry.

The very low toxicity and effective antitumor activity of this Ti complex make it attractive for further studies by exploring related complexes and increased doses.

Table 2. In Vivo Biological Activity of Ti Complex−Liposome Adduct: Life Span Values of Average Treated/Untreated (control) Mice

	AJ mice			CF-1 mice			control
doses	days	SEM	T/C (%)	days	SEM	T/C (%)	T/C (%)
1	11.6/6	1.93 (0.10)	193	27.0/13	2.08 (0.11)	208	100
2	9.3/6	1.55 (0.07)	155	25.2/13	1.94 (0.10)	194	100
3	11.3/6	1.88 (0.08)	188	25.0/13	1.92 (0.12)	192	100
4	17.6/6	2.93 (0.10)	293	26.0/13	2.00 (0.08)	200	100
5	15.0/6	2.54 (0.08)	254	20.0/13	1.54 (0.10)	154	100

Experimental Section

Synthesis and Spectroscopic Characterization of *cyclo*-Tetrakis[bis(1-phenyl-3-methyl-4-benzoylpyrazol-5-onato)-μ-oxotitanium(IV)]. All reagents and solvents were obtained from commercial sources and were used without further purification. The proligand 1-phenyl-3-methyl-4-benzoylpyrazol-5-one was prepared according to the literature.[16] The sample for microanalysis was dried in vacuum to constant weight (20 °C, about 0.1 Torr). Elemental analyses (C,H,N) were performed in-house with a Fisons Instruments 1108 CHNS-O elemental analyzer. IR spectra were recorded from 4000 to 100 cm^{-1} with a Perkin-Elmer System 2000 FT-IR instrument. ^1H NMR spectra were recorded on a VXR-300 Varian spectrometer operating at room temperature (300 MHz). Melting point was determined on an IA 8100 Electrothermal instrument.

cyclo-Tetrakis[bis(1-phenyl-3-methyl-4-benzoylpyrazol-5-onato)-μ-oxotitanium(IV)] was prepared by reaction of 1 mmol of $TiCl_4$ with 2 mmol of 1-phenyl-3-methyl-4-benzoylpyrazol-5-one in refluxing ethanol or CH_2Cl_2. Recrystallized from ethyl acetate/*n*-hexane under aerobic conditions (85% yield based on Ti): mp 195 °C dec; ^1H NMR ($CDCl_3$, 300 MHz, 293 K) δ 1.3m, 1.4m, 1.6m, 1.7m (br) (6H, 3-CH_3), 6.2br, 6.5br, 6.7br,

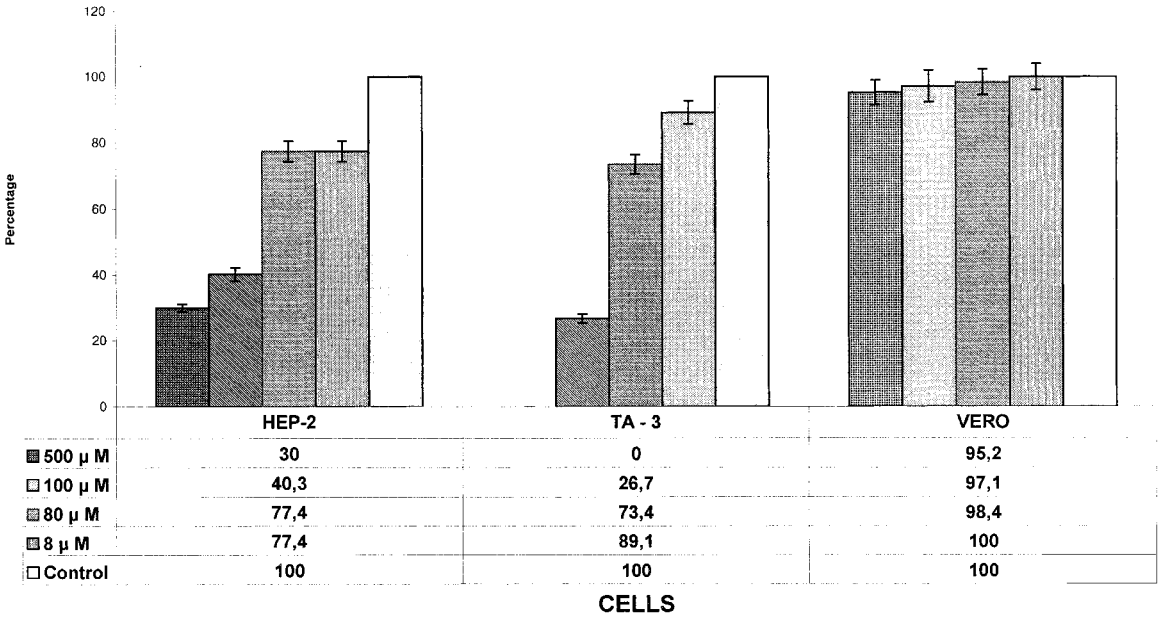

Figure 5. In vitro percentage of live cells after treatment with Ti complex−liposome.

7.0br, 7.5br, 8.0br (20H, C_6H_5); IR (Nujol mull, cm^{-1}) 1606s, 1580s, 1568s, 1531s (C···O, C···C and C···N), 554m, 518m, 504m, 460s, 448vs, 410sh, 398s, 357s (Ti−O). Anal. ($C_{34}H_{26}N_4O_5$-Ti) C, 66.03; H, 4.24; N, 9.06.

X-ray Diffraction. A Siemens $P2_1$ diffractometer was used for the measurements of the cell constants and for the data collection. A set of 23 reflections with high θ-angle was used to obtain refined cell parameters. Monitoring of six reflections, taken every 100 reflections, indicated no decay. Negligible absorption anisotropy was measured with a φ-scan reflection. This correction was applied along with that for Lorentz and polarization effects as described.[17] The molecular structure was solved using direct methods running SHELX86 program.[18] Additional calculations were done using the CAOS system[19] as follows: refinement based on the minimization of the function $\Sigma w(|F_o| - |F_c|)^2$ with the weighting scheme $w = 1/(a + F_o + cF_o^2)$, where a and c are of the order of $2F_o$(min) and $2/F_o$(max),[20] respectively. After refinement convergence H atoms were introduced at fixed positions with a C−H distance of 0.96 Å and H isotropic displacement parameters were kept fixed until final refinement convergence was reached. Atomic scattering factors and anomalous dispersion terms were taken from the literature.[21]

Liposome Formation and Characterization. DPPC was purchased from Sigma and used without further purification. 1 mL of a solution of DPPC in chloroform (2.2×10^{-3} M) was added to 1 mL of solution of the titanium complex in chloroform (1.3×10^{-2} M). A lipid film was prepared by evaporating the organic solvent under nitrogen. The dried lipids were suspended in 25 mL of physiological 0.9% NaCl serum (520 μM) and vortexed for 15 min at room temperature. The cloudy suspension was sonicated and then centrifuged in a Eppendorf equipment. The formation of the Ti complex−DPPC liposome was controlled by UV−vis spectroscopy.

Antitumor Activity. The cytotoxicity of the encapsulated titanium complex in the DPPC liposome was studied using CF-1 (30−35 g) and AJ (20−25 g) female mice, 5−8 weeks old, by treatment with 0.1 mL of the encapsulated complex in NaCl (0.9%), with concentrations ranging from 10^{-4} to 10^{-2} M. All the mice survived these treatments and so it was not possible to obtain IC_{50}.

TA-3 (mouse mammary adenocarcinoma) ascitic[22] tumor cells were locally maintained in female mice 6−8 weeks old, weighing 30 ± 5 g by serial weekly passage in CF-1 mice and 20 ± 5 g by serial weekly passage in AJ mice.[23] TA-3 cells were ip inoculated and mice abdomen increase was observed.[24−25]

The complex−liposome adduct, 1.5×10^{-2} M (in Ti complex), was injected ip 3 days after implantation of 10^6 tumor cells. Five groups of mice (5−7 animals each) were treated, injecting with 0.1 mL of the Ti complex solution every day for 1−5 consecutive days, depending on the group (only one injection in the first group, two in the second, three in the third, etc.). Tumor increase was measured by total mice weight increase.

In vitro study of the Ti complex in the DPPC liposome was carried out in 0.9% NaCl against TA-3, HEP-2 (human epithelial larynx carcinoma) and VERO (African green monkey kidney) cell lines[26] using the trypan blue exclusion technique. Cells were grown in a medium composed of L-15 supplemented with 5% fetal calf serum, 200 UI/mL sodium penicillin G and 0.04 mg/mL gentamicin at 37 °C and 100% humidity.[27] The sensitivity of a cell line to the Ti complex was assessed as follows: cells were cultured in monolayers in the described medium, Ti complex/DPPC/0.9% NaCl (concentration range 1−100 μM) was added and cells were incubated for 72 h at 37 °C. Assays were performed 5 times. The monolayer was observed under a microscope (Ernst Leitz Wetzear optical microscope) in order to analyze the state of the cells, treated with the Ti complex and without (control cells). The viability of cells was determined by washing with PBS (pH = 7.2) and adding 1 mL of trypsin solution (1%)/vercene (0.05 g/mL) and, after about 60 s, 0.5 mL of PBS and 0.5 mL trypan blue (0.4%). The live cells were counted in a Neubauer chamber. The percentage of live cells/mL was determined by means of the following formula:[28−29] % treated viable cells/mL = (no. of treated viable cells/mL)/(no. of control viable cells/mL) × 100.

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Supporting Information Available: Tables containing a summary of crystal data and refinement details, atomic coordinates, anisotropic displacement parameters, and full list of structural data. This material is available free of charge via the Internet at http://pubs.acs.org.

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