G-quadruplex stabilizers such as telomestatin and HXDV bind with exquisite specificity to G-quadruplexes, but not to triplex, duplex, or single-stranded DNAs. Studies have suggested that the antiproliferative and possibly anti-tumor activities of these compounds are linked to their inhibitory effect on telomerase and/or telomere function. In the current studies, we show that HXDV, a synthetic analog of telomestatin, exhibits antiproliferative activity against both telomerase-positive and -negative cells and induces robust apoptosis within 16 h of treatment, suggesting a mode of action independent of telomerase. HXDV was also shown to inhibit cell cycle progression causing M-phase cell cycle arrest, as evidenced by accumulation of cells with 4N DNA content, increased mitotic index, separated centromeres, elevated histone H3 phosphorylation at Ser-10 (an M-phase marker), and defective chromosome alignment and spindle fiber assembly (revealed by time-lapse microscopy). The M-phase arrest caused by HXDV paralleled with reduction in the expression level of the major M-phase checkpoint regulator Aurora A. All these cellular effects appear to depend on the G-quadruplex binding activity of HXDV as its non-G-quadruplex binding analog, TXTLeu, is completely devoid of all these effects. In the aggregate, our results suggest that HXDV, which exhibits anti-proliferative and apoptotic activities, is also a novel M-phase blocker, with a mode of action dependent on its G-quadruplex binding activity.

A G-quadruplex is a four-stranded structure consisting of a stack of G-quartets (1). Accumulating evidence has suggested the existence of the G-quadruplex conformation for telomeric DNA sequences both in vitro and in vivo (1–4). Recent studies have also demonstrated that a set of telomere-derived RNA transcripts containing mainly UUAGGG repeats with heterogeneous size ranging from 100 bases to 9 kilobases were found at the telomere regions (see review, see Ref. 5). These telomeric RNA sequences have also been proposed to exist in their G-quadruplex conformation (5). Other DNA (e.g. a c-MYC promotermoter sequence) (6) and RNA sequences (e.g. fragile X mental retardation protein (FMRP) mRNA) (7) have also been demonstrated to readily adopt the thermodynamically stable G-quadruplex conformation in vitro, although their existence in vivo remains unclear. Additionally, the existence of numerous G-quadruplex binding proteins (e.g. FMRP and yeast RAP1) (7, 8) and G-quadruplex-specific enzymes (e.g. WRN helicase and G-quadruplex-specific endonuclease) (9, 10) has also been well documented. Thus, the notion that the G-quadruplex conformation may play an important role in biology is emerging.

G-quadruplex stabilizers, which stabilize the G-quadruplex conformation in vitro, are known to exhibit cytotoxicity against tumor cell lines (11–14). A large number of G-quadruplex stabilizers have been reported in the literature (reviewed in Refs. 2 and 15), among them, telomestatin, a naturally occurring macrocycle isolated from Streptomyces anulatus (16), appears to be the most specific (17). Telomestatin, which contains seven oxazole and one thiazoline rings, has been shown to induce apoptosis of tumor cells and exhibit anti-tumor activity in mice (11, 12, 18). It has been suggested that telomestatin kills tumor cells through its perturbation of telomerase function and/or telomere structure (e.g. shortening of 3’ G-overhangs) (11, 12). The scarcity of telomestatin, coupled with difficulty in its total synthesis (19), have led to the development of a facile synthetic scheme, leading to the synthesis of structurally similar macrocycle-based compounds (20–22), among which HXDV, a hexaoxazole macrocycle (Fig. 1A), has been shown to exhibit exquisitely specific binding to G-quadruplexes in vitro through a proposed capping mechanism (see supplemental Fig. S1A) (16).

Here we study the mode of action of this novel G-quadruplex stabilizer, HXDV. Surprisingly, HXDV was found to exhibit anti-proliferative activity against many tumor cells independently of their telomerase status. In addition, HXDV induced robust apoptosis within 16 h of treatment. These results suggest that HXDV inhibits cell growth and induces apoptosis through a telomerase-independent mechanism. HXDV was also shown to perturb cell cycle progression, resulting in accumulation of cells at the M-phase of the cell cycle based on results obtained from multiple approaches. Interestingly, the major M-phase checkpoint regulator Aurora A was down-regulated by HXDV. All these cellular effects appear to depend on the G-quadruplex binding activity of HXDV. In the aggregate, our results suggest that HXDV is a novel M-phase blocker, with a mode of action dependent on its G-quadruplex binding activity.
G-quadruplexes and Cell Cycle Progression

![Chemical structure of HXDV](figure1a)

**FIGURE 1.** HXDV exhibits telomerase-independent anti-proliferative activity. A, the chemical structure of HXDV. A macrocyclic compound contains six oxazole moieties. B, the length of the telomeric 3' overhang is not affected by HXDV treatment. Lanes 1, no genomic DNA. Lane 2, (TTAGGG)_6 (1 nmol) was used as template and served as a positive control. Lanes 2 and 4, genomic DNA was treated without (labeled C) or with (labeled C+ExoI) exonuclease I prior to the oligonucleotide ligation assay. Lanes 5-8, genomic DNA isolated from cells treated with 5 μM VP16 (lanes 5 and 6) or 3 μM HXDV (lanes 7 and 8) for 1 day (labeled 1D) or 2 days (labeled 2D). The sizes of the ligated products are indicated on the right. C, HXDV does not cause Pot1 redistribution. Upper panel, a plasmid expressing POT1-EGFP under the regulation of the tetracycline-controlled promoter. Middle panel, transfectants of HEK 293 cells were first treated with tetracycline (2 μg/ml, labeled Tet) or with (labeled Tet+) for 24 h, followed by immunoprecipitation (IP) with either anti-hemagglutinin (HA) (lanes 1 and 2) or anti-GFP (lanes 3 and 4) antibody. The molecular mass of the fusion protein is around 100 kDa. Bottom panel, the distribution of the POT1-EGFP fusion protein in HET 293T cells after HXDV (3 μM) treatment was monitored by time-lapse microscopy.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Plasmids containing histone H2B-EGFP and PA-GFP-α-tubulin cDNA were purchased from Addgene (plasmid 11680, 12296, Addgene Inc.). The DNA fragment encoding the α-tubulin fusion protein was excised (by XhoI/BamHI double digestion) from the plasmid DNA expressing the PA-GFP-α-tubulin fusion protein and cloned into pEYFP-C1 vector. Plasmids expressing H2B-EGFP and EYFP-tubulin were transfected into HEK 293 cells followed by selection with G418 for 2 weeks. The POT1 cDNA fragment was obtained by PCR of the pcDNA5/FRT/hPOT1/V5-His DNA using primers (see below) containing a BamHI restriction enzyme site (underlined). The sequence of the forward primer is 5'-GCGAGAATGTAATGGGAT-CCTGCCATCCTAAATTAGA, and, the reverse primer 5'-TGCTAAA-TTGGATGGCGAGATCCGAAT-TACATCTTCTGC. The PCR product was digested with BamHI, and cloned into the pcDNA5/FRT/TO/HA/MCS/EGFP vector, then transfected into HEK 293 cells. Mouse monoclonal anti-γ-tubulin and anti-γ-H2AX antibodies were purchased from Sigma and Upstate, respectively. Polyclonal anti-H3 S10P, Aurora A/AIK, phospho-CENP-A (Ser-7), phospho-Aurora A (Thr-288)/Aurora B (Thr-232)/ Aurora C (Thr-198), and anti-Cdc2 Y15P antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal anti-PARP1 antibody was purchased from Santa Cruz Biotechnology. HXDV was synthesized as described previously (20).

**Caspase Activation and Cytotoxicity Assays**—About 2.5 x 10^5 cells were treated with HXDV for 18 h. Transfected cells were then stained with FITC-VAD-fmk (10 μM; Promega, Madison, WI) for 15 min for detecting caspase activation. For double staining, FITC-VAD-fmk-stained cells were washed with PBS and fixed with 66% ethanol at 4 °C for 30 min. Cells were then treated with RNase A (0.1 mg/ml) and stained with propidium iodide (25 μg/ml) for 15 min. The stained cells were then subjected to analysis by fluorescence-activated cell sorting (FACS). Cytotoxicity was measured by a 4-day continuous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (23).

**Indirect Immunofluorescence**—KB-3-1 cells were seeded on glass coverslips at about 30% confluence and cultured in a 5% CO_2 incubator for 16 h, followed by HXDV treatment. Cells were fixed with ice-cold acetone/methanol (1:1) fixation reagent for 20 min, washed with 1× PBS twice, then incubated in a blocking solution (1% bovine serum albumin in PBS buffer) for 30 min at 37 °C. Cells were incubated in mouse anti-γ-tubulin antibody, washed, then incubated in anti-mouse IgG-Cy3 antibody for 1 h at room temperature. After the PBS wash, mounting medium was added to the coverslip and sealed.

**Mitotic Spread/Index**—Cells treated with or without HXDV were trypsinized, washed with 1× PBS buffer twice, then incubated in 75 mM KCl solution at 37 °C for 10 min. Cells were

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3 The abbreviations used are: EGFP, enhanced green fluorescent protein; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; FMRP, fragile X mental retardation protein; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; HEK, human embryonic kidney; EYFP, enhanced yellow fluorescent protein; CENP-A, centromeric protein A; PA, photo-activatable.
pelleted and resuspended in 0.5 ml of a fixation reagent (3:1, methanol/acetic acid) and 4',6-diamidino-2-phenylindole (final concentration 0.5 µg/ml). Cells in the fixative were then dropped from about 18 inches high onto angled, humidified microscope slides, followed by air drying for at least 30 min. The mounting medium was added to the slide, followed by covering with a coverslip.

Time-lapse Microscopy—Before time-lapse imaging, HEK 293 cells expressing GFP fusion proteins were plated on a 35-mm culture dish and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in a CO₂ incubator for 12 h. After HXDV addition, the plate was placed on the viewing stage of an inverted microscopy (Olympus) with a motored XY precision stage surrounded by a chamber. To maintain cell activity, the temperature was controlled at 37 °C and 5% CO₂ was provided inside the chamber. The shutter of the digital camera was controlled using IPLab 3.2 software, and digital pictures were taken every 3 min over a 16-h period.

Oligonucleotide Ligation Assay—To measure the length of telomeric 3’ G-tails, genomic DNA was isolated from KB-3-1 cells treated with or without HXDV, followed by an oligonucleotide ligation assay according to the published procedure with slight modifications (24). Briefly, reactions (20 µl each) containing genomic DNA (5 µg), [γ-32P]ATP-labeled CA18 ((CCCTAA)₃)( 2,6-diamidino-2-phenylindole (DAPI), and Taq DNA ligase (2 µl, 40 units/µl, New England BioLabs) in 1× Taq DNA ligase buffer (20 mM Tris, pH 7.6, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM NAD, and 0.1% Triton X-100) were incubated at 45 °C for 5 h, followed by addition of 15 µl of an alkaline formamide dye solution (90% formamide, 10 mM sodium hydroxide, and 1 mM EDTA) and electrophoresis analysis in 8% urea gel.

RESULTS

HXDV Exhibits Anti-proliferative Activity against Tumor Cells Independently of Their Telomerase Status—HXDV was shown to exhibit anti-proliferative activity against many tumor cells with IC₁₀₀ values varying in a narrow range of 0.2 to 0.6 μM (Table 1). The cell lines employed in this study were divided into two groups: telomerase-positive (labeled TERT+), telomerase-negative (labeled TERT−), and telomere-negative (labeled TERT−). SAOS-2 (a human epithelial-like osteosarcoma cell line), GM847 (an SV-40 transformed human fibroblast cell line), and 2RA (a subline derived from SV40-transformed human lung fibroblasts, WI38) are known to be telomerase-negative (TERT−) and employ the alternative lengthening of the telomere pathway for telomere maintenance (25–27). Other cell lines are telomerase-positive (TERT+). As shown in Table 1, both TERT+ and TERT− cells were equally sensitive to HXDV, suggesting that the anti-proliferative activity of HXDV is independent of the telomerase status, a result different from that of telomestatin (18). The notion that HXDV inhibits cell growth through a telomerase-independent mechanism was also supported by the finding that HXDV was able to induce robust apoptosis (see Fig. 2) and cell cycle arrest (see Fig. 3) in HeLa and KB-3-1 cells within 16 h of treatment because telomerase inhibition is expected to cause slow inhibition of cell growth after multiple cycles of cell division (28).

It has been suggested that telomestatin could target the telomeric 3’ G-rich overhang (e.g. by stabilizing its G-quadruplex conformation) and thereby perturb telomere structure and function (e.g. Pot1 redistribution and shortening of the 3’ overhang) (11). However, unlike telomestatin, HXDV (3 μM for 1 and 2 days) treatment did not cause significant shortening of the length of the 3’ G-overhangs (Fig. 1D). In addition, no significant Pot1 redistribution was observed in cells treated with HXDV (3 μM) as revealed by time-lapse microscopy (see Fig. 1C, compare images taken at 0.5 and 16 h).

It has been shown that perturbation of the telomere structure (e.g. expression of dominant-negative TRF2) induces ATM-dependent apoptosis, presumably as a result of telomere deprotection and the consequent formation of DNA double strand breaks at chromosome ends (29). However, as shown in Table 1, HXDV exhibited about equal antiproliferative activity against ATM+ (Y25 and L40) and ATM− cells (PEB7 and L3). These results provide indirect support for the notion that HXDV does not induce telomere deprotection.

HXDV Induces Apoptotic Cell Death—Several G-quadruplex stabilizers including telomestatin have been shown to induce apoptosis (11, 12, 18). To determine whether HXDV induces apoptosis, several apoptosis assays were performed. First, PARP1 cleavage was measured and shown to increase with time in KB-3-1 cells treated with HXDV (Fig. 2A). PARP1 cleavage was shown to depend on caspase activation because co-treatment with the pan-caspase inhibitor, Z-VAD-fmk, largely abolished PARP1 cleavage (Fig. 2A, labeled CI). However, the non-G-quadruplex binding analog, TXTLeu (see supplemental Fig. S1B, for the structure), did not induce any detectable apoptosis signals even at a concentration as high as 10 μM (Fig. 2A, right panel). Additionally, KB-3-1 cells treated with HXDV for 16 h were shown to elevate the population of cells with caspase activation from 3.8 to 36%, as monitored by FACS analysis of FITC-VAD-fmk-stained KB-3-1 cells (Fig. 2B). As positive controls, VP-16 and CPT, both of which are well characterized DNA damaging agents and known to induce apoptosis (30), were shown to induce caspase activation under the same conditions.
Finally, DNA fragmentation, a characteristic apoptosis end point, was detected by monitoring the sub-G1 population in HXDV-treated HeLa cells using FACS (Fig. 2C). The sub-G1 population of HXDV-treated HeLa cells increased from 8 to 37% in a caspase-dependent manner (Fig. 2C, labeled Cl). Together, these results suggest that HXDV induces rapid (within 16 h of treatment) and robust apoptosis.

**HXDV Perturbs Cell Cycle Progression**—HXDV was shown to cause perturbation of cell cycle progression of KB-3-1 cells (Fig. 3A). KB-3-1 cells treated with HXDV (3 μM) for 16 h were
shown to exhibit a significant accumulation of the G2/M population (from 20 to 49%) and a slight increase in S-phase population (from 17 to 23%) (Fig. 3A). As a positive control, KB-3-1 cells were also treated with VP-16 (10 μM) for 16 h (topoisomerase II poisoning DNA damaging agent) (31) and shown to accumulate the G2/M population (20 to 50%) as expected. G2/M arrest is known to be caused by either DNA damaging agents (G2 arrest) or tubulin inhibitors (M-phase arrest). To determine whether HXDV-induced G2/M arrest is due to DNA damage, the DNA damage signal, γ-H2AX, was measured in KB-3-1 cells. As shown in Fig. 3B, HXDV, like VP-16, induced γ-H2AX in KB-3-1 cells, indicative of the formation of DNA double strand breaks. However, further analysis indicated that HXDV-induced γ-H2AX, unlike VP-16-induced γ-H2AX, was completely abolished by co-treatment with the pan-caspase inhibitor, Z-VAD-fmk (Fig. 3B, labeled CI). It is well known that massive DNA double strand breaks and hence DNA damage signals such as γ-H2AX could be generated as a consequence of apoptotic nuclease-induced chromosomal DNA fragmentation (32). Consequently, the HXDV-induced γ-H2AX signal may be due to a secondary event, resulting from apoptosis-induced DNA fragmentation rather than a direct HXDV-induced DNA damage.

It has been shown that phosphorylation of histone H3 at Ser-10 (H3S10P) (33) and dephosphorylation of Cdc2 at Tyr-15 (Cdc2Y15P) (31, 34) are indicative of transition between G2- and M-phases. As shown in Fig. 3B, VP-16-treated KB-3-1 cells exhibited dramatic reduction of the H3S10P level, whereas HXDV-treated cells exhibited a concentration-dependent increase of H3S10P, suggesting that HXDV promotes whereas VP-16 inhibits G2/M transition. Consistent with this notion, HXDV was shown to cause a decrease, whereas VP-16 treatment cause an increase, in the phosphorylation of Cdc2 at Tyr-15 (Fig. 3B).

It is well known that the centrosomes are duplicated in S-phase and become separated in M-phase. Consequently, G2-arrested cells are expected to exhibit duplicated but not separated centrosomes, whereas M-phase arrested cells exhibit duplicated and separated centrosomes. As shown in Fig. 3C (see the doublets pointed by arrows in the left panel with higher magnification), HXDV-treated cells with condensed chromatin show separated centrosomes (right panel).
shown to increase the mitotic index (percentage of cells with condensed chromosomes) about 5-fold from 3.1 to 16% (Fig. 4A). It should be noted that chromosome morphology of the HXDV-arrested cells at M-phase is different from the tubulin inhibitor, colcemid (Fig. 4A, arrows). Instead, the morphology of HXDV-treated cells is similar to dimethyl sulfoxide-treated cells that only showed a normal percentage of M-phase population (about 3%).

The possibility that HXDV may arrest cells at the M-phase was further examined by time-lapse microscopy. HEK 293 cells expressing H2B-EGFP fusion protein were employed to monitor chromosome dynamics using time-lapse microscopy. As shown in Fig. 4B, an untreated 293 cell entered mitosis through progressive chromosome condensation (from 0 to 30 min), followed by alignment of chromosomes at the metaphase plate (45 min), separation of the chromosomes (60 min), and de-condensation of chromosomes (75 and 90 min). By contrast, HXDV-treated cells seemed to arrest cells at the stage of chromosome condensation (either prophase or prometaphase). Some cells undergo chromosome alignment and de-alignment, suggesting a major perturbation in chromosome dynamics (Fig. 4B). We also examined the dynamic of spindle fibers during the course of M-phase progression in cells expressing EYFP-α-tubulin fusion protein by time-lapse microscopy (Fig. 4C). The duration of spindle fiber assembly in the untreated HEK 293 cells was around 60 min, and the whole process of mitosis was less than 120 min. By contrast, the HXDV-treated cell extended the duration of mitosis beyond 400 min, and exhibited a collapsed structure of spindle fibers without further progression into telophase that is normally marked by elongated spindle fibers. These results suggest that the G-quadruplex stabilizer, HXDV, can affect chromosome dynamics and spindle fiber function at M-phase of the cell cycle.

The effect of HXDV on chromosome alignment and spindle fiber assembly could suggest a dysfunction of some mitotic checkpoints. We performed experiments to focus on one of the major mitotic checkpoint kinases, the Aurora A. Aurora kinases are major M-phase checkpoint proteins, essential for many aspects of M-phase progression, including spindle assembly and chromosome alignment (35). As shown in Fig. 5A, left panel, HXDV treatment resulted in a decrease of the protein level of Aurora A. In addition, down-regulation of Aurora A by HXDV was associated with reduced phosphorylation of CENP-A at Ser-7 in a concentration-dependent manner in KB-3-1 cells. Because it has been shown that phosphorylation of CENP-A at Ser-7 by Aurora kinases is a critical step in mitosis involving centromere function and chromosome alignment (36), these results suggest that HXDV-induced down-regulation of Aurora A results in reduced CENP-A phosphorylation, which could explain in part the observed mitosis defect. Also shown in Fig. 5A, right panel, treatment of KB-3-1 cells with the tubulin inhibitors, Taxol and vinblastine, increased, rather than decreased, the protein level of Aurora A, as well as the level of phosphorylated CENP-A (Ser-7). These results suggest that HXDV arrests cells at M-phase possibly by a mechanism distinctly different from that of tubulin inhibitors. The different morphology of condensed

![Figure 4. HXDV arrests cells at the M-phase of the cell cycle.](image-url)
lysates were immunoblotted using different antibodies (anti-c-Myc, anti-Aurora A) at late phase of mitosis (37, 38). As shown in Fig. 5A, the mitotic index assay also supports this conclusion. These results suggest that the cellular effects of HXDV are likely to be the consequence of its G-quadruplex binding activity.

**DISCUSSION**

Considering the extraordinary specificity of telomestatin and HXDV in their binding to G-quadruplexes, and the likely existence of the G-quadruplex conformation associated with the human telomeric sequences (both DNA and RNA) (3–5), it seems quite likely that telomeres could be a target of telomestatin and HXDV. Indeed, a number of studies have suggested that telomestatin kills tumor cells by inhibiting telomerase and/or perturbing telomere function (18, 40). However, several lines of evidence argue against telomere dysfunction being the major mechanism for the anti-proliferative activity of HXDV. First, as mentioned earlier, the anti-proliferative activity of HXDV is independent of the telomerase status in cells (Table 1). In addition, HXDV can induce efficient apoptosis within a short period of time (e.g. 16 h), which cannot be explained by telomerase inhibition that causes critically shortened telomeres only after many rounds of cell divisions (28, 41). Furthermore, HXDV also exhibits anti-proliferative activity against Drosophila Schneider S2 and Kc cells that are known to lack traditional telomeres (i.e. no G-rich telomeric repeats) and maintain their chromosome ends through transposable elements (42). Second, the length of the single-stranded telomeric 3' overhang from the genomic DNA of cells treated with HXDV for 1 to 2 days shows no significant shortening (Fig. 1B). Third, telomestatin has been shown to delocalize telomeric protein Pot1 from telomeres in HT1080 cells (11). However, the delocalization of Pot1 proteins was not apparent in HXDV-treated HEK 293 cells when many cellular events (e.g. apoptosis and perturbation of cell cycle progression) have already occurred (Fig. 1C). In the aggregate, these results suggest that telomeres are not the major cytotoxic target of HXDV in the cell lines employed in the current study. The observed differences in their anti-proliferative mechanisms between telomestatin and HXDV could be due to the difference in cell lines employed in the studies. Alternatively, the observed differences could underscore the potential multitude of G-quadruplex conformations in vivo. These G-quadruplexes may distinguish subtle structural differences of their binding ligands.

Our results show that HXDV has a major effect on cell cycle progression, resulting in accumulation of cells at the M-phase of the cell cycle. G2/M arrest is often associated with DNA damage. Indeed, both VP-16 (a topoisomerase II poison that induces DNA double strand breaks) and HXDV are shown to induce γ-H2AX, suggesting that HXDV might cause DNA damage and therefore arrest cells at G2/M phase. However, it should be noted that HXDV-induced γ-H2AX, unlike VP-16-

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**G-quadruplexes and Cell Cycle Progression**

**A.** HXDV down-regulates the mitotic checkpoint Aurora A kinase. A, left panel, KB-3-1 cells were treated with different concentrations of HXDV (1, 3, and 10 μM) for 16 h. Right panel, KB-3-1 cells were treated with Taxol (lanes 2–4), vinblastine (lanes 5–7), or VP-16 (lanes 8–10) for 16 h. Cell lysates were analyzed by immunoblotting using various antibodies as indicated. B, HXDV reduces the protein level of Aurora A but increases its phosphorylation at Thr-288. KB-3-1 cells were treated with HXDV (1 and 3 μM) for 9 h. Cell lysates were immunoblotted using different antibodies (anti-c-Myc, anti-Aurora A T288P, anti-Aurora A, anti-H3S10P or anti-α-tubulin antibody).

Phosphorylation of Aurora A protein at Thr-288 is known to result in its degradation by anaphase-promoting complex (Cdh1) at late phase of mitosis (37, 38). As shown in Fig. 5B, HXDV treatment was shown to elevate the phosphorylation level at Aurora A (Thr-288), which paralleled the decrease in Aurora A levels, suggesting that HXDV-induced down-regulation of Aurora A may be in part due to proteasomal degradation of Aurora A. It has been suggested that G-quadruplex stabilizers can stabilize a G-quadruplex at the c-MYC promoter (6), resulting in reduced c-Myc expression (39). However, as shown in Fig. 5B, HXDV treatment did not change the protein level of c-Myc under the condition that Aurora A was down-regulated (Fig. 5B), suggesting that down-regulation of Aurora A is not the result of decreased expression of c-Myc.

The Cellular Effects of HXDV Correlate with Its G-quadruplex Binding Activity—TXTLeu (supplemental Fig. S1B), a close analog of HXDV, which has been shown to exhibit no detectable G-quadruplex binding activity (15, 16), was shown to exhibit no detectable anti-proliferative activity against tumor cell lines (see supplemental Fig. S1C). The lack of the G-quadruplex binding activity of TXTLeu compared with HXDV can be explained by a modeling study. As shown in supplemental Fig. S1, panels A and B, isolated docking models illustrate the interaction of G-quadruplexes (four potassium ions labeled in orange are modeled in the complex, each located above the central hole of a G-quartet) with HXDV (labeled magenta) and TXTLeu (labeled green) at both ends. The docking results for the isolated dockings to either top or bottom face of the G-quadruplex reveal that HXDV (top face $E_{int} = -41.4$ kcal/mol; bottom face $E_{int} = -40.6$ kcal/mol) has a lower interaction energy compared with the TXTLeu (top face $E_{int} = -33.4$ kcal/mol; bottom face $E_{int} = -36.4$ kcal/mol).

Our results show that HXDV has a major effect on cell cycle progression, resulting in accumulation of cells at the M-phase of the cell cycle. G2/M arrest is often associated with DNA damage. Indeed, both VP-16 (a topoisomerase II poison that induces DNA double strand breaks) and HXDV are shown to induce γ-H2AX, suggesting that HXDV might cause DNA damage and therefore arrest cells at G2/M phase. However, it should be noted that HXDV-induced γ-H2AX, unlike VP-16-

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induced γ-H2AX, is completely abolished by co-treatment with the pan-caspase inhibitor (Fig. 3B, labeled CI), suggesting that HXDV-induced DNA damage may be a secondary effect of apoptosis due to apoptotic nuclease-induced DNA fragmentation (43). A number of other observations (i.e. the phosphorylation status of Cdc2 and histone H3, the mitotic index increase, the separation of duplicated centrosomes in cells with condensed chromosomes, and altered chromosome alignment and spindle fibers as revealed by time-lapse microscopy) have suggested that HXDV, unlike VP-16, causes a major cell cycle arrest at M, rather than the G2-phase of the cell cycle.

Our time-lapse microscopy studies have suggested that both chromosome alignment and assembly of spindle fibers are altered in HXDV-treated cells. One possibility is that HXDV alters the attachment of the spindle fibers to the kinetochores. The assembly of the kinetochore is regulated by Aurora kinases and their substrates such as CENP-A. Indeed, our results show that the HXDV treatment perturbs Aurora A phosphorylation and reduces the protein level of Aurora A, suggesting that Aurora A and/or its upstream regulators may be a key target(s) of HXDV. The HXDV treatment also reduces the level of phosphorylated CENP-A. The centromeric protein A (CENP-A) is a histone H3 variant that replaces the canonical histone H3 in the centromeric heterochromatin region and a key component of the kinetochore inner plate proteins (44, 45). Many functions of CENP-A are dependent on its phosphorylation by Aurora kinases (e.g. A and B) because it has been demonstrated that CENP-A is initially phosphorylated at Ser-7 by Aurora A kinase in prophase, and this phosphorylation event is essential for recruiting Aurora B kinase into the centromere region and for kinetochore function (36).

The Aurora kinases are universal regulators of mitosis (35, 46). Aurora A is involved in centrosome function, including maturation and separation, and spindle assembly, whereas Aurora B is involved in the kinetochore-microtubule interaction and sister-chromatid segregation (35, 46). It has been shown that overexpression of Aurora A is linked to centrosome amplification and genomic instability, whereas its down-regulation by RNA interference or its inhibition can lead to mitotic arrest and apoptosis (47, 48). Thus, the reduced Aurora A protein level in HXDV-treated cells could be responsible for both HXDV-induced M-phase arrest and apoptosis. It is interesting to note that Aurora A fits the criteria of an oncogene: its gene is amplified in many tumor cell lines and primary tumors, and its transfection into mouse NIH-3T3 cells can lead to tumors when injected into nude mice (35).

At present, it is unclear how HXDV affects the Aurora A protein level. Considering its extraordinary specificity for binding to G-quadruplexes, the molecular target(s) of HXDV is very likely to be a G-quadruplex(es). So far, three classes of nucleic acid sequences have been suggested to have the potential to adopt the G-quadruplex conformation in vivo (2): the G-rich strand of telomeric DNA repeats, the G-repeat containing strand of some oncogene promoters (e.g. the purine-rich strand of the nuclease-hypersensitive sensitive site region of the c-MYC promoter), and the G-repeat-containing RNA sequences (e.g. the mRNA of FMRP and telomeric RNA transcripts). As discussed above, the telomeric DNA is unlikely to be the major cytotoxic target of HXDV at least in the cells employed in the current study. We also showed that HXDV induced Aurora A down-regulation as early as 9 h without affecting the c-Myc protein level (Fig. 5B). Consequently, it seems unlikely that Aurora A down-regulation is due to HXDV-mediated stabilization of the G-quadruplex conformation of the c-MYC promoter. By default, it seems more likely that the major cytotoxic activity of HXDV (as well as the M-phase inhibitory effect of HXDV) may result from its stabilization of G-quadruplex motif-containing RNAs. RNA G-quadruplexes are likely to be more prevalent than DNA G-quadruplexes because RNAs are single-stranded and RNA G-quadruplexes are in general more thermodynamically stable than their counterpart DNA G-quadruplexes (49, 50). For example, an RNA G-quadruplex motif (5′-GGGAGGGCCGGG-CUGGG-3′) has been identified in the 5′-untranslated region of human NRAS proto-oncogene mRNA, and shown to regulate the level of protein translation (49). In addition, the mRNA of the FMRP contains a G-rich motif that can be folded into a stable G-quadruplex recognized by the G-quadruplex binding motif of FMRP (7). Perhaps, HXDV may target Aurora A and/or its upstream regulators (e.g. PP2A, the phosphatase known to regulate Aurora A degradation by dephosphorylating Aurora A) (51) through its stabilization of an RNA G-quadruplex conformation. In this regard, it is interesting to point out that an excess of G-quadruplex motifs exists in both the 5′- and 3′-untranslated region of many mRNAs (52). Indeed, G-quadruplex motifs are present in the 5′-untranslated region of the Aurora A mRNA (NCBI reference sequence, NM_003600.2). In view of the abundance of RNA G-quadruplexes (52), we favor the hypothesis that HXDV may stabilize the G-quadruplex conformation of a G-quadruplex motif-containing RNA(s) leading to its altered function. We are currently testing the possibility that HXDV may inhibit Aurora A (and its upstream regulator) mRNA translation both in vivo and in vitro. Clearly, further studies are needed to identify the molecular target(s) of HXDV.

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