

Apoptosis-inducing Vanadocene Compounds against Human Testicular Cancer

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ABSTRACT

We systematically assessed the cytotoxic effects of five metallocene dichlorides containing vanadium (vanadocene dichloride), titanium (titanocene dichloride), zirconium (zircodocene dichloride), molybdenum (molybdocene dichloride), and hafnium (hafnocene dichloride) as the central metal atom and 19 other vanadocene complexes. These compounds were tested against the human testicular cancer cell lines Tera-2 and Ntera-2 using both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and apoptosis assays. Notably, only the vanadium(IV)-containing metallocenes exhibited significant cytotoxicity against Tera-2 and Ntera-2 cells and induced apoptosis within 24 h. Vanadocenes with dithiocyanate [$\text{VCp}_2(\text{SCN})_2 \cdot 0.5 \text{H}_2\text{O}$] and diselenocyanate [$\text{VCp}_2(\text{NCSe})_2$] as ancillary ligands were identified as the most potent cytotoxic compounds. Vanadocenes, especially the lead compound $\text{VCp}_2(\text{NCSe})_2$, may be useful in the treatment of testicular cancer.

INTRODUCTION

Testicular cancer is the most common nonhematological malignancy among young men in the 20–40-year age group with an estimated 7100 new cases diagnosed each year (1, 2). The cytotoxic antitumor drug cisplatin has revolutionized the treatment of testicular cancer (3). Contemporary platinum-based combination chemotherapy has an overall cure rate of >90% (4). Unfortunately, such regimens also damage the normal germinal epithelium, leading to infertility or subfertility in young men (5, 6). Therefore, new agents that are less toxic to germinal epithelium are urgently needed. Among the metal complexes that exhibit striking similarities to cisplatin are the transition metal-containing bis(cyclopentadienyl) complexes (7, 8). Metallocene diacido complexes containing transition metals, such as

titanium, vanadium, niobium, zirconium, and molybdenum, also exhibit variable antitumor activity for a wide spectrum of murine and human tumors with reduced toxicity when compared with cisplatin (9–13).

The disubstituted metallocene derivatives are known as “bent-sandwich” complexes, where bis-cyclopentadienyl moieties are positioned in a tetrahedral symmetry and in a bent conformation with respect to the central metal atom (8, 13, 14). These metallocenes containing transition metals in oxidation state IV, linked to organic ligands by direct carbon-metal bonds, exhibit antitumor properties both *in vivo* and *in vitro*; however, their mode of action differs from that of cisplatin (11, 13). Unlike cisplatin, which forms covalent DNA adducts that are potentially mutagenic (15), metallocenes inhibit DNA synthesis and are antimetabolic (10, 11, 16–18). Of these metallocenes, the neutral dihalo complexes, *e.g.*, TDC² and VDC, have emerged as promising alternatives to cisplatin (11, 13, 18–22). Because the interaction between the central metal atom and its coordinating ligands contributes to the redox potential altering ability of metallocenes (23, 24) as well as to their stability in aqueous solutions (7, 25–27), different ligands have been selected for lead optimization effects.

In a systematic effort aimed at identifying new cytotoxic agents with potent activity against testicular cancer cells, we examined the cytotoxic effects of 24 metallocenes on the human testicular cancer cell lines, Tera-2 and Ntera-2. The metallocene panel included several derivatives of Cp_2VX_2 vanadocene com-

² The abbreviations used are: TDC, titanocene dichloride, TiCp_2Cl_2 ; VDC, vanadocene dichloride, VCp_2Cl_2 ; HDC, hafnocene dichloride, HfCp_2Cl_2 ; MDC, molybdocene dichloride, MoCp_2Cl_2 ; ZDC, zircodocene dichloride, ZrCp_2Cl_2 ; VD(acac), vanadocene diacido acetylacetonato monotriflate, $\text{VCp}_2(\text{acac})(\text{O}_3\text{SCF}_3)$; VD(H), vanadocene diacido acethydroxamato monotriflate, $\text{VCp}_2(\text{H})(\text{O}_3\text{SCF}_3)$; VDPH, vanadocene diacido *N*-phenyl benzhydroxamato monotriflate, $\text{VCp}_2(\text{PH})(\text{O}_3\text{SCF}_3)$; VD(bpy), vanadocene diacido bipyridino ditriflate, $\text{VCp}_2(\text{bpy})(\text{O}_3\text{SCF}_3)_2$; VD(cat), vanadocene diacido catecholato, $\text{Cp}_2\text{V}(\text{cat})$; VD(dtc), vanadocene diacido dithiocarbamate, $\text{VCp}_2(\text{dtc})(\text{O}_3\text{SCF}_3)$; VMDC, monomethyl-substituted vanadocene dichloride, $\text{V}(\text{MeCp})_2\text{Cl}_2 \cdot 0.5 \text{H}_2\text{O}$; VPMDC, pentamethyl-substituted vanadocene derivative, $\text{V}(\text{Me}_5\text{Cp})_2\text{Cl}_2$; VPMOC, pentamethyl-substituted vanadocene derivative, $\text{V}(\text{Me}_5\text{Cp})\text{OCl}$; VDSe, vanadocene diselenocyanate; VDB, vanadocene dibromide, VCp_2Br_2 ; VDI, vanadocene diiodide, VCp_2I_2 ; VDA, vanadocene diazide, $\text{VCp}_2(\text{N}_3)_2$; VDCN, vanadocene dicyanide, $\text{VCp}_2(\text{N}_3)_2$; VDO, vanadocene dioxycyanide, $\text{VCp}_2(\text{NCO})_2$; VDOCN, vanadocene chlorooxycyanide, $\text{VCp}_2(\text{Cl})(\text{OCN})$; VDS, vanadocene dithiocyanate, $\text{VCp}_2(\text{SCN})_2 \cdot 0.5 \text{H}_2\text{O}$; VDFe, vanadocene monochloromonooxycyanide tetrachloroferrate, $\text{VCp}_2\text{Cl}(\text{CH}_3\text{CN})(\text{FeCl}_4)$; VDT, vanadocene ditriflate, $\text{VCp}_2(\text{O}_3\text{SCF}_3)_2$; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; m.p., melting point(s); Cp⁻, cyclopentadienyl anion; acac, acetylacetonate; bpy, 2,2'-bipyridine; cat, catecholato; dtc, diethyl dithio carbamate; PH, *N*-phenyl benzhydroxamic acid; H, acethydroxamic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TdT, terminal deoxynucleotidyltransferase; TUNEL, TdT-mediated dUTP nick-end labeling assay; PI, propidium iodide; CLSM, confocal laser scanning microscopy.

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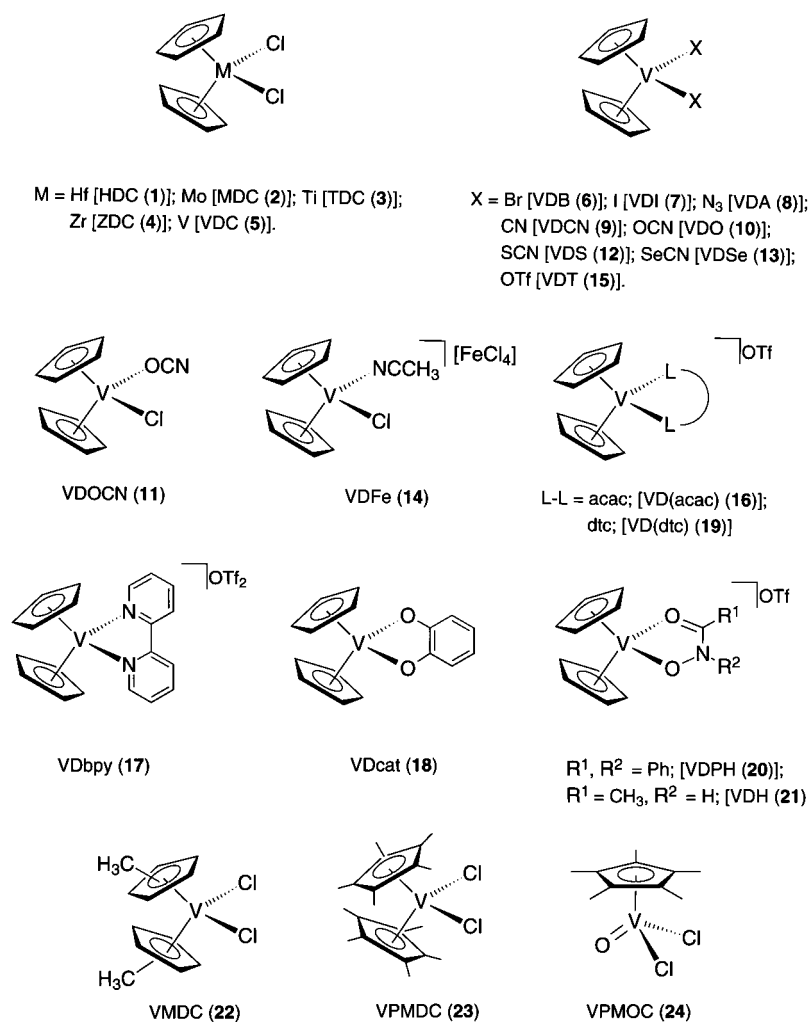


Fig. 1 Generalized structures of vanadium(IV) metallocene complexes.

plexes, where two *cis*-X ligations were achieved via different monodentate or bidentate ligands and X = Cl⁻, Br⁻, I⁻, N₃⁻, CN⁻, OCN⁻, SCN⁻, or SeCN⁻, as well as the Cp₂V(L-L')ⁿ⁺ series species, where L-L' = acac⁻, hexafluoroacetylacetonate⁻, cat⁻, dtc⁻, hydroxamic acids, or bpy-type bidentate ligands and n = 1 or 2. Specifically, we systematically assessed the effects of 20 different vanadocene complexes: 11 vanadocene diacido complexes, 6 chelated complexes [VD(acac), VDH, VDPH, VD(bpy), VD(cat), and VD(dtc)], 1 monomethyl-substituted VDC (VMDC), and 2 pentamethyl-substituted vanadocene derivatives (VPMDC and VPMOC) on the survival of Tera-2 and Ntera-2 cells. Four other metallocene complexes containing titanium, zirconium, molybdenum, and hafnium as the central metal atom (*i.e.*, TiCp₂Cl₂, ZrCp₂Cl₂, MoCp₂Cl₂, and HfCp₂Cl₂) were also tested for comparison. Our results presented herein provide unprecedented evidence that vanadocenes induce apoptosis in human testicular cancer cells. Vanadocenes, especially the lead compound VDSe, may be useful in the treatment of cancer.

MATERIALS AND METHODS

Chemistry

All of the metal tetrachlorides (TiCl₄, VCl₄, MoCl₄, HfCl₄, and ZrCl₄) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Other reagents used were of commercially available reagent grade quality. Unless otherwise stated, all solvents were used as received from Aldrich Sure Seal bottle (with <0.005% water). THF was dried by distillation over sodium. Dichloromethane, reagent grade, was purified by using the following procedure. It was stirred overnight with concentrated sulfuric acids, after which it was separated from acid layer, washed with saturated aqueous NaHCO₃, followed by aqueous KOH/KCl and distilled water, and then dried over anhydrous MgSO₄, the final stage being distilled from KOH (28). All of the solvents were deoxygenated by purging with argon, and reactions were carried out under an argon atmosphere by using standard Schlenk techniques.

The infrared spectral data were recorded on a FT-Nicolet model Protege 460. The solid samples were taken in a KBr

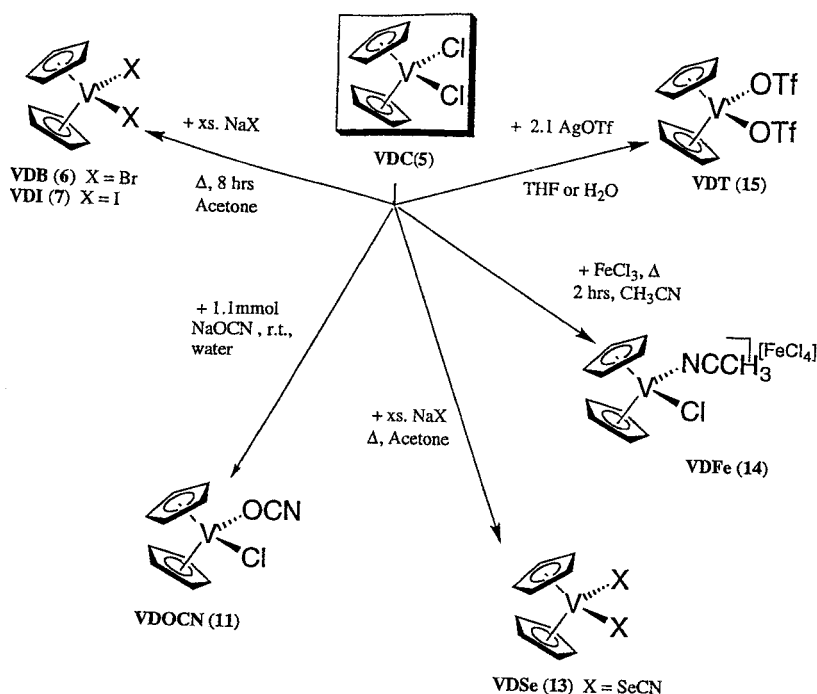


Fig. 2 Synthetic scheme 1.

pellet, and the frequencies were generally in the range of 4000–500 cm^{-1} . UV-visible spectra were recorded in a quartz cell or cuvette on a Beckman model DU 7400 spectrophotometer, and the ranges of the spectral bands were registered between 250 and 800 nm. NMR spectra were recorded in CDCl_3 or $\text{Me}_2\text{SO}-d_6$ on a Varian (300 MHz) NMR spectrometer. Chemical shifts were reported as the δ values downfield from an internal standard of Me_4Si . m.p. were determined with a Melt-Temp apparatus (Melt-Temp Laboratory Devices, Inc.) attached to a Fluke 51 K/J thermometer. All elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, Georgia), and the analytical results are supplied as supporting information. Unless otherwise stated, all operations were carried out at room temperature.

Synthesis of Compounds

The chemical structures of the 24 organometallic compounds [*i.e.*, bis-(cyclopentadienyl) ancillary coordinated metal complexes] analyzed in this study are depicted in Fig. 1. All metallocene dichloride complexes (type 1 series compounds), VCp_2Cl_2 , TiCp_2Cl_2 , ZrCp_2Cl_2 , and MoCp_2Cl_2 , were prepared by following literature procedures (29–31), and their purity was confirmed by ^1H NMR, infrared spectroscopy, and elemental analysis. HfCp_2Cl_2 was purchased from Aldrich Chemical Co. VCp_2Cl_2 was purified under partial vacuum by anaerobic Soxhlet extraction with CH_2Cl_2 at 44°C. TiCp_2Cl_2 was recrystallized from THF. The characterization data for type 1 compounds are given below:

Type 1 Series Compounds

HfCp_2Cl_2 (HDC, compound 1). Yield: 75%; m.p., 330–335°C.

MoCp_2Cl_2 (MDC, compound 2). Yield: 37%; m.p., 220°C.

TiCp_2Cl_2 (TDC, compound 3). Yield: 45%; m.p., 290°C (decomposes).

ZrCp_2Cl_2 (ZDC, compound 4). Yield: 78%; m.p., 240–245°C (decomposes).

VCp_2Cl_2 (VDC, compound 5). Yield: 55%; m.p., 248–255°C (decomposes).

Type 2 Series Compounds

Type 2 series compounds that were new and/or were in some cases modified procedures were used where described, as shown in Fig. 2:

VCp_2Br_2 (VDB, compound 6). This compound was synthesized with slight modification of the procedure reported in the literature (32). To a 20-ml acetone solution of VCp_2Cl_2 (0.2 g, 8 mmol), 0.7 g (80 mmol) of solid LiBr was added with stirring, and the reaction mixture was allowed to reflux for 4 h. The solvent was removed under vacuum and dried. The green product was extracted with 50 ml of boiling CHCl_3 , and the solution was saturated with dry HBr gas before it was left overnight at -20°C for crystallization. The bright green crystals were collected on a frit and washed with hexane and diethyl ether. Yield: 90%; m.p., decomposes and turns darker gradually at 250–350°C.

VCp_2I_2 (VDI, compound 7). To a 25-ml of anhydrous THF, 0.15 g (6 mmol) of VCp_2Cl_2 and 0.99 g (60 mmol) of potassium iodide were added. This reaction mixture was refluxed overnight under argon. The resulting dark red-brown solution was separated from the salts by filtration, and the solvent was evaporated under vacuum. The dark red residue was washed with dry hexane. The solid was dried under vacuum and

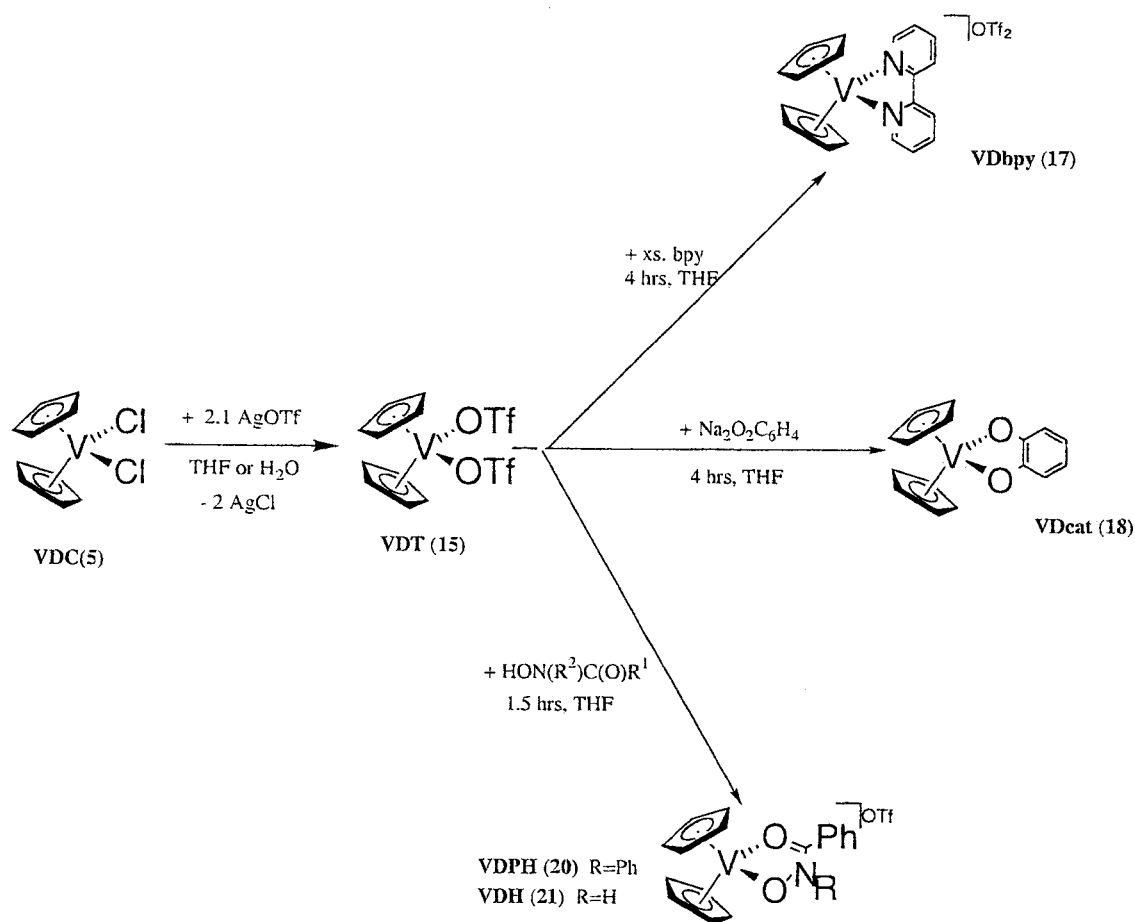


Fig. 3 Synthetic scheme 2.

stored under argon. This compound is extremely sensitive to moisture and readily decomposes in halogenated solvents, but it is stable in DMSO. Yield: 55%; m.p., could not be measured (compound gets sticky during handling in air).

VCp_2X_2 . The pseudo-halide derivatives with $\text{X} = \text{N}_3^-$ (VDA, compound 8), CN^- (VDCN, compound 9), OCN^- (VDO, compound 10), and SCN^- (VDS, compound 12) were prepared by following the literature methods described by Doyle and Tobias (32). The pure compounds were isolated by either recrystallization or Soxhlet extraction. The purity of these complexes was confirmed by elemental analysis, m.p. analysis, and UV-visible and infrared spectra analyses. The results are given below:

$\text{VCp}_2(\text{N}_3)_2$ (VDA, compound 8). Yield: 65%; m.p., sublimes at 173°C (decomposes).

$\text{VCp}_2(\text{CN})_2$ (VDCN, compound 9). Yield: 75%; m.p., sublimes at 173°C (decomposes).

$\text{VCp}_2(\text{NCO})_2$ (VDO, compound 10). Yield: 55%; m.p., 287°C (decomposes).

$\text{VCp}_2(\text{OCN})\text{Cl}$ (VDOCN, compound 11). Dark brown powder was isolated by following the procedure that was described for the titanium analogue (33).

$\text{VCp}_2(\text{SCN})_2 \cdot 0.5 \text{H}_2\text{O}$, (VDS, compound 12). Yield: 75%; m.p., the compound sublimes at 150°C (decomposes).

$\text{VCp}_2(\text{NCSe})_2$ (VDSe, compound 13). The corresponding diselenocyanate complex, $\text{VCp}_2(\text{NCSe})_2$, was isolated in the following manner. To a stirring solution of VCp_2Cl_2 , 0.4 g (1.6 mmol) in 25 ml of anhydrous acetone under argon and 0.85 g (8 mmol) of solid KNCSe was added. The reaction mixture was allowed to stir for 4 h at room temperature. The resulting red brown solution was subjected to rotatory vaporization, and the pure microcrystalline red compound was isolated from the crude product through Soxhlet extraction using dichloromethane as a solvent. Yield: 60%; m.p., the compound slowly turns black and decomposes at 250°C - 275°C .

VCp_2Cl (CH_3CN)(FeCl_4) (Vanadocene monochloroacetone nitrilotetra chloroferrate, compound 14). This compound was prepared essentially by following the procedure described for the corresponding titanium complex (34), except that a 1:1.1 stoichiometric molar ratio of VCp_2Cl_2 :anhydrous FeCl_3 solution was used in an acetonitrile solution. A dark green precipitate was isolated from the solution after standing overnight at -20°C . Yield: 90%.

$\text{VCp}_2(\text{O}_3\text{SCF}_3)_2$ (VDT, compound 15). The generation of $\text{VCp}_2(\text{O}_3\text{SCF}_3)_2$ in THF solution was induced by following the procedure that was described for the titanium complex (35). The precipitated silver chloride was removed by filtration, and the filtrate was evaporated to dryness. The solid green residue

Table 1 Characterization of new vanadocene compounds

No.	Compound	UV-visible [λ (nm); solvent]	IR spectral data ^a (cm ⁻¹)	Elemental analysis [Found (calculated)]
7	VDI	620, 552, 352, 296, 232 (CH ₂ Cl ₂)	3095(s), 1425(s), 1373(m), 1182(m), 1024(m), 1014(m), 825(vs)	C, 28.1 (27.58) H, 2.42 (2.3) I, 58.4 (58.9)
11	VDOCN	710, 490, 257, 227 (CH ₂ Cl ₂)	3110(m), 2657(w), 2117(vs), 1444(m), 1330(s), 1261(w), 1018(m), 950(m), 833(vs), 635(vs), 424(w)	C, 51.35 (51.06) H, 3.97 (3.87) N, 5.65 (5.41) Cl, 13.45 (13.73)
14	VDFe	648, 575, 362, 311, 265, 240 (CH ₂ Cl ₂)	3109(m), 2924(m), 2318(s), 2289(m), 1622(m), 1447(s), 1435(m), 1358(w), 1027(s), 1012(s), 856(s), 846(s)	C, 31.2 (31.6) H, 2.56 (2.9) N, 3.48 (3.1) Cl, 40.31 (39.98)
15	VDT	740, 640, 370, 309, 270, 230 (CH ₂ Cl ₂)	3118(s), 1564(vs), 1440(s), 1350(s), 1218(s), 1194(w), 1149(vs), 1032(s), 959(w), 843(vs), 638(vs)	C, 44.81 (44.45) H, 3.99 (3.96) S, 7.52 (7.46)
17	VD(bpy)	780, 326, 272, 241 (CH ₂ Cl ₂)	3135(m), 3099(s), 1605(s), 1504(m), 1477(m), 1452(s), 1437(vs), 1307(m), 1257(vs), 1232(vs), 1028(vs), 862(vs), 771(vs), 636(vs)	C, 52.48 (53.1) H, 3.72 (3.69) N, 2.51 (2.58) S, 5.73 (5.9)
18	VD(cat)	711, 438, 337, 292, 275, 259 (CH ₂ Cl ₂)	3100(w), 3080(w), 2951(m), 2945(w), 2860(w), 1468(s), 1438(m), 1404(m), 1359(w), 1261(vs), 1012(w), 804(vs), 638(w)	C, 66.79 (66.45) H, 4.93 (4.88)
20	VDPH	680, 501, 377, 314, 261, 233 (CH ₂ Cl ₂)	3117(s), 1600(m), 1539(s), 1495(m), 1450(m), 1300(m), 1281(s), 1244(s), 1173(s), 999(m), 758(m), 694(m), 638(s)	C, 36.85 (36.83) H, 2.64 (2.56) N, 6.97 (7.1)
21	VDH	710, 550, 401, 300, 261, 233 (CH ₂ Cl ₂)	1695(mb), 1635(m), 1500(vs), 1450(s), 1280(s), 1260(s), 1215(vs), 1144(s), 959(m), 758(m), 635(m), 540(w), 480(m)	C, 38.12 (38.61) H, 3.72 (3.46) N, 3.26 (3.46)

^a s, strong; m, medium; w, wide; vs, very strong.

Table 2 *In vitro* cytotoxic activity of vanadocene compounds against testicular cancer cells

Compound	IC ₅₀ (μM) ^a		% apoptosis at 100 μM	
	Tera-2	Ntera-2	Tera-2	Ntera-2
Metalloocene dichlorides				
HDC	>250	>250	ND ^b	ND
MDC	>250	>250	ND	ND
TDC	>250	>250	ND	ND
ZDC	>250	>250	ND	ND
VDC	81	74	70 ± 3 (n = 3)	22 ± 4 (n = 4)
Vanadocene diacido compounds				
VDB	154	70	69 (68, 70)	62 ± 21 (n = 4)
VDI	221	204	61 ± 8 (n = 3)	27 ± 22 (n = 4)
VDA	70	68	70 (63, 77)	59 ± 20 (n = 4)
VDCO	50	90	63 ± 10 (n = 3)	88 (87, 89)
VDCN	51	93	65 ± 3 (n = 3)	86 (85, 87)
VDOCN	31	63	66 ± 6 (n = 3)	78 ± 11 (n = 4)
VDS	23	17	65 ± 5 (n = 3)	65 ± 18 (n = 3)
VDS _e	9	22	70 ± 9 (n = 3)	99 ± 0.2 (n = 4)
VDFe	100	113	73 ± 4 (n = 3)	42 ± 34 (n = 4)
VDT	76	137	59 ± 5 (n = 3)	49 ± 25 (n = 4)
Vanadocene chelated compounds				
VD(acac)	64	75	62 ± 13 (n = 4)	42 ± 6 (n = 4)
VD(bpy)	37	53	58 ± 18 (n = 4)	71 ± 7 (n = 4)
VD(dtc)	60	83	68 ± 8 (n = 4)	58 ± 11 (n = 4)
VD(cat)	79	93	46 ± 25 (n = 4)	42 ± 30 (n = 4)
VDPH	>250	61	ND	76 ± 3 (n = 4)
VDH	118	125	67 ± 2 (n = 3)	42 ± 39 (n = 4)

^a Determined using MTT.

^b ND, not determined.

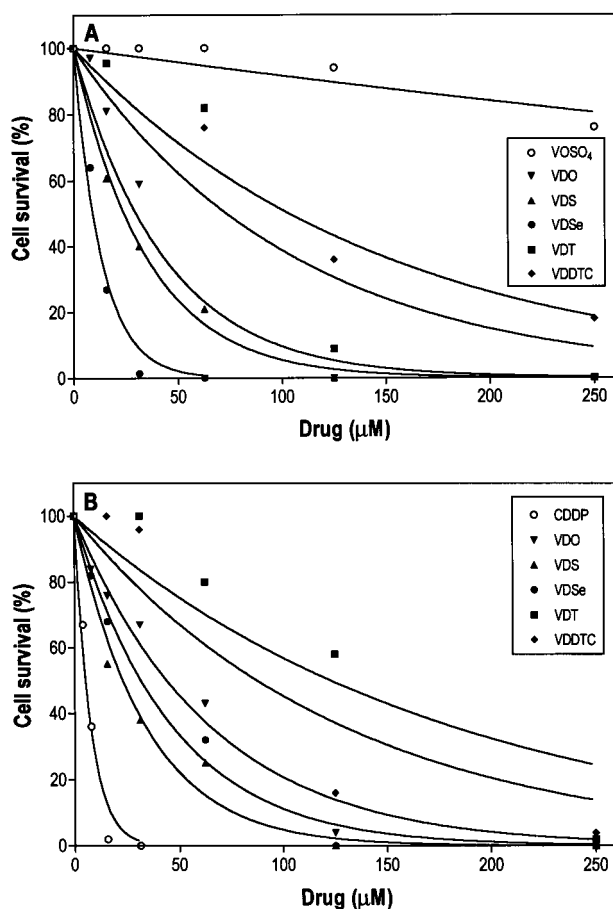


Fig. 4 Cytotoxic activity of vanadocenes on human testicular cancer cell lines. A, Tera-2 cells. B, Ntera-2 cells. Cells were incubated with increasing concentrations (1.9–250 μM) of five representative vanadocenes, VDOCN, VDS, VDSe, VDT, and VD(dtc) or VOSO_4 (in A only) for 24 h, and cell survival was determined by using the MTT assays as described in "Materials and Methods." The data points represent the mean value of triplicate measurements. The SD for each data point was <5% of the mean values. Controls included cells treated with VOSO_4 .

was redissolved in 20 ml of CH_2Cl_2 , filtered again through cannula with one end covered with a filter paper-cotton assembly securely tightened by fine bore copper wire. The dark green precipitate was isolated from dichloromethane using diethyl ether as a cosolvent. The compound is moisture sensitive. Yield: 40%; m.p., decomposition starts at 137°C.

Type 3 Series Compounds

Type 3 series new compounds were synthesized as shown in Fig. 3

$\text{VCp}_2(\text{acac})(\text{O}_3\text{SCF}_3)$ [VD(acac), compound 16]. Dark black-colored large crystals were obtained by following the literature procedure (32). Yield: 45%; m.p., decomposition starts at 247°C.

$\text{VCp}_2(\text{bpy})(\text{O}_3\text{SCF}_3)_2$ [VD(bpy), compound 17]. The synthetic procedure was a modified procedure described for $\text{TiCp}_2(\text{bpy})(\text{O}_3\text{SCF}_3)_2$ (Ref. 35). Light grayish powder was ob-

tained as a precipitate from the THF solution, which was collected by filtration and dried. Yield: 38%; m.p., 305°C.

$\text{Cp}_2\text{V}(\text{cat})$ [VD(cat), compound 18]. One hundred twenty-six mg of Cp_2VCl_2 (0.50 mmol) was placed in a 250-ml flask and dissolved in 100 ml of THF. In another flask, sodium cat was prepared by the addition of NaH (25 mg, 1.0 mmol) to 55.5 mg of catechol (0.50 mmol) in 15 ml of THF. The solution was stirred for 2 h, resulting in a deep blue solution. The cat solution was cannulated into the vanadium solution and stirred for 4 h. The reaction mixture was opened to the air and quickly flash-chromatographed under nitrogen on alumina (neutral; acetonitrile mobil phase). The solvent of the deep blue solution was then removed under vacuum, and the product was collected. Yield: 26%; m.p., decomposition starts at 95°C.

$\text{VCp}_2(\text{dtc})(\text{O}_3\text{SCF}_3)$ [VD(dtc), compound 19]. Bis(cyclopentadienyl)-*N,N*-diethyl dithiocarbamate triflate salt was prepared according to the published procedure (36). Yield: 90%; m.p., 163°C.

$\text{VCp}_2(\text{PH})(\text{O}_3\text{SCF}_3)$ (VDPH, compound 20). The reaction mixture composed of VCp_2Cl_2 (0.2 g, 8 mmol) and AgCF_3SO_3 (0.46 g, 18 mmol) in 10 ml of H_2O was stirred for 2 h and then filtered through fine glass frit. A solution of *N*-phenyl benzohydroxamic acid in 5 ml of ethanol (0.85 g, 4.0 mmol) was added to the filtrate with stirring, and the resulting solution was kept for 4 h to complete the precipitation of the dark-colored compound. The product was collected by filtration and thoroughly washed with diethyl ether and dried for overnight under vacuum. Yield: 38%; m.p., 160°C.

$\text{VCp}_2(\text{H})(\text{O}_3\text{SCF}_3)$ (VDH, compound 21). This reddish-brown compound was prepared following essentially the same procedure as that applied for compound 20 (37). However, the reactions were carried out in dry THF instead of H_2O using acethydroxamic acid as the ligand. Yield: 52%; m.p., decomposes at 55°C.

Type 4 Series Compounds

Type 4 compounds were synthesized as follows:

$\text{V}(\text{MeCp})_2\text{Cl}_2 \cdot 0.5 \text{H}_2\text{O}$ (VMDC, compound 22). The synthetic procedure is described in the literature (14). The bright green microcrystals were separated from the HCl-saturated CHCl_3 solution. Yield: 25%; m.p., 292°C.

$\text{V}(\text{Me}_5\text{Cp})_2\text{Cl}_2$ (VPMDC, compound 23). The green solid was isolated from diethylether from a reaction mixture of $\text{V}(\text{Me}_5\text{Cp})_2$ and PCl_3 as described by Moran *et al.* (38). Yield: 20%; m.p., 155°C.

$\text{V}(\text{Me}_5\text{Cp})\text{OCl}$ (VPMOC, compound 24). This compound was prepared by following the procedure reported by Aistars *et al.* (39). Sublimed green materials of $\text{V}(\text{Me}_5\text{Cp})_2\text{Cl}_2$ (24) were dissolved in dry THF and purged with O_2 for 8 h. The solvent was removed under vacuum, and the compound was recrystallized from hexane. Yield: 80%; m.p., 75°C.

Cell Lines and Culture Conditions

Human testicular cancer cell lines, Tera-2 (embryonal carcinoma) and Ntera-2 (pluripotent embryonal carcinoma), were obtained from the American Type Culture Collection (Rock-

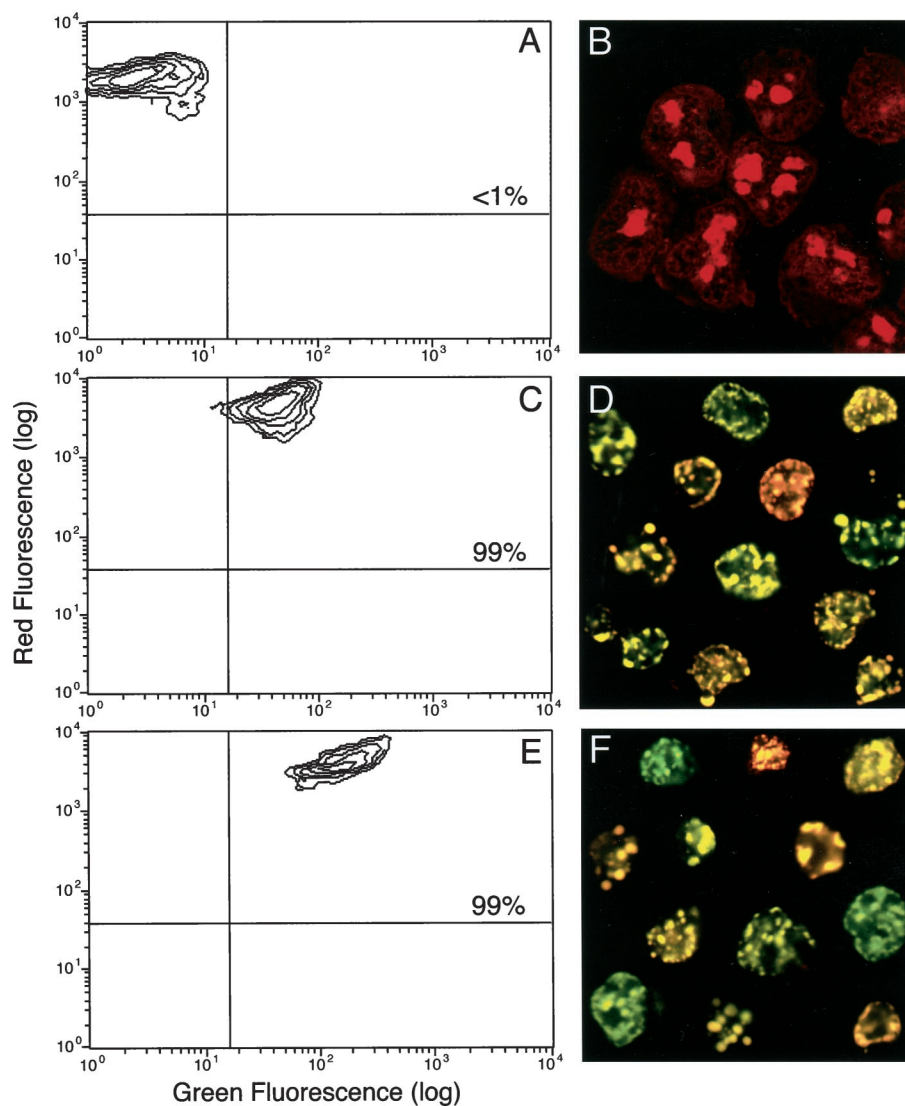


Fig. 5 VDS and VDSe induce apoptosis in human testicular cancer cells. *Left panels*, fluorescence-activated cell-sorting analysis: two-color flow cytometric contour plots of Ntera-2 cells treated with vanadocenes. Cells were incubated for 24 h in either control medium (0.1% DMSO; **A**), medium supplemented with 100 μM VDS (**C**), or VDSe (**E**) in 0.1% DMSO, fixed, permeabilized, and visualized for DNA fragmentation in a TUNEL assay using TdT and FITC-dUTP (green fluorescence) and analyzed with flow cytometry as described in "Materials and Methods." Red fluorescence, nuclei counterstained with PI. Percentages indicate cells with increased dUTP incorporation. *Right panels*, confocal images: two-color CLSM images of control and VDS- and VDSe-treated cells. **B**, control cells visualized for dUTP incorporation. **D** and **F**, apoptotic nuclei of cells treated with VDS and VDSe are recognized by fluorescein-labeled green/yellow (superimposed red plus green) fluorescence. $\times 600$.

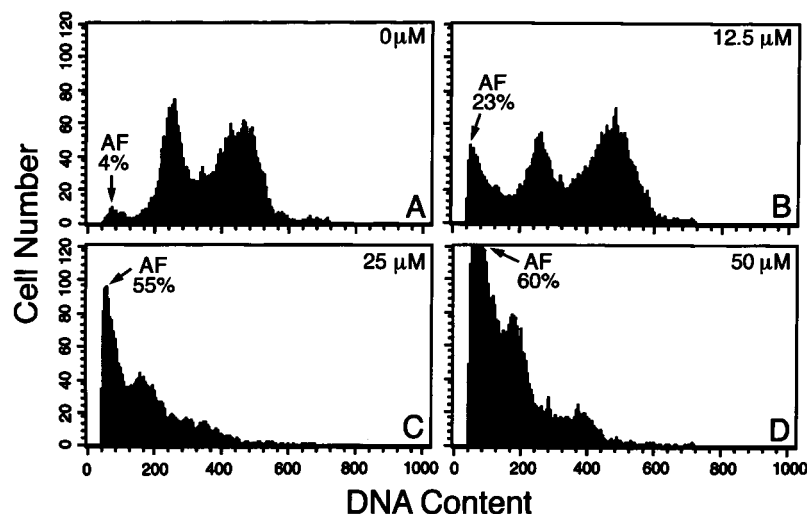
ville, MD) and propagated in McCoy's 5A medium and DMEM, respectively. Both media were supplemented with 10% FCS, 4 mM glutamine, 100 units/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. All tissue culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). Cell lines were cultured for a minimum of two passages after thawing prior to experimentation.

MTT Assays

We used MTT-based colorimetric short-term viability assays (40, 41) for evaluation of the cytotoxicity of vanadocene compounds. Briefly, adherent cells were harvested with 0.125% (w/v) trypsin-0.02% EDTA (Life Technologies, Inc.), and nonadherent cells were harvested with DMEM from the exponential phase and dispensed in triplicate into 96-well tissue culture plates in 100- μl volumes. After 24 h of incubation, the culture medium was discarded and replaced with 100 μl of fresh medium containing serial 2-fold dilu-

tions of drugs in medium to yield final concentrations ranging from 1.9 to 250 μM . All compounds were freshly reconstituted in DMSO to prepare a 100-mM stock solution for each experiment. Culture plates were then incubated for 24 h before adding 10 μl of MTT solution (5 mg/ml in PBS) to each well. The tetrazolium/formazan reaction was allowed to proceed for 4 h at 37°C, and then 100 μl of the solubilization buffer (10% SDS in 0.1% HCl) were added to all wells and mixed thoroughly to dissolve the dark blue formazan crystals. After an overnight incubation at 37°C, the absorbances at $A_{540\text{ nm}}$ and a reference wavelength of 690 nm were measured using a 96-well multiscanner autoreader. To translate the $A_{540\text{ nm}}$ values into the number of live cells in each well, the $A_{540\text{ nm}}$ values were compared with those on standard $A_{540\text{ nm}}$ versus cell number curves generated for each cell line. The percentage of survival was calculated using the formula: % survival = live cell number [test]/live cell number [control] $\times 100$. The $A_{540\text{ nm}}$ values were calculated by nonlinear

Fig. 6 Induction of apoptosis in vanadocene-treated Tera-2 testicular cancer cells. Cells were treated with vehicle, 12.5, 25, or 50 μM VDS_e for 24 h, stained with PI, and analyzed by flow cytometry for DNA content. The percentages indicate the hypodiploid/apoptotic nuclei. *AF*, apoptotic fraction.



regression analysis using Graphpad Prism software version 2.0 (Graphpad Software, Inc., San Diego, CA).

Apoptosis Assays

A flow cytometric two-color TUNEL was used to detect apoptotic nuclei (42). Exponentially growing cells ($10^6/\text{ml}$) were incubated in DMSO alone (0.1%) or treated with 100 μM each of the 20 vanadocenes [VDB, VDC, VMDC, VDI, VDA, VDCN, VDOCN, VDS, VDS_e, VDT, VDO, VDFe, VD(acac), VDH, VDPH, VD(cat), VD(bpy), VD(dtc), VPMDC, and VPMOC] in 0.1% DMSO for 24 h. Cells were washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min on ice. After two washings in PBS, they were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice and washed twice in PBS. Labeling of exposed 3'-OH ends of fragmented nuclear DNA was performed using TdT and FITC-conjugated dUTP according to the manufacturer's recommendations (Boehringer Mannheim, Indianapolis, IN). Cells were counterstained with 5 $\mu\text{g}/\text{ml}$ PI. Control samples included untreated cells as well as cells incubated with the reaction mixture without the TdT enzyme. Cells were analyzed after excitation from an argon laser (488 nm) using a fluorescence-activated cell sorting Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Relative DNA content (PI emission) was measured with band-pass filter 585/42, and dUTP incorporation (FITC emission) was measured with a band-pass filter 530/30. Fluorescence was compensated for in the acquisition software using single-label control samples. Data were acquired in a listmode, gated to 10,000 events/sample, and analyzed using CellQuest Software (Becton Dickinson). Nonapoptotic cells do not incorporate significant amounts of dUTP because of lack of exposed 3'-OH ends and consequently have relatively little or no fluorescence compared with apoptotic cells, which have an abundance of 3'-OH ends (M2 gates). Vanadocene-induced apoptosis is shown by an increase in the number of cells staining with FITC-dUTP. The M1 and M2 gates were used to demarcate nonapoptotic and apoptotic PI-counterstained cell populations, respectively. TUNEL assays were performed using two testic-

ular cell lines, Tera-2 and Ntera-2, after exposure to each of the 20 vanadocenes. Apoptosis was documented by combining TUNEL assays with CLSM. CLSM was performed using a Bio-Rad MRC-1024 Laser Scanning Confocal Microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon mixed gas laser (excitation lines at 488, 568, and 647 nm) and mounted on a Nikon Eclipse E800 series upright microscope equipped with high numerical objectives. Using fluorescence imaging, the fluorescence emission of FITC and PI from nuclei of cancer cells was simultaneously recorded using the 598/40-nm and 680 DF32 emission filter, respectively. Confocal images were obtained using a Nikon $\times 60$ (NA 1.4) objective and a Kalman collection filter. Digitized images were saved on a Jaz disc (Iomega Corp., Roy, UT) and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). Final images were printed using a Fuji Pictography 3000 (Fuji Photo Film Co., Tokyo, Japan) color printer.

Cell Cycle Analysis

Exponentially growing cells were incubated with various concentrations ranging from 1 to 25 μM of oxovanadium compounds for 24 h at 37°C. Cells were harvested by trypsin release and resuspended in DNA staining solution (10 $\mu\text{g}/\text{ml}$ RNase, 0.1% Triton X-100, 0.1 mM EDTA, 0.1% sodium citrate, 50 $\mu\text{g}/\text{ml}$ PI, and 1 mM Tris-HCl) 1–2 h before flow cytometric analysis. The fluorescence of 10,000 cells was measured with a Becton Dickinson flow cytometer with excitation of 488 nm. The percentages of cells in the G₁, S, and G₂-M phases of the cell cycle were determined using CellQuest software, version 3.1.

RESULTS AND DISCUSSION

Synthesis and Characterization of Vanadocene Compounds. A total of 24 metallocene compounds including 8 novel vanadocene compounds (*i.e.*, 7, 11, 14, 15, 17, 18, 20, and 21) were prepared and analyzed as described in "Materials and Methods." Fig. 1 illustrates the chemical structures of the com-

pounds. The critical physicochemical data for the novel vanadocene compounds are detailed in Table 1.

Cytotoxic Effects of Vanadocenes on Human Testicular Cancer Cells. We used standard 24-h MTT viability assays to examine the cytotoxic activity of five metallocene dichlorides containing vanadium (vanadocene dichloride), titanium (titanocene dichloride), zirconium (zirconocene dichloride), molybdenum (molybdocene dichloride), or hafnium (hafnocene dichloride) against the human testicular cancer cell lines Tera-2 and Ntera-2. Only vanadium-containing metallocene VDC was cytotoxic against both cell lines, with IC_{50} s of 81 and 74 μ M, respectively. Surprisingly, other metallocene dichlorides containing titanium, zirconium, molybdenum, or hafnium as the central metal atom (oxidation state IV) had no effect on cell viability, even at 250 μ M (Table 2).

We next examined 20 structurally similar compounds with differing substituents around the ancillary position of the Cp_2 -vanadium (IV) unit for cytotoxic activity against Tera-2 and Ntera-2 cells (Table 2). Each one of these 20 vanadocene compounds exhibited a concentration-dependent cytotoxicity against both Tera-2 and Ntera-2 cells, with IC_{50} s ranging from 9 to 221 μ M (Table 2). The most potent vanadocene compound was VDSe, which killed Tera-2 and Ntera-2 cells with IC_{50} s of 9 and 22 μ M, respectively. Fig. 4 demonstrates the concentration-response curves obtained for Tera-2 (A) and Ntera-2 (B) cells, respectively, when exposed to 1.9–250 μ M of five representative vanadocenes, VDOCN, VDS, VDSe, VDT, and VD(dtc), for 24 h.

The variable potency of vanadocenes suggests that the various monodentate and bidentate ligand groups affect the cytotoxic activity of these compounds against testicular cancer cells. We also tested the potential cytotoxic effects of vanadium [vanadyl(IV) sulfate] at the same concentrations. In sharp contrast to the organometallic compounds containing vanadium(IV), inorganic vanadium (oxidation state IV) salt lacked cytotoxic activity, even at 250 μ M (Fig. 4 and Table 2).

Vanadocenes Induce Apoptosis in Human Testicular Cancer Cells. We next set out to determine whether the cytotoxicity of vanadocenes against testicular cancer cells is associated with apoptosis. Tera-2 and Ntera-2 cells were cultured with vanadocenes (100 μ M) for 24 h at 37°C and then subjected to flow cytometric analysis for dUTP incorporation by the TdT-mediated TUNEL assay. The TdT-dependent incorporation of FITC-dUTP was dramatically increased in vanadocene-treated cells. Fig. 5 depicts the two-color flow cytometric contour plots as well as confocal microscopy images of cells from representative TUNEL assays. Among the 20 vanadocene complexes evaluated by the flow cytometric TUNEL assay, 18 (*i.e.*, all except VDPH and VDMOC) caused a marked increase in TUNEL-positive nuclei ranging from 35 to 88% for Tera-2 cells and 20 to 99.6% for Ntera-2 cells, respectively (Table 2). Apoptosis after vanadocene treatment was also evident from the concentration-dependent emergence of a hypodiploid (<2N) peak in the DNA histograms of PI-stained Tera-2 cells, which was accompanied by nonselective loss of G_0/G_1 -, S-, and G_2 -M-phase cells (Fig. 6).

In summary, we systematically assessed the cytotoxic effects of 24 metallocene-disubstituted compounds, including 20 vanadocene complexes, against human testicular cancer cells.

Vanadium-containing organometallics exhibited significant cytotoxicity against testicular cancer cells and induced apoptosis within 24 h. Although the cytotoxic effect of vanadocenes was primarily dependent upon the central vanadium(IV) ion, the two cyclopentadienyl units attached to vanadium(IV) coordination sites and the various monodentate and bidentate ligand groups coordinated to the bis(cyclopentadienyl)vanadium (IV) moiety appear to be also very important for their anticancer activity. Vanadocenes with dithiocyanate (VDS) and diselenocyanate (VDSe) as ancillary ligands were identified as the most potent cytotoxic compounds.

To our knowledge, this is the first report on the antitumor effects of vanadocene diacido complexes against human testicular cancer cells. Metallocene diacido complexes, especially TDC and VDC, have been explored as chemopreventive agents and found to be active in athymic mice xenografted with human cancer cells (19–22). Surprisingly in the present study, unlike vanadocenes, TDC as well as other non-vanadium(IV)-containing metallocenes had no effect on the growth of testicular cancer cells. Therefore, it is likely that the molecular mechanism of vanadocene-mediated cytotoxicity is different from that of titanocenes or other metallocenes (19, 20, 43).

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