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Valproic Acid Inhibits Invasiveness in Bladder Cancer but not in Prostate Cancer Cells[†]

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Valproic Acid Inhibits Invasiveness in Bladder Cancer

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d) NONSTANDARD ABBREVIATIONS:

CAR – coxsackie and adenovirus receptor
CFDA SE – Carboxyfluorescein Diacetate, Succinimidyl Ester
ECL – electrochemical luminescence
H3Ac – Acetylated Histone H3
HAT – histone acetyltransferase
HDACI – histone deacetylase inhibitor
MMP – matrix metalloproteinases
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PET – polyethylene terephthalate
PVDF – polyvinylidene fluoride
SAHA – suberoylanilide hydroxamic acid
VPA – valproic acid, 2-propylpentanoic acid

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ABSTRACT

Histone deacetylase inhibitors (HDACIs) represent a promising new class of antineoplastic agents that affect proliferation, differentiation, and apoptosis in both solid and haemotologic malignancies. In addition, HDACIs can alter the expression of at least one cellular adhesion molecule, the Coxsackie and Adenovirus Receptor (CAR), in bladder cancer. Since HDACIs can increase expression of a known cellular adhesion molecule, we hypothesized that migration and/or invasion may also be affected. We evaluated this hypothesis using valproic acid (VPA), a commonly prescribed anticonvulsant recently shown to have potent HDACI activity, in the bladder cancer cell lines T24, TCC-SUP, HT1376, and RT4. Analyses of cell migration and invasion were both qualitative (fluorescent microscopy) and quantitative (static and dynamic migration/invasion assays). Our results show that acute VPA treatment (72 hours) causes a dose dependent decrease in invasion for all bladder cancer cell lines, except RT4, a noninvasive papilloma. Migration, in contrast, was not affected by VPA treatment. The inhibitory effect of VPA may be cancer type specific as there was no difference in invasion between treated and untreated prostate cancer cell lines, LNCaP, PC3 and DU145. Furthermore, when administered chronically (34 days), VPA significantly inhibits growth of T24t tumor xenografts. Our data suggest that VPA exerts some of its antineoplastic effect by inhibiting invasion, as well as tumor growth, and thus may represent a novel adjuvant strategy for patients at high risk of recurrence and/or progression of muscle invasive bladder cancer.

INTRODUCTION

Histone-DNA complexes form the basic structural unit of chromatin known as the nucleosome. In addition to organizing the eukaryotic genome, histones, via chemical modification, also play a key role in the epigenetic regulation of gene transcription (Marmorstein, 2001). Although this phenomenon was first observed over three decades ago (Allfrey, 1966), understanding of the mechanisms involved in histone modification and gene transcription has evolved only in recent years (Yoshida et al., 1990; Strahl and Allis, 2000). We now know that histone acetylation status is determined by the interplay of two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDAC). Acetylation of the histones, especially H3 and H4, by HATs is associated with active transcription, while deacetylation by HDACs is linked to gene repression. Furthermore, treatment of cancer cells with histone deacetylase inhibitors (HDACIs) results in increased histone acetylation and ultimately, induction of growth arrest, apoptosis, and cell differentiation. These effects have been demonstrated in both haematologic (leukemias, lymphomas) and epithelial/solid (breast, prostate, lung, neuronal) cancer cell lines (Marks et al., 2001) and are selective for transformed cancer cells. Since much of the previous research has focused on the anti-proliferative and differentiation properties of HDACIs, comparatively little is known about their effects on cancer cell migration, invasion, and metastasis, which collectively result in the morbidity and mortality of cancer.

In our previous work we have shown that treatment of bladder cancer epithelium with HDACIs can alter the expression of at least one cell-cell adhesion molecule, the Coxsackie and Adenovirus Receptor (CAR) (Sachs et al., 2004). CAR is a member of the

Ig-type super family of cellular adhesion molecules linked to cell motility and tumor invasiveness (Bruning and Runnebaum, 2003; Philipson and Pettersson, 2004), and is expressed robustly in normal urothelium, but down regulated in a tumor stage and grade dependent fashion in clinical bladder cancer specimens (Sachs et al., 2002). This repressed expression level seemed to be regulated transcriptionally via epigenetic events (Li et al., 1999; Okegawa et al., 2001) as treatment of the bladder cancer cells with VPA *in vitro* effectively restored CAR expression (Höti et al, submitted). Hence, we postulated that treatment with VPA may lead to inhibition of bladder cancer cell migration and invasion. We also predicted that VPA might have the same effect on prostate cancer cells as well.

We now show that VPA treatment inhibits invasion in multiple bladder cancer cell lines including T24, TCC-SUP and HT1376. These effects were dose dependent, cell line specific, and independent of cell migration, which was not affected by treatment. When the same hypothesis was tested in multiple prostate cancer cell lines, there was no difference in migration or invasiveness between treated and untreated cells. These results demonstrate that the effects of VPA on invasion are cancer type specific and further suggest that the pathways critical to the development of invasion differ among various cancer types. We also show that acute (72 hour) treatment with VPA *in vitro* results in decreased in bladder cancer cell viability, especially at higher doses, and that chronic administration (34 days) of lower dose (0.05-0.4 mM) VPA significantly inhibits tumor xenograft growth *in vivo*. Collectively, these findings suggest that VPA may represent a novel adjuvant strategy for patients at high risk of recurrence and/or progression of muscle invasive bladder cancer.

METHODS

Cell Lines and Chemicals. T24, TCC-SUP, HT1376 and RT4 human bladder cancer cell lines and LNCaP, DU145 and PC3 human prostate cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in the media recommended by the ATCC following standard procedures until 70% confluent. The T24t cell line is a subline of T24, which has been multiply passaged through xenografts in athymic mice to facilitate a high tumor take rate, and was obtained as a gift from Jer-Tsong Hsieh, Ph.D in the Department of Urology at the University of Texas Southwestern Medical Center. Normal human bladder tissue was obtained from children undergoing urological procedures for benign conditions, after Institutional Review Board approval. A 1cm × 1cm piece of the dome of the bladder was obtained, and washed with Hank's Balanced Salt Solution (Cellgro, Herndon, VA) before micro-dissecting the urothelium. The urothelium was then minced into small pieces and digested with 100 Units/ml of Collagenase IV (Sigma, St Louis, MO) for 4 hours. Cells were next suspended in Eagle's minimum essential medium with L-Glutamine (Cellgro, Herndon, VA), supplemented with Insulin 1.0 U/ml, heat inactivated fetal bovine serum (FBS) 10% (GIBCO, Carlsbad, CA), a 1% mixture of antibiotic/antimycotic (containing Penicillin base 100 U/ml, Streptomycin base 100 μg/ml, and Amphotericin B 0.25 μg/ml), and 10mM HEPES buffer (Sigma, St Louis, MO). Once urothelial cells were 90% confluent, they were harvested with 0.05% trypsin/EDTA (Cellgro, Herndon, VA) and routinely passaged. A stock solution of valproic acid sodium salt (solubility: 50 mg/ml) (Sigma, St. Louis, MO) was prepared in water and filter sterilized. VPA treatment consisted of 0.6, 1.2, 2.4 and 5.0 mM VPA containing media for 72 hours. TSA

treatment (Trichostatin A) (Biomol, Plymouth Meeting, PA) consisted of 50 ng/ml containing media for 48 hours.

Cell Viability Assay. Bladder cancer cell lines T24, TCC-SUP, HT1376, and RT4 were seeded at $1x10^4$ cells/well in 96-well culture plates and incubated overnight with the appropriate media containing 10% FBS. The cells were then treated with media containing VPA (0, 0.6, 1.2, 2.4 and 5.0mM) for 72 hours. Doses of 0.6 to 5.0 mM were selected based on preliminary studies of cell viability in the applied cell lines as well as previously published studies in other non-bladder cell lines (Courage-Maguire et al., 1997; Kaiser et al., 2006). The fraction of cells surviving after acute VPA treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay (Roche, Basel Switzerland) as per manufacturer's recommendation. Reduction of MTT to a formazan salt by viable cells was quantified by SPECTRAmax plus (Molecular Devices) at 570 nm. The experiments were performed at least in quadruplicates.

Caspase Assay. Equal numbers of T24 cells were seeded and incubated overnight in T75 flasks to allow attachment. The cells were then treated with media containing 0, 0.6, 1.2, 2.4, 5.0 mM VPA for 16 hours, washed, and lysed using the 1X Cell Lysis Buffer from the ApoAlert™ kit (BD Biosciences, San Jose, CA). Cell lysates were then transferred to a 96-well plate and mixed with Reaction Buffer/DTT Mix according to the manufacturer's protocol. Caspase activity was determined by measuring fluorescence from converted substrate using the CytoFluorII Series 4000 fluorometer (Applied Biosystems, Foster City, CA) (excitation: 380 nm, emission: 460 nm).

Western Immunoblotting. T24 cells (70 to 80% confluent) in T75 flasks were treated with media containing 0, 0.6, 1.2, 2.4 and 5.0 mM VPA or TSA 50 ng/ml for 72 hours. The cells were harvested with 0.05% trypsin/ 0.53 mM EDTA, washed in PBS and re-suspended in 100 µl M-PER (Mammalian Protein Extraction Reagent) (Pierce, Rockford, IL). Tissue from T24t tumor xenografts was homogenized with the Dounce homogenizer and also re-suspended in M-PER. The BCA protein assay kit (Bio-Rad, Hercules, CA) was used to determine total protein concentration and purified BSA (Sigma, St. Louis, MO) was used to generate the standard curve. Proteins were separated on a 12% Tris-HCL Polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to PVDF Membrane (Bio-Rad, Hercules, CA). The membrane was blocked for an hour in Blocking Buffer (100 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk, then incubated overnight with a 1:1000 dilution of rabbit anti-acetyl histone H3 (Lys 9) antibody (Cell Signaling Technology, Danvers, MA) followed by anti-rabbit IgG Peroxidase Conjugate (1:20,000) (Sigma, St. Louis, MO) for 1.5 hours at room temperature. Immunoreactive bands were detected using the ECL Plus Western Blotting detection System (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions. Monoclonal anti-β-actin (1:20,000) (Sigma, St. Louis, MO) and anti-mouse IgG-peroxidase (1:20,000) (Sigma, St. Louis, MO) were used to detect β-actin in the same blots. Anti-Cip1/WAF1/P21 mouse monoclonal IgG (Upstate, Charlottesville, VA) and anti-mouse IgG-peroxidase (Sigma, St. Louis, MO) were used to test p21 expression.

Qualitative Invasion Assessment by Fluorescent Microscopy. A modified Boyden chamber invasion assay (Woodward et al., 2002) was performed using a mixed

culture of human fibroblasts and primary urothelial cells. These cells were tagged with carboxy SNARF®-1 (Molecular Probes, Eugene, OR) and plated (1×10^4 cells/well) on top of a Matrigel-coated, fluorescence blocking, 8μm pore, polycarbonate membrane (BD BioCoat-Fluroblok™) (BD, Bedford, MA). The BioCoat-Fluroblok prevents fluorescent signal from traveling from the upper to lower chambers during microscopy. Next, T24 cells were treated with 0 or 5mM VPA for 24 hours and tagged with Vybrant™ CFDA SE (Molecular Probes, Eugene, OR). The cells were then collected by brief (~1 min.) trypsinization followed by blockage with an equal volume of trypsin inhibitor (Gibco-Invitrogen, Carlsbad, CA). Using the Trypan blue exclusion dye, 1×10^5 viable cells were added on top of the urothelial cells in each well of the upper chamber. The T24 cells were evaluated for invasion through the urothelial cell layer, coated Matrigel layer and porous membrane to reach 10% FBS enriched media in the lower wells. Fluorescent microscopy (Ziess) pictures were taken at different time intervals (1, 2, 4, 8 and 24 hours) from the bottom side of fluorescence blocking membrane, and therefore showed only the invading cells.

End-Point (Static) Quantitative Migration and Invasion Assays. Migration and invasion studies were performed simultaneously for optimization and comparison using the ChemoTx® system (Neuro probe, Gaithersburg, MD) which consisted of a 96-well plate covered by an 8μm porous, polycarbonate membrane. Both migration and invasion assessments were performed on the same plate, with readings taken at 0.5 and 8 hours. The T24, TCC-SUP and HT1376 cell lines were treated for 72 hours with VPA (0, 1.2 or 5.0mM) or TSA (50ng/ml) then tagged with Vybrant™ CFDA SE (Molecular Probes, Eugene, OR) as per manufacturer's recommendations. The cells were then harvested by

brief (~1 min) trypsination followed by blockage with an equal volume trypsin inhibitor (Gibco-Invitrogen, Carlsbad, CA). To evaluate migration, 5×10^4 cells/well were plated on top of the membrane in triplicates or quadruplicates in designated wells and allowed to migrate towards 20% FBS enriched media in the lower wells. To assess invasion, 1×10^5 cells/well were plated on top of the Matrigel coated membrane in triplicates or quadruplicates and allowed to migrate towards 20% FBS enriched media in the lower wells. A CytoFluor™, fluorescence multi-well plate reader (Perspective Biosystems, Framingham, MA) was used to quantify cell migration and invasion into the lower wells at different time points. The choice of 10% or 20% FBS as a chemo-attractant and 5×10^4 or 1×10^5 cells/well for the migration and invasion assays, respectively, were chosen based upon results of a pilot optimization study (data not shown) on T24 cells.

Real-Time (Dynamic) Quantitative Invasion and Migration Assays. T24 cells were treated with 0, 0.6, 1.2, 2.4 or 5 mM VPA for 72 hours then placed in serum-free media overnight. Next, a 10 μM staining solution was prepared by diluting 10 mM Vybrant™ CFDA SE (Molecular Probes, Eugene, OR) with PBS. Cells were pre-labeled in the solution for 15 minutes at 37°C, then trypsinized, counted, and resuspended in serum-free media. A total of 1×10^5 cells in 500 μl serum-free media were loaded into each well of the upper chamber of a modified Boyden chamber (BD BioCoat™ FluoroBlok™ Invasion System) and 750 μl of 20% FBS enriched media was added to each bottom well. The polycarbonate membrane was either coated or uncoated with Matrigel matrix to simulate invasion and migration, respectively. Cells that crossed through the 8μm pore size polycarbonate membrane were detected in real-time using a multi-plate fluorometer (CytoFluor II plate reader) (excitation: 492 nm, emission: 517

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nm). To account for possible effects of CFDA SE on the biological function of cells, each reading was normalized to that taken from a regular 24-well Falcon plate with the same number of CFDA SE-tagged cells. Data are shown as the mean percent cell invasion which is calculated as the mean fluorescent unit (FU) of cell invasion through the Matrigel coated membrane divided by the mean FU of cell migration through the Matrigel uncoated control membrane × 100, as recommended by the manufacturer (BD BioCoat™ FluoroBlok™ Invasion System). Percent cell invasion (invasiveness) was calculated by **Equation #1**:

$$\% \text{ Invasiveness} = \frac{\text{Mean FU of cells invading through Matrigel coated membrane}}{\text{Mean FU of cells invading through Matrigel uncoated membrane}} \times 100$$

Animal Experiments. T24t cells were grown to 80 to 90% confluence and harvested. Cells were resuspended in 1X Phosphate Buffered Saline pH 7.4 (BioSource, Rockville, MD), mixed 1:1 with Matrigel (BD Biosciences, Palo Alto, California) and injected ($1x10^6$ cells/injection) subcutaneously into the lateral flanks of male athymic nu/nu mice. Once palpable tumors were established, animals were randomized into control and treatment arms with the latter receiving 0.4% VPA in drinking water. Tumor measurements were taken three times per week until animals were sacrificed and tumors harvested on day 35.

Statistical Analysis. Data are presented as means± standard error of the mean. All statistical analyses were performed on an IBM compatible computer, running GraphPad Prism™ v4.0 or GraphPadInStat™ v3.0 (GraphPad, Inc) on Windows XP. All experiments were done with at least four and as many as twenty replicates. All error bars

represent the standard error of the mean. Statistical significance was calculated using the Student's t-test, Wilcoxon Rank Sum test, or repeated measures analysis of variance (RMANOVA) with post-hoc testing when appropriate. A value of $p < 0.05$ was considered statistically significant and marked on graphs with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

RESULTS

VPA Increases Histone H3 Acetylation and p21 $^{WAF1/CIP1}$ Expression

T24 bladder cancer cells were treated with VPA (0, 0.6, 1.2, and 2.4 mM) for 72 hours and H3 acetylation was assessed by Western blot. As shown in Figure 1A, cells exhibit a significant increase in levels of acetylated histone H3 compared to the untreated control. The effect was dose dependent with 2.3, 3.5, 4.8, and 4.8 fold increases in H3 acetylation with 0.6, 1.2, 2.4 and 5.0 mM VPA treatment, respectively. Furthermore, the maximal effect (4.8 fold) achieved by the highest VPA doses (2.4 and 5.0 mM) was comparable to that obtained in the positive control sample treated with 50 ng/ml of TSA (3.3 fold).

As secondary confirmation of HDACI activity, expression of p21 $^{WAF1/CIP1}$, a cell cycle kinase inhibitor, was also evaluated since previous studies have demonstrated that hydroxamic acid-based HDACIs, such as suberoylanilide hydroxamic acid (SAHA), can increase both p21 $^{WAF1/CIP1}$ mRNA and protein expression in a time and dose dependent manner (Richon et al., 2000). T24 cells were treated with VPA (0, 1.2, and 5.0 mM) for 4, 8, 16, and 24 hours and analyzed for p21 $^{WAF1/CIP1}$ expression on Western blot. As expected, VPA up regulates p21 $^{WAF1/CIP}$ expression in a time and dose dependent manner with maximal effects at 16 hours (Figure 1B). T24 cells cultured with VPA for greater

than 16 hours undergo cell death with up to a 40% decrease in cell viability with the 5.0mM dose at 72 hours (Figure 2A). We also found that p21 expression increased by over 300% in untreated cells at 24 hours. This may have resulted from cell overgrowth since p21 expression can be regulated by cell contact (Evers et al., 1996; Ritt et al., 2000) and cells were plated at 70 to 80% confluency and allowed to grow overnight. Nonetheless, it is clear that VPA causes a dose dependent increase in p21 expression, a known HDAC inhibition effect.

Effect of VPA on Bladder Cancer Cell Survival

In order to further evaluate HDACI activity of VPA in bladder cancer cells, we performed cell viability assays on a panel of bladder cancer cell lines, including T24 (anaplastic, invasive), TCC-SUP (anaplastic, invasive), HT1376 (invasive) and RT4 (non-invasive papilloma). These cell lines represent the spectrum of disease from highly invasive, anaplastic carcinoma to non-invasive papilloma. Cells were treated with VPA (0, 0.6, 1.2, 2.4, 5.0 mM) for 72 hours then evaluated by MTT. As shown in Figure 2A, cell viability decreases in a dose-dependent manner in all four cell lines. However, the decrease in cell viability with 72 hour treatment is modest except at the higher dose (5.0 mM). Of note, the IC_{50} of VPA is greater than 5.0 mM in most cell lines, which is at the level of dose-limiting neurotoxicity in the clinical setting.

Of the cells that did not survive VPA treatment, we sought to determine whether activation of apoptosis was involved. In this experiment, T24 cells were VPA treated (0, 0.6, 1.2, 2.4 or 5.0 mM) for 24 hours, harvested, and assessed for caspase activity. VPA treatment resulted in induction of both Caspase 2 and 3 enzyme activity (Figure 2B). However, maximal enhancement was less than two-fold even at the highest dose

(5.0mM). In addition, there appeared to be little change in Caspase 8 or 9 activities. This suggests that acute treatment (72 hours) with VPA results in a small fraction of cells undergoing cell death via apoptosis, likely through the intrinsic (mitochondrial) pathway given the relative elevation of Caspase 2. This finding is consistent with previous studies which have demonstrated HDACI induction of apoptosis often occurs via the intrinsic pathway (Peart et al., 2003).

Qualitative Analysis of VPA Effect on T24 Invasion

The effects of HDACIs on cancer cell properties such as invasion and migration have not been well characterized. In our previous work, we have shown that HDACIs can alter the expression of at least one cell-cell-adhesion molecule, CAR, in bladder cancer cells (Sachs et al., 2004). Based on this finding, we sought to determine if VPA, by extension, may affect invasion and migration properties. In order to investigate this hypothesis, we used both qualitative and quantitative methods and multiple bladder cancer cell lines.

A modified Boyden chamber and fluorescently labeled T24 cells were applied to evaluate the effect of VPA on cell invasion *in vitro*. T24 cells were treated with VPA (0 or 5.0mM) for 72 hours, fluorescently tagged, and plated in equal numbers in the upper wells of a modified Boyden chamber. The chamber consisted of a urothelial layer covering a matrigel-coated porous membrane, and a lower well containing FBS enriched media. The urothelial and matrigel layers simulate the lamina propria and basement membrane, respectively. Invading cells were imaged in the lower chamber through fluorescent microscopy. The results are presented as paired, time-lapsed serial photomicrographs of treated vs. untreated T24 cells. As shown in Figure 3, acute VPA

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treatment leads to a qualitative decrease in the invasion rate of T24 cells, an effect which is evident within 2-4 hours and up to 24 hours.

End-point Analysis of VPA Effect on T24, TCC-SUP and HT1376 Invasion

We next performed a static or end-point invasion assay to provide quantitative assessments of VPA effect on bladder cancer cell invasion. T24, TCC-SUP and HT1376 cells were treated with VPA (0, 1.2, 5.0 mM) or TSA (50ng/ml) for 72 hours, fluorescently tagged, then harvested and plated on a matrigel-coated, porous, polycarbonate membrane. Cell invasion to the lower chamber was quantified by fluorescence emission using a multiplate fluorometer (Figure 4). Invasion, expressed as mean RFU, decreases with VPA treatment in a time dependent manner, with maximal effects at 8 hours for both 1.2 and 5.0mM doses, except in HT1376 which exhibited inhibition only at the higher dose. Furthermore, the extent of inhibition is comparable to that achieved with 50ng/ml of TSA. This confirms our observations from the qualitative invasion assay and further demonstrates that VPA can decrease invasion not only in T24 cells, but in multiple bladder cancer cell lines as well.

Real-time Analysis of VPA Effect on T24 Invasion and Migration

To further investigate the effects of VPA on bladder cancer cell invasion and migration, we used the modified Boyden chamber in a dynamic invasion assay to monitor cell movement in real-time. Passage of cells through the matrigel-coated membrane simulated invasion through the basement membrane, while passage through the uncoated membrane simulated cellular migration only. The T24 cell line was selected for initial evaluation as it showed promising results in the static, end-point invasion assays. T24 cells were treated with VPA (0, 0.6, 1.2, 2.4, 5.0 mM) for 72 hours, fluorescently tagged,

then harvested and plated in the upper wells of a modified Boyden chamber. Cell movement was recorded in real-time for up to 72 hours using a multiplate fluorometer. The data are shown in Figure 5 as percent migration, percent invasion, and percent ***invasiveness***. These experiments demonstrate that acute treatment with VPA decreases T24 cell invasion in a dose dependent manner. The reductions in invasion were statistically significant for all tested concentrations and varied from approximately 40 to 85% between the lowest and highest doses, respectively. In contrast, VPA seemed to cause a slight increase in T24 migration at 1.2 and 5.0 mM; however, these effects were neither dose dependent nor statistically significant (Figure 5B). As an additional measure of VPA effect, the percent invasiveness was calculated and compared among treated and untreated cells. The results are shown in Figure 5C and were similar to those for invasion; acute VPA treatment significantly decreases percent invasiveness of T24 cells in a dose dependent manner.

Real-time Analysis of VPA Effect on T24, TCC-SUP, HT1376 and RT4 Invasion and Migration

After testing the effect of VPA on T24 invasion and migration, we then interrogated this effect on the other cell lines including TCC-SUP, HT1376 and RT4, via the dynamic invasion assay. Using the same protocol described in the previous section, the cells were treated with VPA (0, 1.2 or 5.0 mM), fluorescently labeled, and monitored in real-time for migration and invasion. Data are shown in Figure 6 as percent invasiveness of treated cells relative to untreated. Our results show that acute VPA treatment decreases invasiveness in the TCC-SUP and HT1376 bladder cancer cell lines, but has no effect on the non-invasive RT4 cell line. The effect was dose dependent for

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both cell lines and statistically significant after 8 hours for all doses in TCC-SUP, but lacked sustained inhibition in HT1376. The RT4 cell line demonstrated minimal invasion even in the absence of VPA, a phenomenon which may be attributed to its derivation from noninvasive tumor. Taken together, these data confirm that VPA administered acutely for 72 hours can inhibit invasion in multiple bladder cancer cell lines *in vitro*.

Real-time Analysis of VPA Effect on Prostate Cancer Cell Invasion and Migration

In light of our findings from the dynamic invasion assay with bladder cancer cells, we performed the same experiment on prostate cancer cell lines LNCaP, DU145 and PC3 to determine if VPA could mediate inhibition of invasion in other solid tumors of the genitourinary system. Prostate cancer cells were treated with VPA (0, 1.2 and 5.0 mM) for 72 hours, labeled, and evaluated for migration and invasion as described in the previous experiment. Our results show that acute VPA treatment has no effect on prostate cancer cell invasiveness (Figure 7). This finding was true for all cell lines tested and contrasts our results from identical experiments performed in bladder cancer cells. This suggests that VPA effects on invasiveness may be tissue specific.

Effect of VPA on T24t Tumor Xenografts

In order to evaluate the effects of VPA on bladder cancer cells *in vivo*, we established an animal model using tumor xenografts. T24t bladder cancer cells were injected (1×10^6 cells / injection) subcutaneously into the lateral flanks of male, athymic nude mice. Once tumors were palpable, animals were randomized into control and treatment arms, with the treatment group receiving 0.4% VPA in drinking water. Serum VPA levels were measured weekly and ranged from 8 to 67 μg/ml (0.05 to 0.4 mM, respectively). Of note, the upper limit of serum VPA concentration clinically achieved in

the treatment of seizure and bipolar disorders is typically thought to be 135 μg/ml (0.94 mM); above this level, there is significant risk for toxicity including thrombocytopenia and somnolence. We were therefore in a clinically applicable dose range. Tumor volumes were measured 3 times per week until time of sacrifice during week 5. Our results show that VPA administered chronically and at a clinically relevant dose significantly inhibits T24t tumor xenograft growth with a 40% reduction in treated animals when compared to untreated controls (Figure 8A).

Further confirmation of VPA activity was obtained through Western blot analysis of proteins harvested from tumor tissue. As shown in Figure 8B, tumors treated with VPA had up regulation of acetylated histone H3 and p21 expression compared to those in the untreated controls. Relative induction of acetylated histone H3 and p21 was 3.3 and 6.5 fold, respectively, demonstrating that the effect of tumor volume reduction correlates to HDACI activity in vivo.

DISCUSSION

Bladder cancer is the fourth most common malignancy diagnosed in men and the ninth most common in women, with more than 63,210 new cases in 2005 (American Cancer Society, 2005) and an estimated annual expenditure of $2.9 billion in the US alone (Brown et al., 2002). Approximately 70% of bladder tumors present as superficial lesions, and 10% to 20% of these progress to muscle-invasive lesions (Ro et al., 1992). Once invasion outside the bladder occurs, outcomes in the absence of systemic therapy are poor with overall five year survival rates ranging from 4 to 35% (Herr, 1994). Given the unfavorable prognosis associated with nonorgan confined disease, therapeutic

approaches targeted towards inhibiting invasion should have potentially beneficial effects in the prevention of muscle-invasive disease.

HDAC inhibitors are a new class of drugs shown to have anti-neoplastic activity in haematologic and solid malignancies (Sandor et al., 2002; Takai et al., 2004). A well known anticonvulsant recently discovered to have potent HDACI activity is valproic acid (VPA) (Phiel et al., 2001). Commonly used in the treatment of seizure and bipolar disorders (Loscher, 2002), VPA is well tolerated in patients and has an established safety profile (Gottlicher et al., 2001; Blaheta and Cinatl, 2002). In our previous work, we have shown that HDACIs can alter expression of at least one cell adhesion molecule, CAR, in bladder cancer (Sachs et al., 2004). In addition, loss of CAR expression has been associated with aggressive bladder cancers (Sachs et al., 2002; Matsumoto et al., 2005). For these reasons, we chose to investigate the effects of VPA on bladder cancer cell viability, motility and invasiveness. We further examined whether these effects were cancer type specific by testing prostate cancer cell lines and applicable *in vivo* using tumor xenografts.

We first sought to confirm the HDACI activity of VPA through evaluation of acetylated H3 and $p21^{WAF1/CIP1}$ expression in VPA treated T24 cells. p21 is a well known cell cycle regulatory protein and target gene of HDAC inhibition (Sowa et al., 1999; Mei et al., 2004). We found that acute (72 hour) VPA treatment induces a dose dependent increase in both acetylated H3 and p21, with maximal induction for the 5.0 mM dose at 72 and 16 hours, respectively (Figure 1). T24 cells cultured with VPA for greater than 16 hours undergo cell death, contributing to the relative decrease in p21 at 24 hours.

We next determined the effect of acute VPA on bladder cancer cell viability *in vitro*. We found that 72 hour treatment with VPA results in a dose dependent decrease in cell viability for all tested cell lines (Figure 2A). Furthermore, caspases 2 and 3, effectors of the intrinsic (Guo et al., 2002) and final common pathways, respectively, demonstrated increased activities (Figure 2B). However, enhancement was modest, suggesting that apoptosis comprises a small fraction of VPA-mediated cell death.

Following confirmation of VPA's HDACI and cytotoxic activities, we studied the effect of VPA on invasion. Cancer invasion is a complex process involving activation of proteolytic activity (i.e. matrix metalloproteinases, collagenases, etc.), alterations in cell-cell interactions and coordinated motility. Recent evidence suggests that HDAC inhibitors may affect expression of some prominent effectors in these processes (Kim et al., 2000). Using time lapse video fluorescent microscopy, we found that acute VPA treatment leads to a qualitative decrease in the invasion rate of treated versus untreated T24 cells (Figure 3). This effect was evident within 2 to 4 hours and for up to 24 hours. Subsequent quantitative end-point invasion assays confirmed these findings in multiple bladder cancer cell lines including T24, TCC-SUP and HT1376 (Figure 4). These effects were significant for the 5.0 mM dose in TCC-SUP and HT1376, and approached significance in the T24 cell line. Taken together, these data demonstrate a clear trend towards decreased invasion with the acute administration of VPA in multiple bladder cancer cell lines.

Since diminished invasion may be a nonspecific phenomenon depending on the effect of treatment on cell migration or motility (Kassis et al., 2002), we studied the effects of VPA on both migration and invasion using a real-time invasion assay.

Regardless of the approach, the results were consistent. Namely, VPA does not effect migration, but has a significant effect on invasion. Both invasion and invasiveness of T24 cells were clearly reduced in a dose dependent manner, while motility was unaffected (Figure 5A–C). Similarly, invasiveness in the TCC-SUP and HT1376 bladder cancer cell lines was reduced, though the effects were somewhat diminished and not sustained over time in HT1376 (Figure 6A–C). The lack of effect in RT4 was expected since it was derived from non-invasive papilloma. These data confirm our previous findings that VPA inhibits invasion in a dose dependent and cell line specific manner and further demonstrates that VPA directly affects invasion and not cell motility.

Given the varying potency of VPA to prevent invasion in different bladder cancer cell lines, we also interrogated whether this effect was cancer type specific. We chose to evaluate prostate cancer cell lines LNCaP, PC3 and DU145, which vary in androgen dependence and aggressiveness. In contrast to our findings in bladder cancer, VPA had no effect on invasion or migration for all tested prostate cancer cells. This may be due to the fact that invasion is the net result of alterations in multiple pathways and not merely a single pathway, ultimately leading to enhanced destruction of basement membrane and extracellular matrix. Therefore, invasion may still occur if the pathways modified by VPA are not critical to or constitute a small component of the events of invasion. Together, these findings suggest that VPA exerts its effect in a cancer type specific manner, inhibiting invasion in bladder, but not prostate cancer cells.

Since the doses required for optimal activity *in vitro* often exceed the toxicity threshold in clinical use, the therapeutic significance of VPA *in vivo* remains to be seen. In order to assess the feasibility of VPA as an adjuvant, we performed a limited animal

xenograft experiment with established T24t tumors to determine if non-toxic levels of VPA would have measurable activity *in vivo*. At serum concentrations of 8 to 67 ug/ml (0.05 to 0.4 mM, respectively) – well below the maximum threshold for safety in humans (135 ug/ml (0.9 mM)) – VPA treated animals demonstrated a 40% reduction in tumor volume when compared with untreated controls. This sensitivity to such low doses of VPA may be due to chronic (34 days) VPA treatment, in comparison to acute 3 day treatment *in vitro* (Figure 2). We have previously found that prostate cancer cells are more sensitive to lower VPA doses when given chronically rather than acutely (Xia et al., 2006). Nevertheless, the lower VPA doses achieved *in vivo* were still capable of inducing significant HDACI activity supported by increased histone H3 acetylation and p21 induction (Figure 8B & C).

Prior studies of HDACIs have focused on the inhibition of cell growth and induction of apoptosis as primary mechanisms of their anti-cancer effects. However, newer insights have revealed that the effects of HDAC inhibition on both histone and nonhistone proteins may be even more far reaching than originally thought, modulating cancer cell differentiation, migration, invasion, metastasis and angiogenesis (Liu et al., 2006). For example, VPA has been reported to alter the chemokine expression profile of endothelial cells, potentially effecting angiogenesis and neutrophil infiltration (Engl et al., 2004). In our previous work, we demonstrated that HDACI treatment *in vitro* can up regulate expression of the cellular adhesion molecule, CAR (Sachs et al., 2004). We now demonstrate that chronic low dose VPA treatment also induces CAR expression *in vivo* and that this treatment has profound effects on tumor growth (Figure 8). It has been previously shown that over-expression of CAR in a glioma tumor model resulted in over

80% decreases in tumor volume *in vivo*, decreased cell invasion *in vitro*, and that this inhibition of tumor growth required the carboxy-terminal domain of CAR (Huang et al., 2005). Interestingly, there was no reported effect of CAR over-expression on glioma cell growth *in vitro*, suggesting additional microenvironment-specific effects of CAR in tumors. On the other hand, over-expression of CAR in T24 bladder cancer lines has been reported to significantly inhibit cell growth *in vitro*, and this inhibition of cell growth also required the carboxy-terminal domain of CAR (Okegawa et al., 2001). Collectively, it is apparent that VPA effects on tumor growth and invasion are mechanistically complex, that they may be tissue specific, and that in bladder cancer, VPA mediated CAR upregulation may play an important role in controlling bladder tumor growth *in vitro* and *in vivo.*

In summary, our study provides initial evidence that VPA can inhibit the growth and invasion of bladder cancer cells. These data suggest a potential role for VPA as an adjuvant therapy for patients with recurrent, progressive, or muscle-invasive disease.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Western Blot Analysis of Acetylated Histone H3 and p21$^{WAF/CIP1}$ Expression in VPA Treated T24 Cells. (A) T24 cells were treated with VPA (0, 0.6, 1.2, 2.4, or 5.0 mM) or TSA (50 ng/ml) for 72 hours and analyzed for histone H3 acetylation by Western blot. Untreated and TSA-treated (50 ng/ml) cells were used as negative and positive controls, respectively. Relative fold increase was determined by scanning densitometry of the Western blot normalized to β-actin. VPA treatment results in a dose dependent increase in acetylated H3 expression with up to a 4.8 fold increase with 1.2 and 5.0mM doses **(B)** T24 cells were treated with VPA (0, 1.2 and 5.0 mM) for 4, 8, 16 and 24 hours then evaluated for p21 expression by Western blot. VPA increases p21 $^{WAF1/CIP1}$ expression in a time and dose dependent manner with maximal effects at 16 hours.

FIGURE 2. (A) The Effect of VPA Treatment on Bladder Cancer Cell Viability. Bladder cancer cell lines, TCC-SUP, T24, RT4 and HT1376, were treated with VPA (0, 0.6, 1.2, 2.4, or 5.0 mM) for 72 hours and the surviving fraction was assayed for by MTT. Viabilities of treated and untreated cells were compared for each cell line and data are presented as percent viability of untreated cells. VPA treatment decreases the number of viable cells in a dose dependent manner for all cell lines. **(B)** T24 cells were treated with VPA (0, 0.6, 1.2, 2.4, 5.0 mM) for 16 hours and harvested for evaluation of Caspase 2, 3, 8, and 9 activities. Caspase 2 and 3 activities were increased by VPA treatment, but only at the highest dose (5.0 mM). Caspase 8 and 9 activities remained relatively unchanged with VPA treatment. Comparison of all groups was performed by repeated measures

analysis of variance (RMANOVA) with post-hoc testing and marked with an asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) when statistically significant for all comparisons relative to untreated cells.

FIGURE 3. Qualitative Analysis of T24 Invasion by Fluorescent Microscopy.

T24 cells were treated with VPA (0 or 5.0 mM) for 24 hours then labeled with CFDA SE fluorophore, harvested, and plated on top of a simulated cellular-biological barrier in a modified Boyden chamber. Paired pictures of invading cells from VPA treated and untreated groups were taken at different time intervals (1, 2, 4, 8 and 24 hours) from the bottom side of a fluorescence blocking, human urothelial cell-covered, Matrigel-coated, porous membrane. VPA treatment results in a qualitative decrease in the number of invading cells between treated and un-treated groups. Photomicrographs were taken at 10X magnification with a calibration mark showing 20 μm.

FIGURE 4. End-point Quantitative Analysis of T24, TCC-SUP and HT1376 Invasion.

T24, TCC-SUP, and HT1376 cells were treated with VPA (0, 1.2 or 5.0 mM) or TSA (50 ng/ml) for 72 hours, then labeled with CFDA SE fluorophore, harvested, and plated in equal numbers on top of a Matrigel coated PET membrane of the ChemoTx® system 96-well plate. Cells were allowed to migrate towards FBS-enriched media in the lower wells and total fluorescence of invading cells was measured at 0.5 and 8 hours. While there was no difference in the number of invading cells at 0.5 hours, there was significant inhibition of invasion between treated and untreated cells at 8 hours with all doses in TCC-SUP and with 5.0 mM VPA in the HT1376 cell line. Of note, there was an approximately 4-fold

difference in invasion between treated and untreated T24 cells for all doses; however, these differences did not achieve statistical significance. Comparison of groups was performed by student's t-test and marked with an asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) when statistically significant for all comparisons relative to untreated cells.

FIGURE 5. Real-time Quantitative Analysis of T24 Invasion and Migration.

T24 cells were treated with VPA (0, 0.6, 1.2, 2.4, 5.0 mM) for 72 hours then harvested, labeled with CFDA SE fluorophore, and loaded into the upper chamber of a modified Boyden chamber with FBS-enriched media in the bottom wells. Cells were allowed to cross through Matrigel coated and uncoated PET membranes, simulating invasion and migration, respectively, and then detected in real-time with a multi-plate fluorometer (excitation 492nm and emission 517nm). The detection of each reading was normalized to those from equal numbers of CFDA SE-tagged T24 cells in a regular 24-well Falcon plate. Data are presented as **(A)** Percent Invasion **(B)** Percent Migration and **(C)** Percent Invasion/Migration or Invasiveness. VPA significantly decreases T24 cell invasion and percent invasiveness in a dose dependent manner. At lower concentrations (0.6 and 1.2 mM), VPA had no effect on migration, while at higher doses (2.4 and 5.0 mM) migration was slightly decreased and increased, respectively. However, these effects lost significance over time with the exception of the 72 hour time point for 5.0mM VPA. Comparison of groups was performed by student's t-test and marked with an asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) when all values for a specific time point are statistically significant relative to untreated cells. If all values for a time point are not significant, then the asterisk is not used.

FIGURE 6. Real-time Quantitative Analysis of T24, TCC-SUP, HT1376 and RT4 Invasion and Migration. **(A)** TCC-SUP **(B)** HT1376 and **(C)** RT4 cells were treated with VPA (0, 1.2 or 5.0 mM) for 72 hours then harvested, labeled with CFDA SE fluorophore, and loaded into a modified Boyden chamber. Chemotactic activities were recorded up to 72 hours with readings normalized to those from equal numbers of CFDA SE-tagged cells in 24-well falcon plates. Data presented as percent invasiveness shows that VPA inhibits invasion in T24, TCC-SUP and HT1376 cells, but has no effect on the non-invasive RT4 cell line. The effect was dose dependent in all cell lines and statistically significant after 8 hours in TCC-SUP. Comparison of groups was performed by student's t-test and marked with an asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) when all values for a specific time point are statistically significant relative to untreated cells.

FIGURE 7. Real-time Quantitative Analysis of LNCaP, PC3 and DU145 Invasion and Migration. **(A)** LNCaP, **(B)** PC3 and **(C)** DU145 cells were treated with VPA (0, 1.2 or 5.0 mM) for 72 hours then harvested labeled with CFDA SE fluorophore, and loaded into a modified Boyden chamber. Chemotactic activities were recorded up to 72 hours with readings normalized to those from equal numbers of CFDA SE-tagged cells in 24-well falcon plates. Data are shown as percent invasiveness. VPA had no effect on invasion or migration for all tested prostate cancer cell lines. There was a mild decrease in invasion for the PC3 cell line at the earliest time points; however, this was not consistent over time. Comparison of groups was performed by student's t-test and

marked with an asterisk (*p<0.05, **p<0.01, ***p<0.001) when statistically significant for all comparisons relative to untreated cells.

FIGURE 8. The Effect of VPA on T24t Tumor Xenografts.

(A) Subcutaneous T24t tumor xenografts were established in male athymic mice. Animals in the treatment arm received 0.4% VPA in drinking water. Tumor volumes were measured 3 times per week for 34 days and compared using Wilcoxon Rank and Sum tests and 2-way ANOVA with post-hoc testing. VPA treated animals demonstrated a 40% reduction in tumor volume when compared with untreated controls. Asterisks (*) mark curves with statistical significance (p<0.05)**. (B)** Western blot with corresponding densitometry data of the harvested tumor shows increased expression of acetylated histone H3, p21 and CAR in VPA treated tumors versus controls.

TABLE 1. Densitometry Data for Western Blot Analysis of Acetylated Histone H3.

VPA	0 mM	0.6 mM	1.2 mM	2.4 mM	5.0 mM	TSA 50 ng/ml
Fold Intensity	1.0	2.3	3.5	4.8	4.8	3.3

Correlating densitometry data for Figure 1A demonstrating fold increase in acetylated H3 expression.

TABLE 2. Densitometry Data for Western Blot Analysis of $p21^{WAF/CIP1}$.

VPA	4 hrs	8 hrs	16 hrs	24 hrs
0 mM	1.0	1.0	1.0	1.0
1.2 mM	1.1	1.3	6.8	0.4
5.0 mM	1.6	4.8	58.6	7.8

Correlating densitometry data for Figure 1B demonstrating fold increase in p21 expression.

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Figure 1

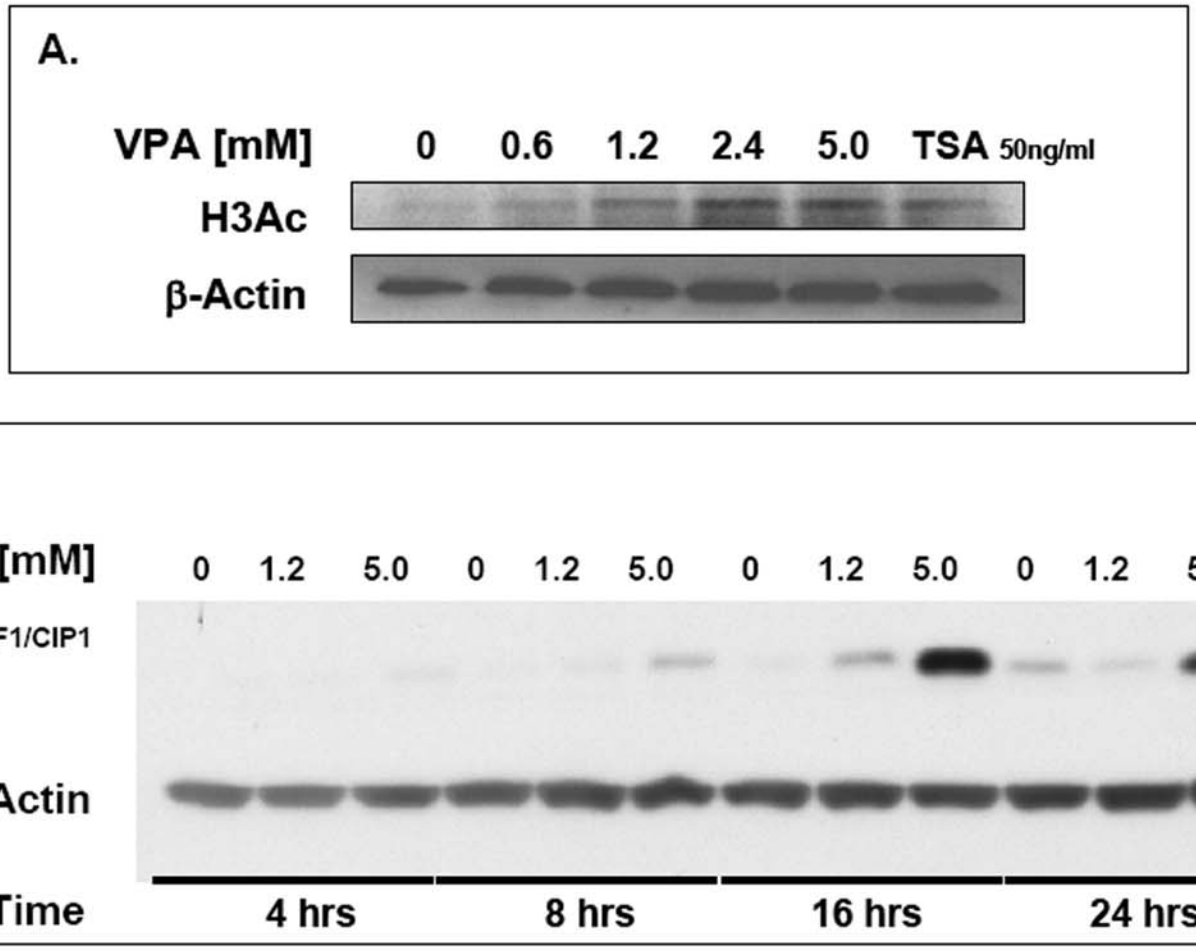

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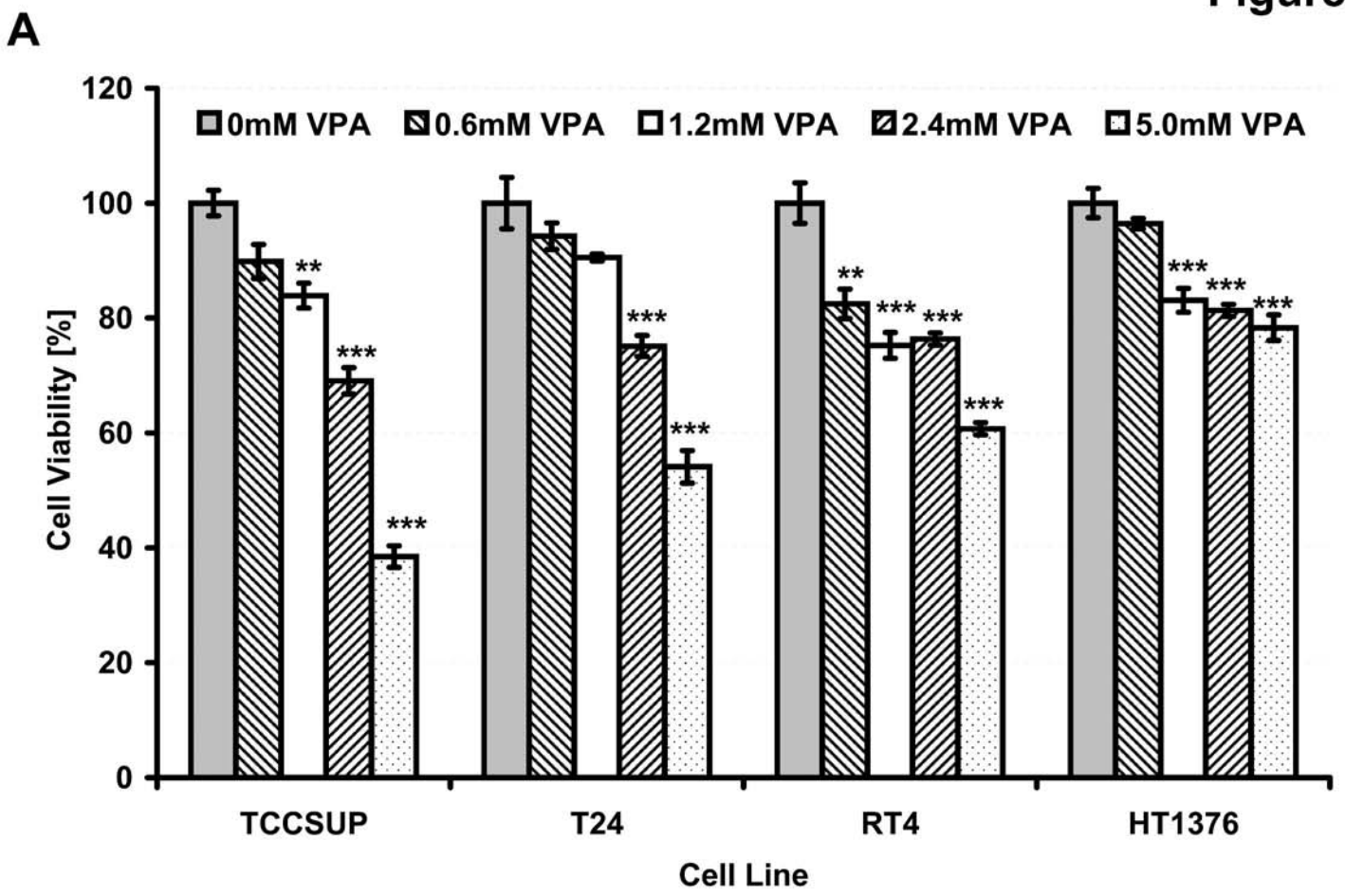
Figure 2



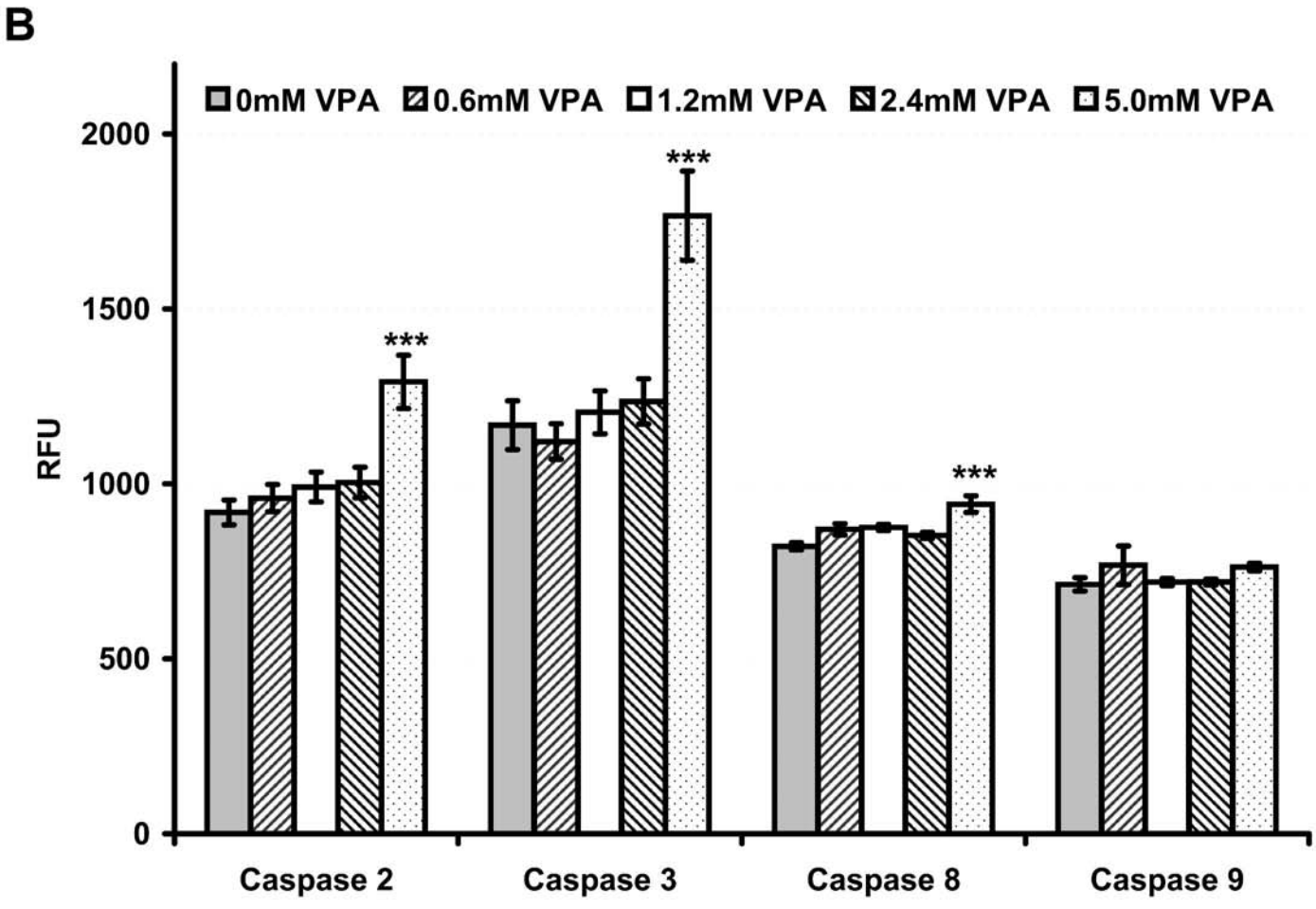


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Figure 3

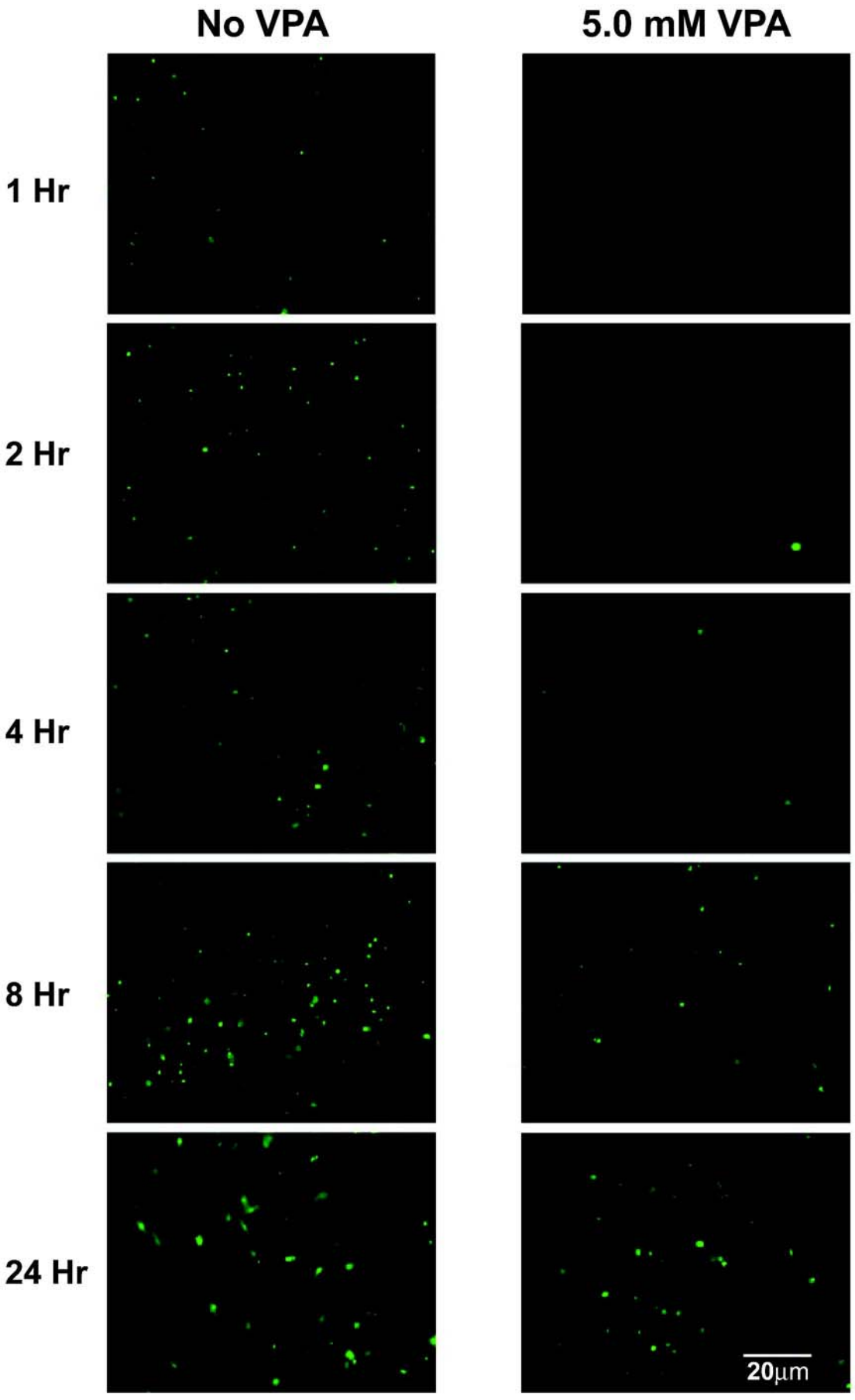

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Figure 4

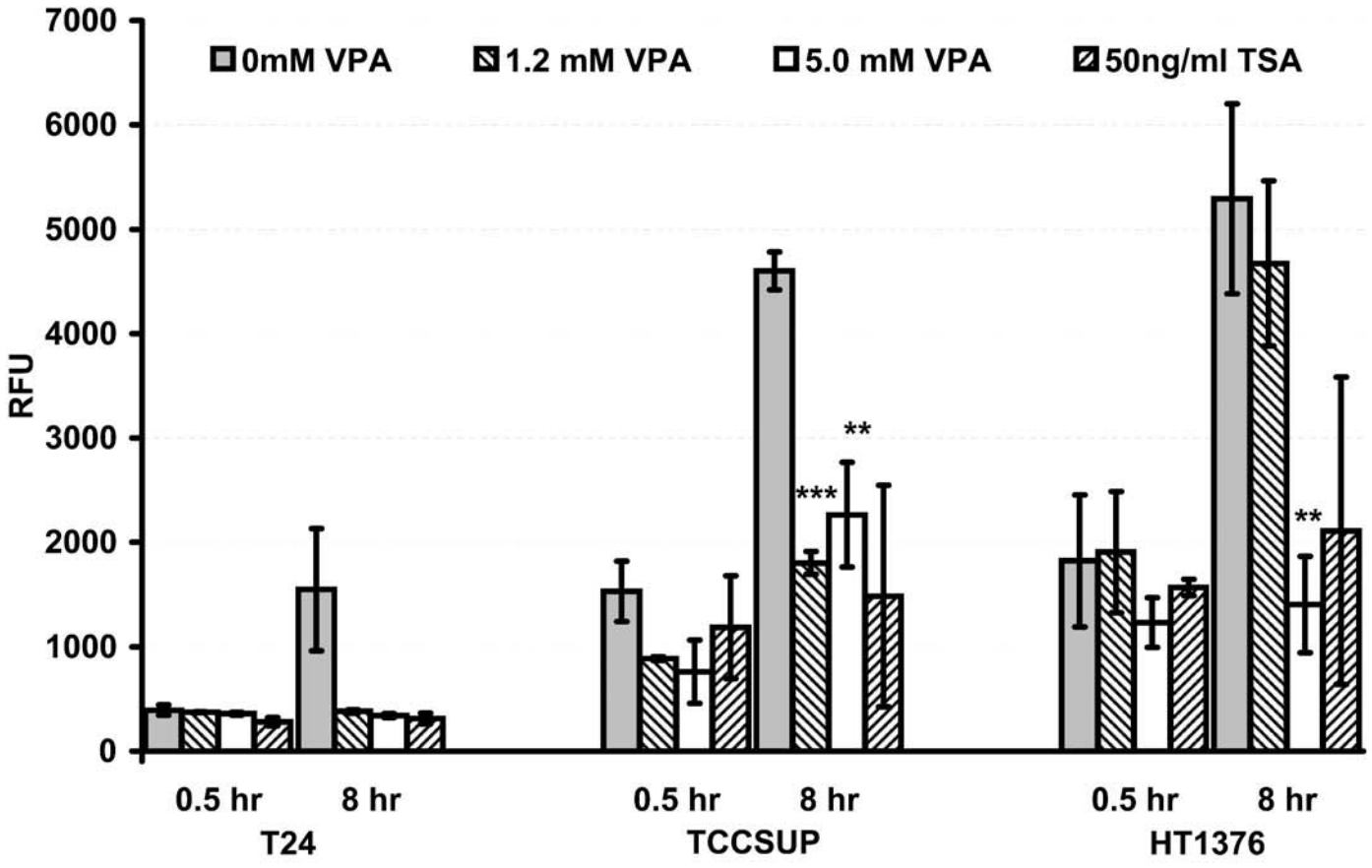

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Figure 5

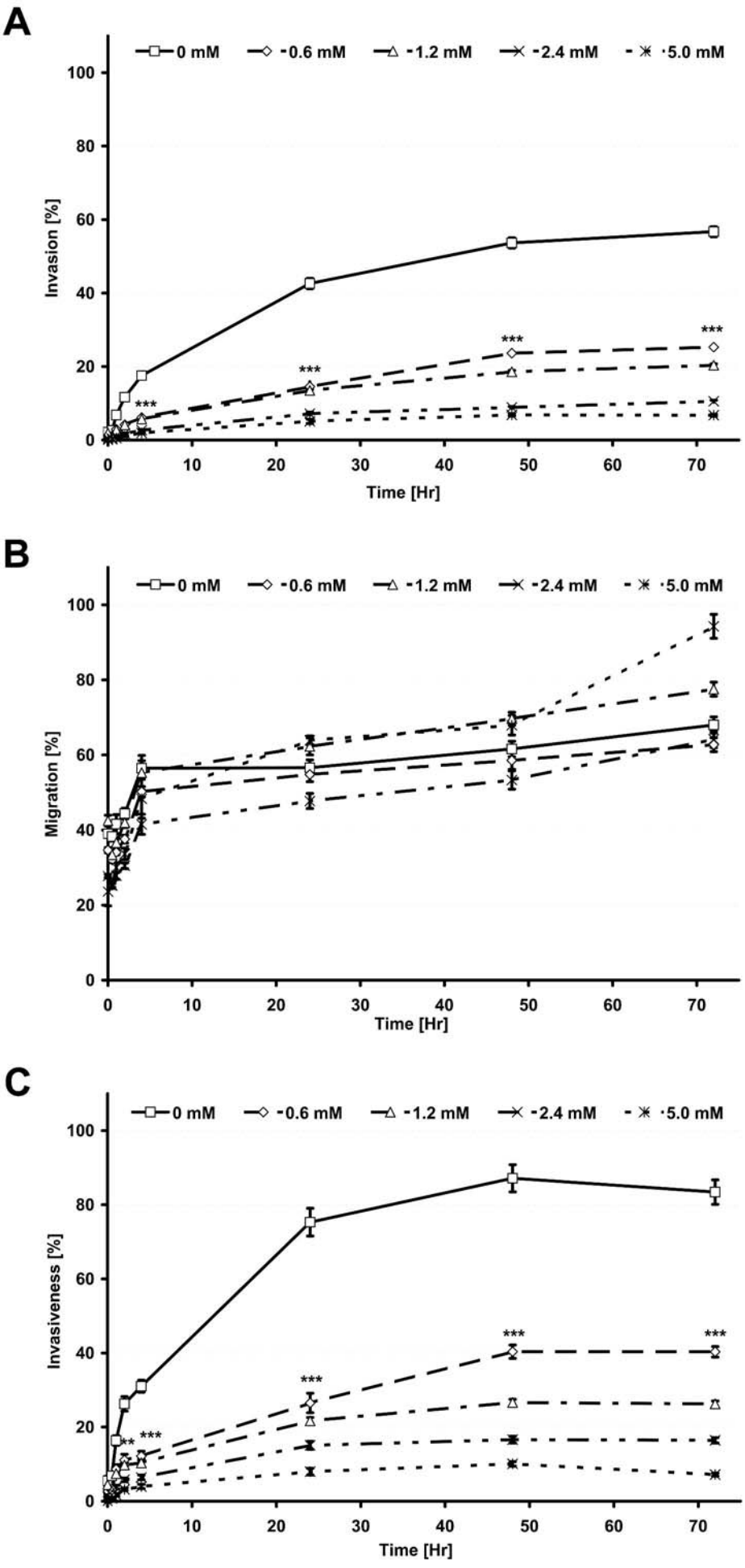

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Figure 6

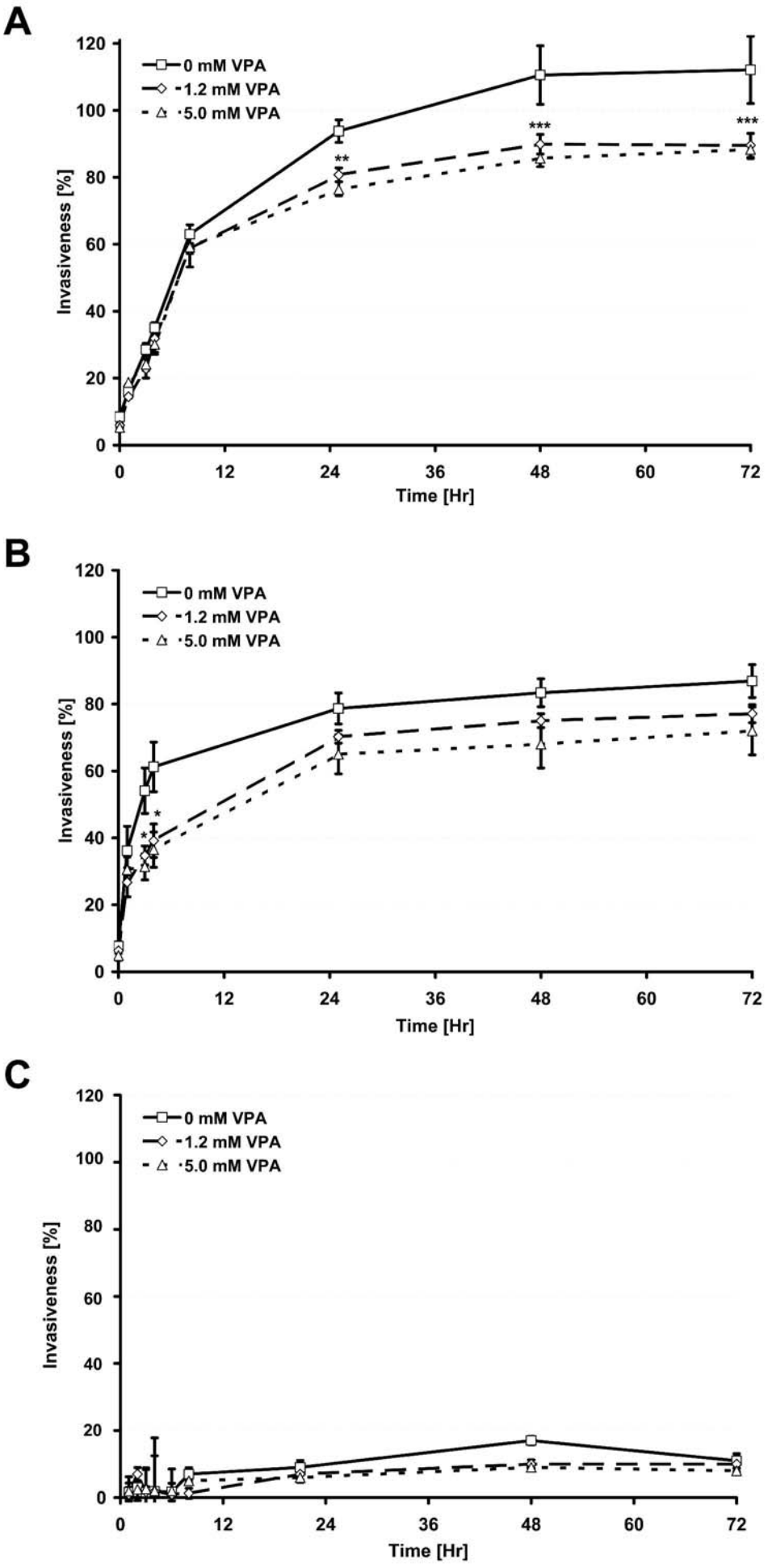

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Figure 7

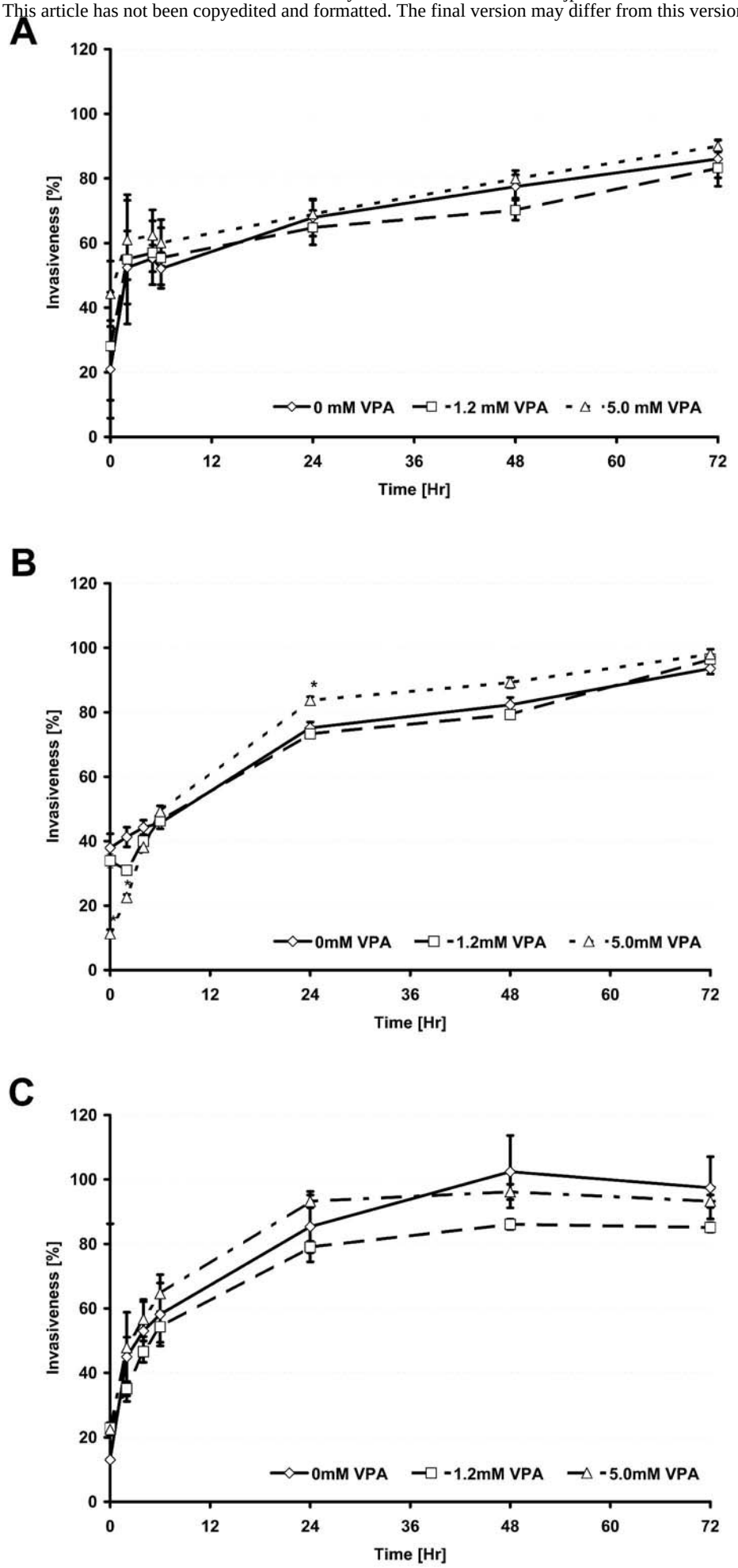

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Figure 8