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Report

Vanadocenes as potent anti-proliferative agents disrupting mitotic spindle formation in cancer cells

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We present experimental data which establish the organometallic compounds vanadocene dichloride (VDC) and vanadocene acetylacetonate (VDacac) as potent anti-proliferative agents. We first examined the effects of VDC and VDacac on the rapid embryonic cell division and development of Zebrafish. Both compounds were capable of causing cell division block at the 8–16 cell stage of embryonic development followed by total cell fusion and developmental arrest. We next examined the effect of VDC and VDacac on proliferation of human breast cancer and glioblastoma cell lines using MTT assays. VDC inhibited the proliferation of the breast cancer cell line BT-20 as well as the glioblastoma cell line U373 in a concentration-dependent fashion with IC_{50} values of 11.0, 14.9 and 18.6 μM, respectively. VDacac inhibited cellular proliferation with IC_{50} values of 9.1, 26.9 and 35.5 μM, respectively. Whereas in vehicle-treated control cancer cells mitotic spindles were organized as a bipolar microtubule array and the DNA was organized on a metaphase plate, vanadocene-treated cancer cells had aberrant monopolar mitotic structures where microtubules were detected only on one side of the chromosomes and the chromosomes were arranged in a circular pattern. In contrast to control cells which showed a single focus of γ-tubulin at each pole of the bipolar mitotic spindle, VDC- or VDacac-treated cells had two foci of γ-tubulin on the same side of the chromosomes resulting in a broad centrosome at one pole. All monopolar spindles examined had two foci of γ-tubulin labeling consistent with a mechanism in which the centrosomes duplicate but do not separate properly to form a bipolar spindle. These results provide unprecedented evidence that organometallic compounds can block cell division in human cancer cells by disrupting bipolar spindle formation. In accordance with these results vanadocene treatment caused an arrest at the G_2/M phase of the cell cycle. This unique mechanism of anti-mitotic function warrants further development of vanadocene complexes as anti-cancer drugs. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cell cycle, centrosome, mitotic spindle, vanadium, vanadocene, Zebrafish.

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Introduction

Vanadocene dichloride (VDC) has been shown to arrest tumor cells in G_2/M of the cell cycle.[1] Arrest in G_2/M of the cell cycle suggests defects in proper spindle formation and arrest of the cell cycle by triggering the mitotic spindle checkpoint(s). Cells must properly form a bipolar mitotic spindle with bivalent chromosomes properly attached to each pole of the spindle (for reviews, see Gorbsky[2] and Hardwick[3]). Cells which do not form a correct mitotic spindle arrest at metaphase of mitosis indefinitely or progress into apoptosis. Several proteins from yeast and mammals have been implicated in this process; MAD1 (mitotic arrest deficient), MAD2 and MAD3,[4] BUB1 (budding uninhibited by benzimidazole), BUB2 and BUB3.[5] Mammalian counterparts for these proteins include HsMAD2[6] and hBUB1.[7]

One of the most crucial and tightly regulated events during mitosis is centrosome duplication.[8,9] The centrosome is an organelle consisting of a pair of centrioles surrounded by an amorphous electron-dense material and represents the mammalian equivalent of the yeast spindle pole body. This organelle serves as a site of microtubule organization in the cell. During cell cycle progression the centrosome duplicates, separates and functions as the poles for the mitotic spindle. It is crucial for proper chromosome segregation and fidelity that centrosome replication be tightly regulated, doubling just once during each cell cycle. Centrosome regulation is tightly linked to the S-phase checkpoint.[10,11] For example, blocking cells at the beginning of S-phase leads to the formation of multiple centrosomes.[12]

The primary purpose of the present study was to investigate the effects of the vanadocenes VDC and vanadocene acetylacetonate (VDacac) on mitotic spindle formation in human cancer cells. Our results

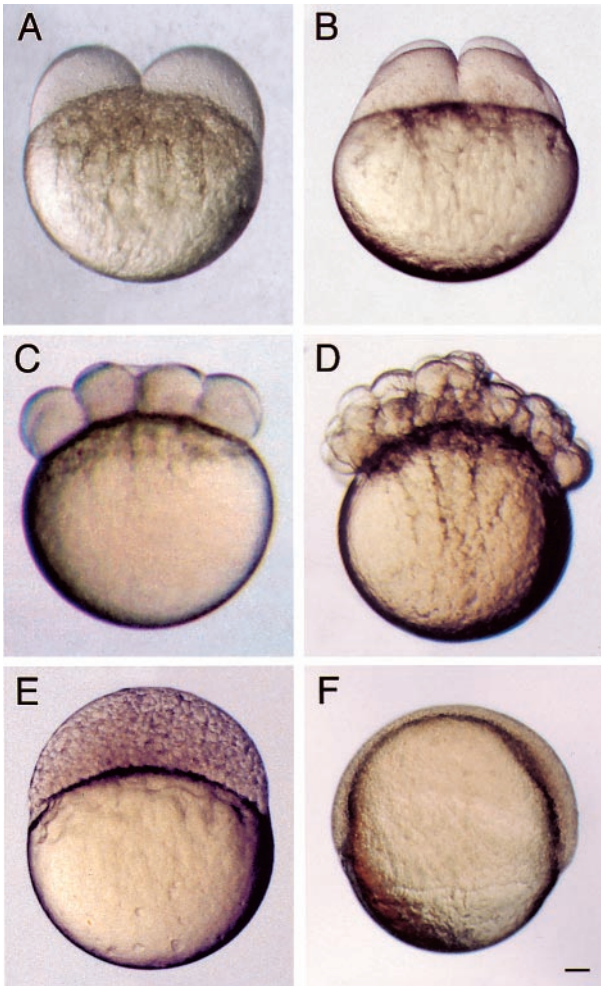
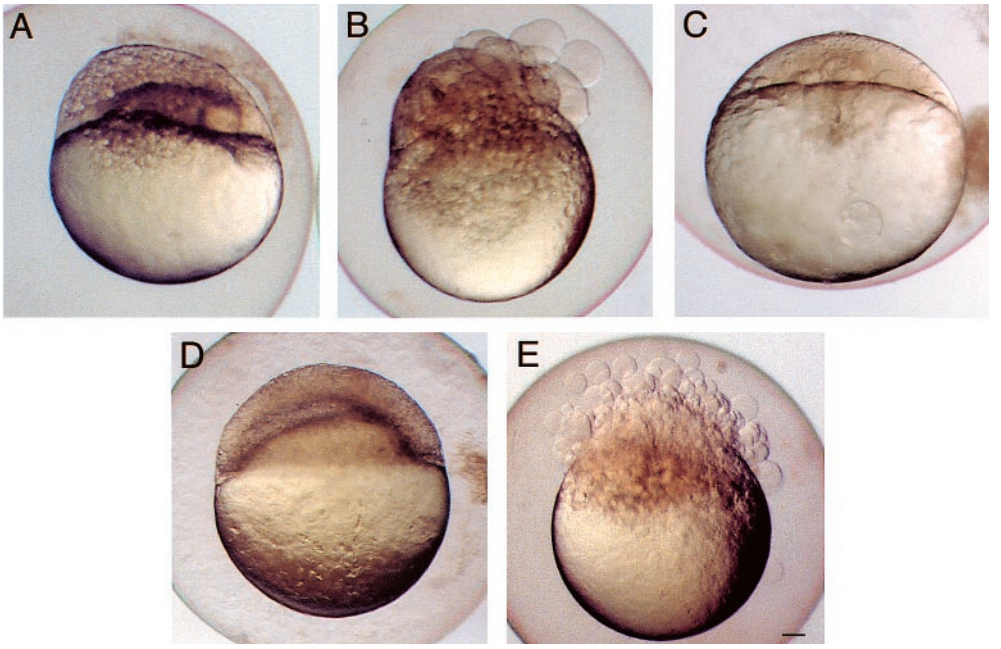


Figure 1. Cell division and development in the Zebrafish embryo. Two-cell stage Zebrafish embryos (A) were dechorionated with 1% trypsin–EDTA and treated with 1% DMSO at a temperature of 28.5°C. At 60 min post-fertilization the embryo reached the 4-cell stage (B). Fifteen minutes later the embryos reached the 8-cell stage (C). Two hours after fertilization the embryos reached the 64-cell stage (D) and 200 min after fertilization the embryos reached the blastula stage. Just 7 h after fertilization the embryo is midway through the gastrula stage and has completed 70% epiboly. Bar=100 μm.

Figure 2. Microinjected VDC and VDacac affect cell division and development in the Zebrafish embryo. Microinjection of VDC into the Zebrafish embryo caused concentration-dependent defects in embryonic development. Zebrafish embryos were microinjected with 0.64 (A), 32 (B) and 58 (C) pmol of VDC. The lowest concentration caused formation of a blastocoel-like cavity in the blastoderm and prevented gastrulation. A higher concentration led to cell division slow-down and total disarray of cell localization and developmental arrest, and the highest concentration caused total cell fusion within 30 min of microinjection. Similar results were observed after microinjection of 1 (D) and 49 (E) pmol VDacac. Lower concentrations caused formation of a blastocoel-like cavity in the blastoderm and prevented gastrulation. Higher concentrations led to deterioration of cell localization and developmental failure. Bar=100 μm.



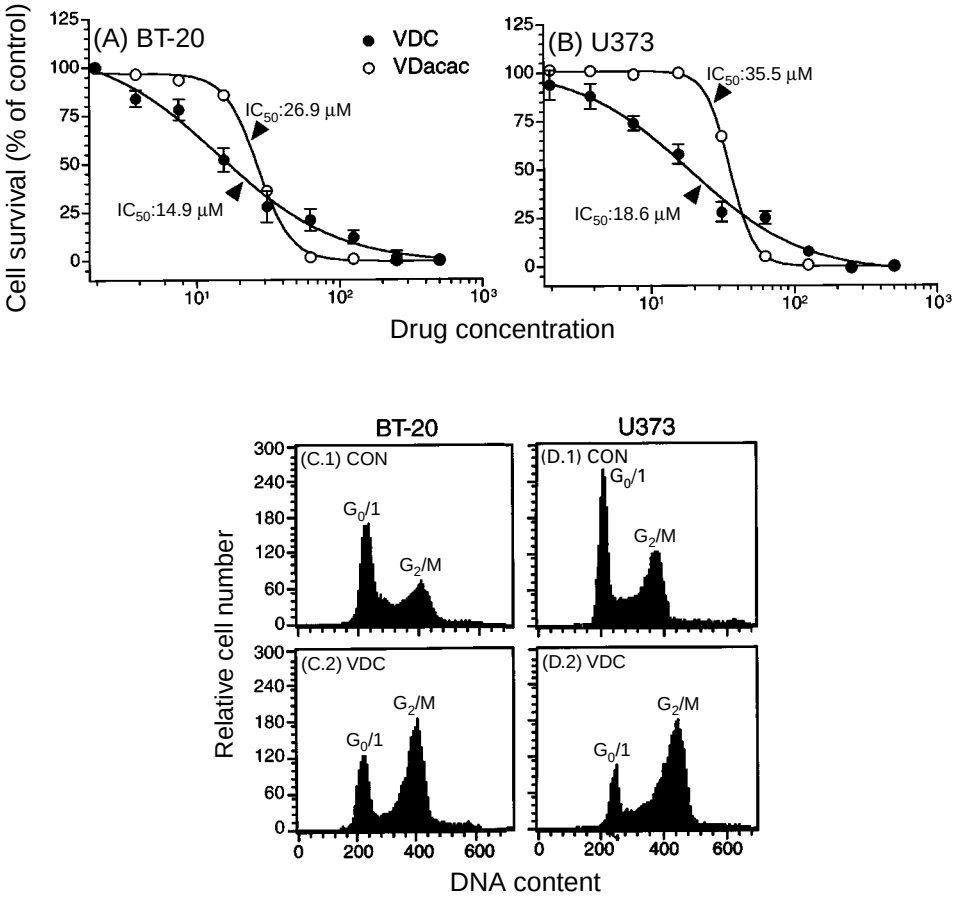


Figure 3. VDC inhibits proliferation of cancer cell lines. (A and B) Two cancer cell lines were tested including the breast cancer cell line BT-20 (A) and the brain tumor cell line U373 (B). Cell proliferation was assessed using MTT assays. VDC inhibited the proliferation in a concentration-dependent fashion with micromolar IC_{50} values (BT-20, 14.9 μM; U373, 18.6 μM). Treatment with VDacac resulted in IC_{50} values of 26.9 and 35.5 μM for BT-20 and U373, respectively. (C and D) Flow cytometric analysis of cell cycle distribution after vanadocene treatment. Shown are DNA histograms obtained 72 h after treatment with VDC. Both cell lines have an accumulation of cells at the G_2/M phase of the cell cycle after treatment with VDC (BT-20, 48.0%; U373, 52.8%) relative to control cells (BT-20, 19.0%; U373, 31.1%).

provide unprecedented evidence that these vanadocenes block cell division by disrupting bipolar mitotic spindle formation. In vanadocene-treated breast cancer cells, centrosomes duplicated but did not separate properly to form a bipolar spindle.

This study establishes vanadocenes as a new class of anti-proliferative agents which cause monopolar spindle formation by inhibiting centrosome separation.

Materials and methods

Vanadocene synthesis

VDC: (VCp_2Cl_2). VCp_2Cl_2 was prepared by following literature procedures,[13] and its purity was confirmed by ^{1}H-NMR, IR spectroscopy and elemental analysis. The compound was purified under partial vacuum by anaerobic Soxhlet extraction with CH_2Cl_2 at 44°C.

VDacac: [VCp_2(acac)](CF_3SO_3). Bis(π-cyclopentadienyl) (acetyl acetonato) vanadium (IV) triflate, [VCp_2(acac)](CF_3SO_3), was prepared according to a published procedure described for the corresponding ClO_4^- salt.[14] Anal. calcd for $VC_{16}H_{18}O_5F_3S$: C, 44.65; H, 4.1; S, 7.44. Found: C, 44.81; H, 3.99; S, 7.52. M.P. 247°C.

Fish and embryos

The adult wild-type Zebrafish were maintained generally according to *The Zebrafish Book* recommendations.[15] Males and females were kept in 10 G tanks (70 fish per tank) with a constant slow flow of conditioned

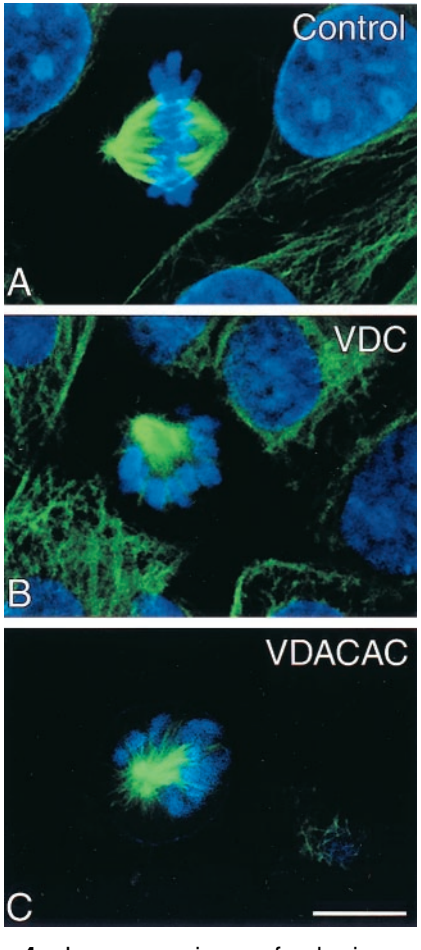


Figure 4. Laser scanning confocal microscopy of treated and untreated mitotic cells. Untreated mitotic cells (A) had bipolar spindles as assessed by microtubule staining (green). Chromosomes (blue) are aligned on the metaphase plate. In contrast mitotic cells treated with 25 μM VDC (B) or 0.3 μM VDacac (C) were frequently monopolar with all tubulin (green) staining on one side of the condensed chromosomes (blue). Green=anti-tubulin staining, blue=DNA. Scale bar=10 μm.

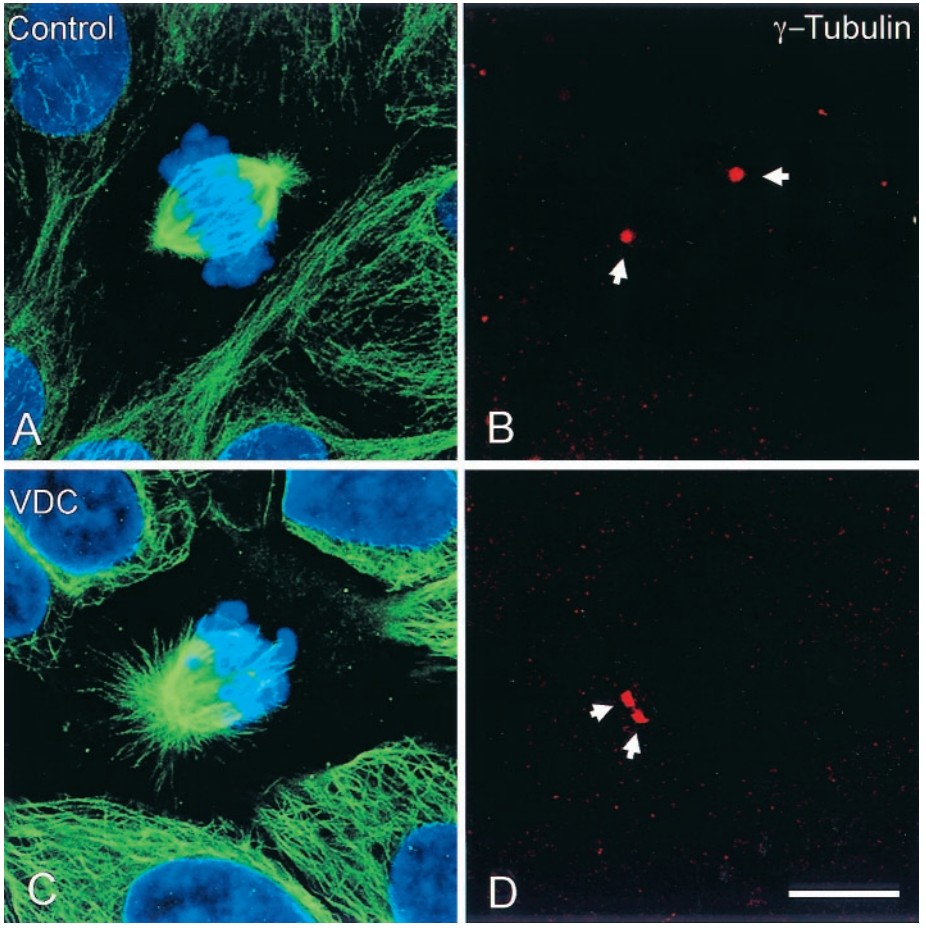


Figure 5. Immunocytochemical detection of the centrosomal protein γ-tubulin in treated and untreated cells. Untreated mitotic cells (A and B) had one foci of γ-tubulin staining (B, arrows) at each pole of the mitotic spindle labeled with anti-tubulin (A, green) and toto-3 for DNA (A, blue). Monopolar mitotic spindles generated by treatment with 25 μM VDC (C and D) also had two foci of γ-tubulin (D, arrows) but both were located at the same spindle pole. Green=anti-tubulin staining, Blue=DNA, red=γ-tubulin staining. Bar=10 μm.

water at 26°C and a controlled 14 h day/10 h night cycle. The fish were fed twice a day with live brine shrimp (Ocean Star International, Snowville, UT). Breeding of one and the same group of fish occurred once in 2 weeks. With this breeding schedule, the Zebrafish embryos were obtained daily through (i) natural spawning at 28.5°C in the breeding tanks with a netted false bottom or (ii) fertilization *in vitro* using eggs and milt collected from the mature females and males anesthetized with Tricaine (Sigma, St Louis, MO).

Zebrafish embryo model system

Zebrafish eggs were removed from their chorions by mild digestion in 1% Trypsin–EDTA (Sigma, St Louis, MO) for 10 min at 28.5°C [standard temperature (ST)], washed 3 times in 'egg water' and twice in 'embryonic medium' (EM).[15]

Microinjections were performed with the help of a SMZ-10A stereo microscope (Nikon, Melville, NY) and Transjector 5246 (Eppendorf, Westbury, NY) at room temperature. The dechorionated Zebrafish embryos at the 2-cell stage were transferred to the Petri dish filled with EM. The bottom of the Petri dish was covered with an agar layer and the embryos were placed in the grooves in agar, exactly as described.[15] All microinjections were performed under visual control. At the 2–4-cell stage, approximately 2 nl of the compound dissolved in Hank's balanced salt solution (HBSS) (Gibco, Rockville, MD) with 10% DMSO was injected into the cytoplasm of one of the blastomeres through a constantly flowing micropipette with a splinted sharp tip of 2–3 μm in diameter. Sham-treated control embryos were injected with 2 nl of HBSS containing 10% DMSO. After the treatment, the embryos were transferred to ST.

Observations of cell division and development of the ZF embryos were carried out using a SMZ-10A stereo microscope, once every 30 min within the first 3 h after injection, and at 6, 12 and 24 h, as well. The drug effect was considered to be revealed when all embryos were affected in a characteristic manner in three independent experiments. The stereo microscope was fitted with a specially designed transparent heating tray in order to keep embryos at ST during observations. Pictures of the embryos were taken with a H-III Photomicrographic System (Nikon) using Ektachrome 64X film (Kodak, Rochester, NY).

Cell culture and treatment

BT-20 breast cancer cell line (HTB-19; ATCC, Rockville, MD) and U373 glioblastoma cell line (HTB-17; ATCC) grown in MEM media modified with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Rockville, MD) were used for these experiments. Cells were seeded onto sterile 22 mm^2 coverslips in six-well plates. Cells on coverslips were kept in an incubator for 24 h prior to treatment. The following day, VDC or VDacac was added from a stock solution made in DMSO to yield final concentration ranging from 3.9 to 500 μM (final DMSO concentration=0.025%). Cells were returned to a 37°C incubator for 24 h.

Cytotoxicity assay

The cytotoxicity of these compounds against human tumor cell lines was assayed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim, Indianapolis, IN). Briefly, exponentially growing tumor cells were seeded into a 96-well plate at a density of 2.5×10^4 cells/well and incubated for 36 h at 37°C prior to drug exposure. On the day of treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing VDC or VDacac at concentrations ranging from 3.9 to 500 μM. Triplicate wells were used for each treatment. The cells were incubated with the vanadocenes for 48 h at 37°C in a humidified 5% CO_2 atmosphere. To each well, 10 μl of MTT (0.5 mg/ml final concentration) was added and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbence of each well was measured in a microplate reader (Labsystems, Helsinki, Finland) at 540 nm and a reference wavelength of 690 nm. To translate the OD_{540} values into the number of live cells in each well, the OD_{540} values were compared to those on standard OD_{540} versus cell number curves generated for each cell line. The percent survival was calculated using the formula: % survival=live cell number [test]/live cell number [control] $\times$ 100. The IC_{50} values were calculated by non-linear regression analysis using Graphpad Prism software version 2.0 (Graphpad Software, San Diego, CA).

Cell cycle analysis

Cell cycle distribution was determined using a Becton Dickinson Calibur flow cytometer by measurement of propidium iodide. Control and treated cells were trypsanized, fixed in ice cold ethanol and labeled with propidium iodide.

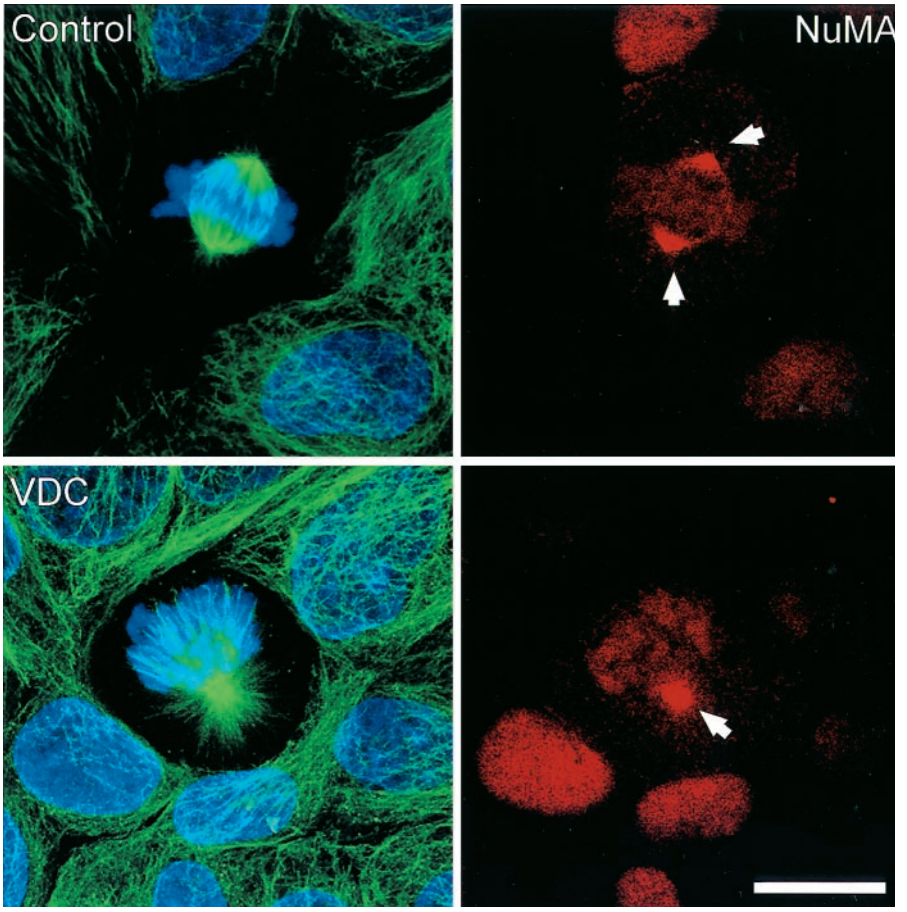


Figure 6. Immunocytochemical localization of the spindle pole protein NuMA. Control mitotic spindles have NuMA localization on both sides of the condensed chromosomes at the spindle poles. NuMA can also be seen in the interphase nuclei. In monopolar spindles from cells treated with 25 μM VDC, NuMA is only detected at the single spindle pole. Green=microtubules, red=NuMA, blue=DNA. Bar=20 μm.

Immunocytochemistry and confocal microscopic analysis

At the appropriate timepoints coverslips containing BT-20 cells were fixed in -20°C methanol for 15 min followed by 15 min incubation in phosphate-buffered saline + 0.1% Triton X-100 (PBS+Tx). Coverslips were next incubated with a primary antibody recognizing α-tubulin (Sigma) γ-tubulin (BAbCo, Berkeley, CA) or NuMA (Calbiochem, San Diego, CA) for 40 min in a humidified chamber at 37°C. Coverslips were washed for 15 min in PBS-Tx followed by a 40 min incubation with a fluorescently labeled secondary antibody (Jackson Immunoresearch, West Grove, PA). For double labeling the respective primary and secondary antibodies were combined. The coverslips were again rinsed in PBS-Tx and incubated with 5 μM Toto-3 (Molecular Probes, Eugene, OR) for 20 min to label the DNA. Coverslips were immediately inverted onto slides in Vectashield (Vector, Burlingame, NH) to prevent photobleaching, sealed with nail polish and stored at 4°C.

Slides were examined using a BioRad MRC-1024 laser scanning confocal microscope mounted on a Nikon Eclipse E800 upright microscope with high numerical aperture objectives. Digital data was processed using Lasersharp (BioRad, Hercules, CA) and Photoshop (Adobe Systems, Mountain View, CA) software, and printed on a Pictrography printer (Fuji Photo, Elmsford, NY).

Results and discussion

Cell division and development of Zebrafish embryos is blocked by VDC

The embryonic development of the Zebrafish (*Danio rerio*) is thoroughly studied and staged.[15–17] In the

Zebrafish meroblastic eggs, rapid cell divisions occur after the ooplasmic segregation on the animal pole of the egg cell, resulting within the first 3 h of development in the generation of a multicellular blastula comprised of several thousand cells. The first series of cell divisions of the initial cleavage stage are approximately synchronous only 15 min apart and each set of the dividing blastomeres is characterized by a distinct pattern of cellular localization. This remarkable proliferation rate of undifferentiated eukaryotic vertebrate cells makes the Zebrafish embryo an attractive experimental model for evaluation of new agents for anti-proliferative activity. In order to determine if the vanadocene compounds VDC and VDacac could affect cell division, we examined their effect on embryonic development of Zebrafish. Zebrafish embryos were dechorionated with Trypsin–EDTA and incubated in embryonic medium. At standard temperature, 2-cell stage control embryos (Figure 1A) reached 4-cell (Figure 1B), 8-cell (Figure 1C) and 64-cell (Figure 1D) stages within 15, 30 and 75 min, respectively. Within 3.5 h post-fertilization, these embryos developed into a high blastula (Figure 1E) and underwent gastrulation approximately 2.5 h later (Figure 1F). When VDC or VDacac were microinjected into the cytoplasm of 2-cell stage Zebrafish embryos, they inhibited cell division in a concentration-dependent fashion (Figure 2). At the dose level of 1 pmol/embryo, both compounds resulted in the formation of a blastocoel-like cavity in the blastoderm at the late blastula–early gastrula stages (VDC, Figure 2A; VDacac, Figure 2D). At a dose level of 40 pmol/cell, both compounds slowed down cell division and resulted in deterioration of the cell localized pattern (VDC, Figure 2B; VDacac, Figure 2E). At 60 pmol/cell, total cell fusion and developmental arrest was observed within 30 min after VDC microinjection (Figure 2C).

Mitotic spindle formation in human cancer cells is disrupted by VDC

We next examined the effect of VDC and VDacac on proliferation of human breast cancer and glioblastoma cell lines using MTT assays. VDC inhibited the proliferation of the breast cancer cell line BT-20 as well as the glioblastoma cell line U373 in a concentration-dependent fashion with IC_{50} values of 14.9 and 18.6 μM, respectively (Figure 3A and B). As shown in Figure 3(C and D), VDC treatment caused an arrest at the G_2/M phase of the cell cycle. In control cells 37.2% (BT-20) and 29.3% (U373) of the cells, respectively, were in the G_0/G_1 phase of the cell cycle, while 19.0% (BT-20) and 31.1% (U373) of the cells were in the G_2/M phase of the cell cycle. In VDC-treated cells these numbers were BT-20; G_0/G_1=20.0%, G_2/M=48.0% and U373; G_0/G_1=11.5%, G_2/M=52.8%.

The ability of these compounds to inhibit the proliferation of human cancer cells and cause a G_2/M arrest in cell cycle progression prompted the hypothesis that they likely affect mitotic spindle formation. To test this hypothesis, we examined mitotic spindles of vehicle-treated and vanadocene-treated BT-20 breast cancer cells using confocal laser scanning microscopy. Whereas in vehicle-treated control cells mitotic spindles were organized as a bipolar microtubule array and the DNA was organized on a metaphase plate (Figure 4A), VDC-treated (Figure 4B) and VDacac-treated (Figure 4C) BT-20 cells had aberrant monopolar mitotic structures where microtubules were detected only on one side of the chromosomes and the chromosomes were arranged in a circular pattern.

In yeast, monopolar spindles have been shown to arise either from a failure of the centrosome to duplicate or a failure of the duplicated centrosomes to separate correctly.[18,19] In order to distinguish between these two possibilities, we labeled BT-20 cells for the spindle pole marker γ-tubulin. As shown in Figure 5, control cells showed a single focus of γ-tubulin (Figure 5B, arrows) at each pole of the bipolar mitotic spindle. In contrast, VDC-treated cells had two foci of γ-tubulin on the same side of the chromosomes (Figure 5D, arrows) resulting in a broad centrosome at one pole (Figure 5C). All monopolar spindles examined had two foci of γ-tubulin labeling consistent with a mechanism in which the centrosomes duplicate but do not separate properly to form a bipolar spindle. To further confirm the absence of a centrosome on the other side of the spindle we examined the localization of the mitotic apparatus protein NuMA. As is shown in Figure 6, in control cells NuMA localizes in a crescent-shaped pattern at both poles of the mitotic spindle. In contrast, in cells treated with VDC, NuMA only localized to one side of the mitotic spindle confirming the lack of a functional centrosome on the other side of the spindle.

The formation of monopolar mitotic spindles can be broken down into two categories: those without replicated centrosomes and those with replicated centrosomes. The first category is exemplified by those caused by mutations of *mps1/mps2*[18] and *cdc31*[20] in yeast. In these cases, the spindle pole body, the yeast equivalent of the animal cell centrosome, fails to duplicate properly and a monopolar spindle is formed. The second category includes the *aur*[19] and *dal*[21] mutations in *Drosophila*. In this case the centrosome duplicates but does not separate properly, forming a monopolar spindle. VDC-generated monopolar spindles fall into the second category.

Labeling cells with the invariant centrosomal marker γ-tubulin clearly shows two centrosomes at the single pole.

These results establish vanadocenes as a new class of antiproliferative agents which block centrosome separation in mitotic cells. These compounds warrant further investigation as antiproliferative agents.

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