

The histone deacetylase inhibitor trichostatin A has genotoxic effects in human lymphoblasts in vitro

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Abstract

Histone deacetylase (HDAC) inhibitors are a class of putative chemotherapeutic agents for which the mechanism of toxicity has not been fully identified. To explore the possibility that HDAC inhibitors are genotoxic, human TK6 lymphoblastoid cells were exposed to trichostatin A (TSA) and genetic damage measured. TSA caused a dose-dependent increase of G1 arrested cells at 24h that correlated with increasing levels of p21 and apoptosis. Significantly elevated frequencies of structural chromosomal aberrations in cells exposed to TSA were observed using both the kinetochore-antibody micronucleus assay and non-banding metaphase chromosome analysis. Increased tail intensities, indicative of elevated levels of DNA damage, were observed using the alkaline comet assay. Elevated levels of phosphorylated histone γ H2AX protein were observed as early as 3h following TSA exposure and peaked at 12h for 200nM TSA. Significant levels of aneuploidy at the 200nM TSA dose were observed using metaphase analysis, but interestingly, kinetochore-positive micronuclei were not detected at any dose using the kinetochore micronucleus assay, suggesting that TSA induces aneuploidy via a non-disjunction event rather than chromosome lagging. Increases in chromosomal loss and breakage were observed using simultaneous FISH metaphase analysis of chromosomes 5, 7, 8, and 21, consistent with data obtained from the micronucleus and metaphase chromosome analyses. We conclude that TSA is both a clastogen and aneugen in the TK6 cell line and propose that the observed cytostatic and apoptotic properties of TSA may partially be due to this genotoxicity.

Introduction

Histone deacetylase (HDAC) inhibitors are a class of putative chemotherapeutic agents that induce cell cycle arrest, apoptosis, and differentiation in a variety of cancer cell lines (Minucci and Pelicci 2006). These characteristics, along with the fact that many HDAC inhibitors synergistically enhance cell death to DNA damaging agents, have pushed them forward as lead cancer treatment compounds with many entering, or currently undergoing, clinical trials (Minucci and Pelicci 2006). The mechanisms by which HDAC inhibitors elicit this cytotoxicity, however, is not completely understood (Drummond *et al.* 2005) and continues to be a topic of investigation. One common explanation is that HDAC inhibitors alter transcription and that cellular cytotoxicity is just a by-product of these transcriptional changes. While this hypothesis remains to be verified (Johnstone and Licht 2003), it is supported by the observation that tumor suppressor proteins, like p21, increase following chemical exposure to HDAC inhibitors (Blagosklonny *et al.* 2002). Another hypothesis is that HDAC inhibitor -induced cytotoxicity is a consequence of heterochromatic hyperacetylation and genotoxicity (Johnstone and Licht 2003). It is theorized that the proteins normally associated with the heterochromatin are released when it becomes decondensed, giving rise to chromosome missegregation and subsequent aberrant mitosis (Johnstone and Licht 2003; Warrenner *et al.* 2003). The finding that the mutation of histone deacetylases in fission yeast reduces the fidelity of chromosome segregation supports this hypothesis (Grewal *et al.* 1998). Interestingly, this phenotype in fission yeast is mimicked by exposure to the HDAC inhibitor TSA (Ekwall *et al.* 1997; Grewal *et al.* 1998).

Despite the possibility that HDAC inhibitors may be genotoxic, a thorough investigation of their ability to cause chromosomal damage has not been conducted to our knowledge. To address this need, human TK6 lymphoblastoid cells were exposed to the classic HDAC inhibitor TSA, which shares structural similarity to the clinically relevant HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). The studies presented here demonstrate that TSA has both clastogenic and aneugenic properties. Using multiple approaches, including the kinetochore-micronucleus assay, metaphase chromosome analysis (both with and without fluorescence *in situ* hybridization {FISH}), single cell gel electrophoresis, and phosphorylated histone γ H2AX protein analysis, we demonstrate that exposure to TSA causes chromosome breakage in this cell line. Increases in aneuploidy are observed using metaphase and FISH chromosome analysis, but not with the kinetochore-micronucleus assay, suggesting that chromosome missegregation occurs via a non-disjunction event. In addition, TSA causes dose-dependent increases in G1 cell cycle arrest and apoptosis with accompanying increases of p21 but not caspase 3 protein levels. We conclude that TSA is both a clastogen and aneugen in the TK6 cell line and posit that the observed cytostatic and apoptotic characteristics of TSA may partially be due to this genotoxicity.

Materials and Methods

Cell culture and chemical treatments

The human TK6 lymphoblastoid cell line (ATCC) was chosen because HDAC inhibitors have been proposed for the treatment of lymphomas and leukemias. The cell line was maintained in RPMI1640 medium (GIBCO, San Diego, CA) containing 10% FBS

(Omega Scientific, Tarzana, CA) and 1% penicillin and streptomycin (Omega Scientific, Tarzana, CA) under standard conditions. TSA (CAS# 58880-19-6, MW 302.37, >99% purity; Sigma Aldrich, St. Louis, MO) was dissolved in DMSO for all experiments. Cells were dosed with 0.1% DMSO and 25, 50, 100, 200, or 400nM TSA at a concentration of $3.5\text{--}4 \times 10^5$ cells/ml.

Cell cycle analysis and apoptosis assay

For cell cycle analysis, asynchronous TK6 cells were exposed to TSA for 24h and 1×10^5 cells were isolated from culture and washed twice with ice-cold PBS and subsequently fixed for 15 min in 1% paraformaldehyde/PBS on ice, washed twice with PBS, and resuspended in 1ml of propidium iodide (PI) solution containing 2.5 µg/ml PI in PBS and 25 µl RNase A (1mg/ml) and stained overnight at 4°C. For analysis of apoptosis, TK6 cells were exposed to TSA for 3, 6, and 24h and subsequently resuspended in fresh medium lacking TSA for another 21h. This protocol was chosen specifically to mimic the exposures used for the various genotoxicity assays (described below). TK6 cells were prepared for flow cytometric annexin V analysis following the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA), except the cells were not washed several times with ice-cold PBS as it increased background necrosis and apoptosis levels. At least 1×10^4 TK6 cells were analyzed on a Beckman Coulter EPICS XL-MCL flow cytometer using System II software for each experiment and repeated three times.

Cytogenetic assays

Doses above 200nM TSA were not used for any of the genotoxicity studies due to excessive apoptosis observed at the 24 or 45h time points in the Annexin V assay. The procedure for the cytokinesis-blocked in vitro micronucleus assay was performed as

previously described (Eastmond and Tucker 1989) with minor modifications. Cells were exposed for 20 hours and were prepared for the analysis of micronuclei following a 24h recovery period. A total of 2000 binucleated cells were analyzed per experiment for the presence of micronuclei containing the CREST-kinetochore antibody. Metaphase chromosome preparation and multiple probe FISH was performed as previously described (Smith *et al.* 1998; Zhang *et al.* 1998) with minor modifications enabling for the simultaneous analysis of chromosomes 5, 7, 8, and 21. These specific chromosomes were chosen because previous studies had demonstrated that they are selectively lost and gained upon chemical exposure to benzene and its metabolites benzoquinone and hydroquinone (Smith *et al.* 1998; Zhang *et al.* 1998). Cells were exposed to TSA for 20h and prepared for metaphase analysis following a 24h recovery period. A total of 500 metaphases were analyzed for the presence of structural and numerical chromosomal aberrations for each experiment (total of two experiments) whereas 1000 metaphases were analyzed using multiple probe FISH for only one experiment. The alkaline comet assay was performed as previously described (Singh *et al.* 1988) with some modifications. Cells were exposed to TSA for 3, 6, and 24 hours prior to preparation for analysis of DNA damage in the comet assay. Five hundred randomly chosen cells per slide were scanned and analyzed with CometScan imaging software (MetaSystems, Germany). Cells were subsequently screened manually to exclude cells that did not meet stringent requirements (i.e. poor staining, out of focus, and oddly shaped). The average $\pm$ standard error of the mean for tail intensity, a measure of total DNA damage (Collins 2002), from two independent experiments is shown for the 3 and 24h time periods but for

only one experiment at the 6h time point. All slides for all assays were coded to prevent observer bias.

Immunoblot analysis

Total cell lysates were collected from $1.5\text{--}2 \times 10^6$ cells using 100 μl of radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA). Equal protein amounts (40 μg) were resolved by PAGE, transferred onto nitrocellulose membranes, and immunoblotted for p21, caspase 3, and γH2AX (Upstate, Charlottesville, VA). Proteins were visualized using the ECL method per manufacturer's protocol (Amersham Biosciences, United Kingdom). Film (Kodak BioMax XAR; Eastman Kodak, Rochester, NY) was exposed and developed using the Konica SRX-101 developer (Wayne, NJ). Caspase 3 protein levels did not change following TSA exposure and was used as the loading control for all experiments. Scion Image software (<http://www.scioncorp.com/> Frederick, MD) was used to quantify phosphorylated histone γH2AX and caspase 3 protein levels.

Statistical analysis

Ordinary least squares was used to estimate the effect for all analyses – for those analyses with different numbers of cells evaluated for each observation, we used simply the number of cells as an analytic weight. For each regression we also included categorical “experiment” as a variable to adjust for experimental variability. For trend test, dose was entered as a continuous variable; we also did an analysis of categorical dose, entering the appropriate indicator variables for each dose, except 0 (baseline). For all analyses, the p-values reported are from robust standard errors (MacKinnon and White 1985), which

relaxes the constant residual variance assumptions of the standard inference returned by ordinary least squares.

Results

TSA induces apoptosis and G1 cell cycle arrest in TK6 cells

Flow cytometric analysis measuring annexin V binding to phosphatidylserine demonstrated that apoptosis levels significantly increased (p-values < 0.01) at the 24 and 45h time periods for cells exposed to 400nM TSA (Figure 1A). Increases in apoptosis were also observed with the 200nM TSA dose at the 45h time-point but were not statistically significant (p-value = 0.37). Flow cytometric analysis measuring PI staining of DNA demonstrated that 24h TSA exposures induced a significant (trend test p-value < 0.001) dose-dependent G1 cell cycle arrest that coincided with decreasing levels of G2/M cells (Figure 1B). TSA-induced apoptosis and G1 cell cycle arrest coincided with increasing levels of p21 protein levels (Figure 1C). Doses of 200 and 400nM TSA caused noticeable increases in p21 protein levels as early as 3 hours following exposure, peaking at 12h, and decreasing at the 24h time point. Caspase 3 protein levels for the 200 and 400nM TSA doses did not change throughout the 24h time period analyzed (Figure 1C).

TSA causes chromosomal breakage in the TK6 cell line

The kinetochore antibody micronucleus assay can be used to discriminate between micronuclei that have been formed through either chromosomal loss or breakage. Micronuclei staining negative will have been formed through a chromosome breakage (clastogenic) event whereas micronuclei staining positive are formed through

chromosomal loss, although they may also contain chromosome fragments. Kinetochore-antibody micronucleus assay analysis demonstrated that TSA caused significant increases in chromosomal breakage (micronuclei that do not stain positive for the CREST antibody; trend test p-value < 0.001) in the TK6 cell line (Figure 2A). Significant increases of kinetochore-negative micronuclei were observed for both the 50 and 200nM doses (p-values = 0.01 and <0.001, respectively). Further studies utilizing a second method based upon direct metaphase chromosome analysis were conducted in order to confirm these initial findings. Metaphase analysis revealed that significant increases in structural chromosomal aberrations (breaks, gaps, and rearrangements; trend test p-value = 0.016) occurred following treatment with TSA (Figure 2A), mimicking what was observed using the micronucleus assay. The trend test for SCA was also similarly significant when gaps were excluded (p = 0.015). Simultaneous analysis of chromosomes 5, 7, 8, and 21 using multiple probe FISH also demonstrated that TSA caused increases in chromosomal breakage of these chromosomes (Figure 2B), providing additional evidence that TSA is a clastogen in the TK6 cell line. Elevated levels of phosphorylated histone γ H2AX, a protein that localizes to the site of a DNA double-strand break, regardless of the origin (Pilch *et al.* 2003), were observed following exposure to both 200 and 400nM TSA (Figure 2C). While both doses induced noticeable increases in phosphorylated γ H2AX as early as 3h following exposure, the 200nM dose appeared to peak at 12h whereas the 400nM dose appeared to cause phosphorylated γ H2AX to peak at or after 24h (Figure 2C).

TSA causes DNA breaks

Comet assay analysis of TK6 cells exposed to TSA for 3, 6, and 24h demonstrated that TSA increased DNA damage in a dose- and time-specific manner (Figure 3A). A significant increase in tail intensity was observed at the 6h time point (trend test p-value = 0.02) but was not observed at any other time points. Tail intensities for the 200nM TSA dose increased at the 6h time point but decreased at 24h whereas the 25 and 50nM doses had increased tail intensities at the 24h time point but not at the 3 or 6h (Figure 3A). Differences between studies were observed for the same doses using comet analysis and, as such, are cautiously interpreted. Representative images of damaged and non-damaged TK6 cells are presented in Figure 3B. Quantitative analysis of phosphorylated histone γ H2AX levels of TK6 cells exposed to 200nM TSA (Figure 3C) demonstrated that protein levels fluctuated in a time-dependent manner closely mimicking the levels of DNA damage identified with comet analysis (Figure 3A).

TSA causes aneuploidy

Analysis of TSA exposed TK6 cells with the kinetochore-antibody micronucleus assay failed to identify increases in chromosomal loss (Figure 4A). These data contradict the findings obtained through direct metaphase chromosome analysis (Figure 4A), which identified significant increases of chromosomal loss (trend test p-value = 0.02 and a p-value of 0.03 for the 200nM TSA dose). Further analysis using multi-probe FISH to simultaneously evaluate chromosomes 5, 7, 8, and 21 also demonstrated that aneuploidy of these chromosomes increases following 200nM TSA exposure (Figure 4B).

Discussion

Histone deacetylase inhibitors induce cytotoxicity in a variety of cancer cell lines through a mechanism that has yet to be elucidated. The possibility that these cytostatic and

apoptotic effects may be due to their ability to induce genotoxicity has not yet been fully addressed. We studied the effects of the hydroxamic acid TSA on the human TK6 lymphoblastoid cell line to explore the possibility that HDACi-induced cytotoxicity may partially be explained by DNA breakage and chromosome damage. Using multiple approaches, including protein analysis and several standard genetic toxicology assays, we demonstrate that TSA has both clastogenic and aneugenic properties in the TK6 cell line. While the primary focus of the current study was to identify whether TSA is genotoxic, a secondary aim was to identify if the TK6 cell line exhibited cytostatic effects similar to those observed in other cancer cell lines following TSA exposure. We identified that TSA elevated p21 protein levels, caused G1 cell cycle arrest, and induced apoptosis in a time- and dose-dependent manner, findings consistent with previous studies in other cell lines (Blagosklonny *et al.* 2002; Fandy *et al.* 2005). Caspase 3 protein levels did not change following either the 200 or 400nM TSA 24h exposures, but were not investigated at longer timepoints or using higher doses. Therefore it would be premature to declare that this result is indicative of a caspase 3-independent apoptotic mechanism in the TK6 cell line. Another, more likely scenario, is that the apoptosis, while sufficient to be observed by flow cytometric analysis of annexin V, may not have been high enough to be observed by caspase 3 protein analysis. Thus we cannot currently rule out the possibility that apoptosis in the TK6 cell line occurs through a caspase 3-mediated mechanism following TSA exposure.

TSA has previously been identified to cause chromosome missegregation in fission yeast (Ekwall *et al.* 1997; Grewal *et al.* 1998) through a mechanism that likely involves disruption of protein localization at centromeric heterochromatin (Ekwall *et al.*

1997). The current model suggests that histone deacetylation is a necessary step preceding RNAi-directed heterochromatic methylation and subsequent cohesin recruitment and binding (Ekwall 2004). Inhibition of the initial deacetylation step will thus block a series of ensuing events necessary for proper chromosome segregation. Because many basic biological processes are highly conserved between yeast and mammals, it is possible that histone deacetylases also play a critical role in chromosome segregation in human cells. Data presented in this study support this hypothesis as we observed increased levels of aneuploidy in human TK6 cells exposed to TSA. Using direct metaphase chromosome analysis with and without multi-probe FISH, we identified elevated levels of aneuploidy with a threshold dose of 200nM following a 24h exposure. Interestingly, we failed to identify significant increases in aneuploidy at any dose using the kinetochore-micronucleus assay. This discrepancy can likely be attributed to the fact that the micronucleus assay cannot detect non-disjunction events as the aberrant chromosome will segregate to one of the two daughter nuclei during mitosis and will not form a micronucleus. Direct metaphase chromosome analysis circumvents this problem because total numbers of chromosomes are enumerated. Our findings in the TK6 cell line, supported by several other reports in other mammalian cell lines (Cimini *et al.* 2003; Taddei *et al.* 2001), indicate that TSA induces chromosomal missegregation. While the understanding of the mechanism by which this HDAC inhibitor-induced chromosomal loss occurs in mammalian cells lags behind that for the fission yeast, there is nonetheless accumulating evidence to indicate that the histone deacetylase inhibitor TSA is an aneugen.

We identified that TSA caused DNA damage using the kinetochore micronucleus assay, metaphase chromosome analysis (with and without FISH), alkaline comet assay, and phosphorylated histone γ H2AX protein quantification by western blotting. Significant increases in kinetochore-negative micronuclei were observed at both the 50 and 200nM doses using the micronucleus assay with very similar results being observed with metaphase chromosome analysis (Figure 2A). Additional cytogenetic studies using multiple probe FISH supported these initial findings and indicated that TK6 cells exposed to TSA had elevated levels of damage in chromosomes 5, 7, 8, and 21 (Figure 2B). No threshold effect was observed using the micronucleus assay, but appears to be present above the 100nM TSA dose using metaphase analysis both with and without FISH analysis. Western blot analysis from three separate experiments demonstrated that increases in phosphorylated histone γ H2AX protein levels occurred following 200 and 400nM TSA exposures (Figure 2C). Analysis of the TSA exposed TK6 cells was also performed using the alkaline comet assay to ensure further the validity of these results. Although the results from the comet assay are not as definitive as those obtained from the other assays, increased levels of DNA damage are observed and correlate tightly with the γ H2AX protein levels (Figures 3A and C). The comet assay results were initially puzzling as the 25 and 50nM doses only have elevated levels of damage at the 24h time point and far exceed the amount of damage detected at the same time in the 200nM or 15 μ M etoposide positive control (data not shown). The observation that the γ H2AX protein levels change in a temporal manner very similar to the amount of DNA damage observed with the comet assay indicates that the time- and dose-specific results observed are not likely due to an artifact with the assay. We consider that variations may be due to

a combination of cell cycle kinetics and chromosome decondensation that is dose specific.

Previous studies in *Tetrahymena thermophila* and mouse embryonic fibroblasts have failed to identify that TSA causes chromosome breakage (Duharcourt and Yao 2002; Yaneva *et al.* 2005), results that contrast with the findings presented here using the human TK6 cell line. These data raise the question of whether the clastogenic effect of TSA is specific to TK6 cells. As previously described, the TK6 cells responded in a manner similar to other cell lines exposed to TSA, exhibiting G1 cell cycle arrest, increased p21 protein levels, apoptosis, and chromosome missegregation. Additionally, TSA has been reported to alter chromosome and interphase chromatin condensation in HeLa and PtK1 cells (Cimini *et al.* 2003; Toth *et al.* 2004), results that provide a possible mechanism for our findings that TSA causes chromosome breakage. We thus theorize that it is unlikely that the clastogenic effects of TSA will be limited only to the TK6 cell line. Furthermore, these results highlight the possibility that other HDAC inhibitors may also cause DNA and chromosome damage as TSA is often considered the model HDAC inhibitor.

Using a variety of approaches, we present data demonstrating that the histone deacetylase inhibitor TSA is both a clastogen and an aneugen. Because these effects are observed to occur simultaneously with p21 induction and precede the induction of apoptosis, we propose that the cytotoxicity of TSA may partially be due to its genotoxicity. Further work is required to elucidate the mechanism of TSA-induced genotoxicity and identify whether this, and/or its effects on transcription, plays more of a role in its cytotoxicity.

References

- Blagosklonny, M. V., Robey, R., Sackett, D. L., Du, L., Traganos, F., Darzynkiewicz, Z., Fojo, T., and Bates, S. E. (2002). Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. *Mol Cancer Ther* **1**, 937-41.
- Cimini, D., Mattiuzzo, M., Torosantucci, L., and Degrassi, F. (2003). Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. *Mol Biol Cell* **14**, 3821-33.
- Collins, A. R. (2002). The comet assay. Principles, applications, and limitations. *Methods Mol Biol* **203**, 163-77.
- Drummond, D. C., Noble, C. O., Kirpotin, D. B., Guo, Z., Scott, G. K., and Benz, C. C. (2005). Clinical development of histone deacetylase inhibitors as anticancer agents. *Annu Rev Pharmacol Toxicol* **45**, 495-528.
- Duharcourt, S., and Yao, M. C. (2002). Role of histone deacetylation in developmentally programmed DNA rearrangements in *Tetrahymena thermophila*. *Eukaryot Cell* **1**, 293-303.
- Eastmond, D. A., and Tucker, J. D. (1989). Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ Mol Mutagen* **13**, 34-43.
- Ekwall, K. (2004). The roles of histone modifications and small RNA in centromere function. *Chromosome Res* **12**, 535-42.
- Ekwall, K., Olsson, T., Turner, B. M., Cranston, G., and Allshire, R. C. (1997). Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. *Cell* **91**, 1021-32.
- Fandy, T. E., Shankar, S., Ross, D. D., Sausville, E., and Srivastava, R. K. (2005). Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. *Neoplasia* **7**, 646-57.
- Grewal, S. I., Bonaduce, M. J., and Klar, A. J. (1998). Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. *Genetics* **150**, 563-76.
- Johnstone, R. W., and Licht, J. D. (2003). Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell* **4**, 13-8.
- MacKinnon, J., and White, H. (1985). Some heteroskedasticity consistent covariance matrix estimators with improved finite sample properties. *Journal of Econometrics* **29**, 305-325.
- Minucci, S., and Pelicci, P. G. (2006). Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* **6**, 38-51.
- Pilch, D. R., Sedelnikova, O. A., Redon, C., Celeste, A., Nussenzweig, A., and Bonner, W. M. (2003). Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochem Cell Biol* **81**, 123-9.
- Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175**, 184-91.
- Smith, M. T., Zhang, L., Wang, Y., Hayes, R. B., Li, G., Wiemels, J., Dosemeci, M.,

- Titenko-Holland, N., Xi, L., Kolachana, P., Yin, S., and Rothman, N. (1998). Increased translocations and aneusomy in chromosomes 8 and 21 among workers exposed to benzene. *Cancer Res* **58**, 2176-81.
- Taddei, A., Maison, C., Roche, D., and Almouzni, G. (2001). Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. *Nat Cell Biol* **3**, 114-20.
- Toth, K. F., Knoch, T. A., Wachsmuth, M., Frank-Stohr, M., Stohr, M., Bacher, C. P., Muller, G., and Rippe, K. (2004). Trichostatin A-induced histone acetylation causes decondensation of interphase chromatin. *J Cell Sci* **117**, 4277-87.
- Warrener, R., Beamish, H., Burgess, A., Waterhouse, N. J., Giles, N., Fairlie, D. P., and Gabrielli, B. G. (2003). Tumor cell-specific cytotoxicity by targeting cell cycle checkpoints. *The FASEB Journal* **11**, 1550-2.
- Yaneva, M., Li, H., Marple, T., and Hasty, P. (2005). Non-homologous end joining, but not homologous recombination, enables survival for cells exposed to a histone deacetylase inhibitor. *Nucleic Acids Res* **33**, 5320-30.
- Zhang, L., Wang, Y., Shang, N., and Smith, M. T. (1998). Benzene metabolites induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes. *Leuk Res* **22**, 105-13.

Figure Legends

Figure 1: TSA exhibits cytotoxicity in TK6 lymphoblastoid cells. *A*, TSA increases apoptosis levels in both a time- and dose-dependent manner. A combination of annexin V FITC and PI staining was used to discriminate among apoptotic (highlighted lower right box) and necrotic (upper right box) cells. The average $\pm$ standard error of the mean from three independent experiments is shown. *B*, TSA causes dose-dependent increases in cells arrested in G1 at following 24 hours of exposure with simultaneous decreases in the number of cells in G2/M. PI staining was used to determine DNA content levels. Figures are representative of three independent experiments and include the average $\pm$ standard error of the mean. *C*, TSA exposure increases p21 but not caspase 3 protein levels. Increasing p21 protein levels are clearly visible following 3h of both 200 and 400nM TSA exposure and appear to peak at 12h. Caspase 3 levels do not change following TSA exposure in either of the doses tested and is used as the loading controls. Western blots are representative of three independent experiments.

Figure 2: TSA causes chromosomal damage. *A*, Significant increases of chromosomal damage are observed using two separate assays: the kinetochore-antibody micronucleus assay and metaphase non-banding chromosome analysis. The average $\pm$ standard error of the mean from three independent experiments is shown for the micronucleus data ($R^2 = 0.74$) whereas only two independent experiments were analyzed for the metaphase analysis ($R^2 = 0.54$). SCA is the abbreviation for structural chromosomal aberrations. *B*, Significant increases of structural damage are observed in chromosomes 5, 7, 8, and 21 using multiple probe fluorescence *in situ* hybridizaion (FISH). Similar levels of chromosomal damage to those observed with the metaphase non-banding analysis and

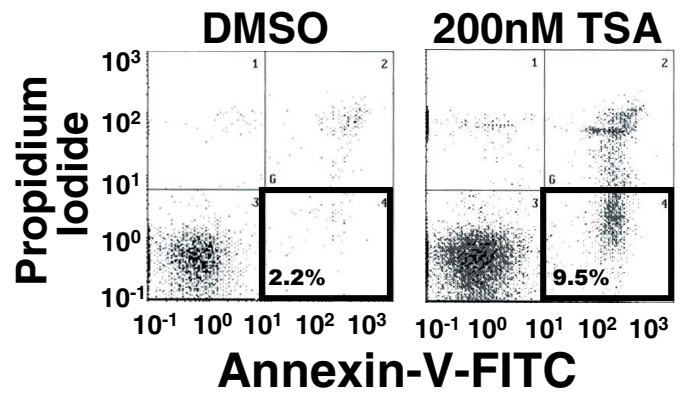
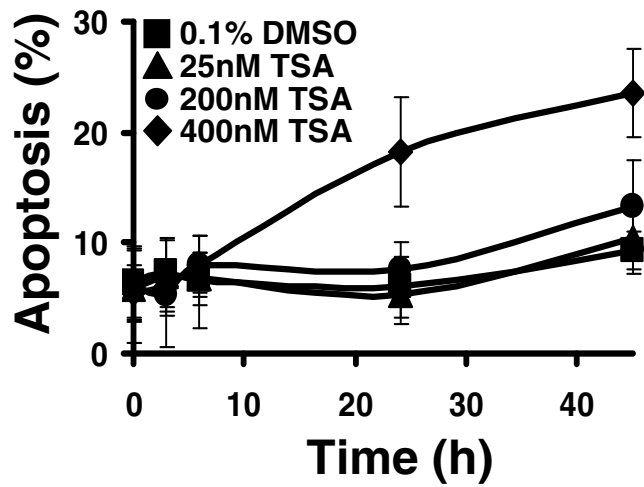
micronucleus assays is seen. The averages from one experiment are shown. *C*, Phosphorylated histone γ H2AX protein levels increase following both 200 and 400nM TSA chemical exposure and appear to peak at the 12h time point for the 200nM dose but continue to increase at 24h for the 400nM dose. Caspase 3 is used as the loading control and western blots are representative of three independent experiments.

Figure 3: TSA causes single-stranded DNA damage in TK6 cells. Analysis by single cell gel electrophoresis demonstrates that TSA increases DNA damage (tail intensity) following 6 and 24h TSA exposures. The average $\pm$ standard error of the mean from two independent experiments is shown for the 3 and 24h time periods but for only one experiment at the 6h time point. *B*, Representative images of control (0.1% DMSO 24h) and treated (50nM TSA 24h) TK6 cells from the alkaline comet assay are shown. *C*, Phosphorylated histone γ H2AX protein levels of cells exposed to 200nM TSA correlate in a dose- and time-dependent manner with tail intensity measurements obtained from comet assay analysis of cells exposed to 200nM TSA. The average $\pm$ standard error of the mean for three independent experiments is shown.

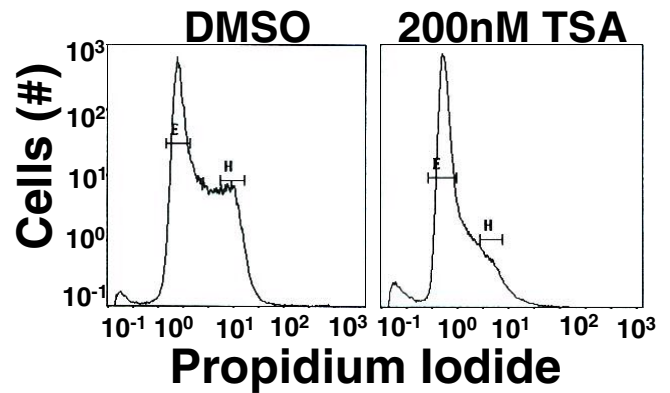
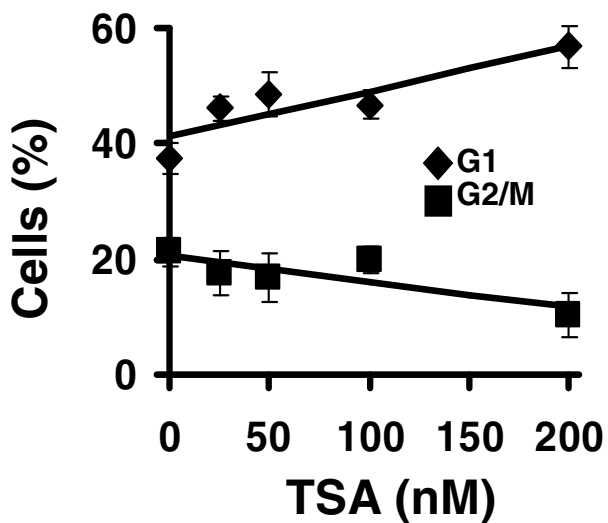
Figure 4: TSA causes aneuploidy via a non-disjunction event. *A*, Significant increases of chromosomal loss are observed at the 200nM TSA dose using metaphase non-banding chromosome analysis but not at any doses using the kinetochore-antibody micronucleus assay. The average $\pm$ standard error of the mean from three independent experiments is shown for the micronucleus data whereas only two independent experiments were analyzed for metaphase analysis. *B*, Significant increases of aneuploidy are observed to affect chromosomes 5, 7, 8, and 21 using multiple probe FISH.

FIGURE 1

A



B



C



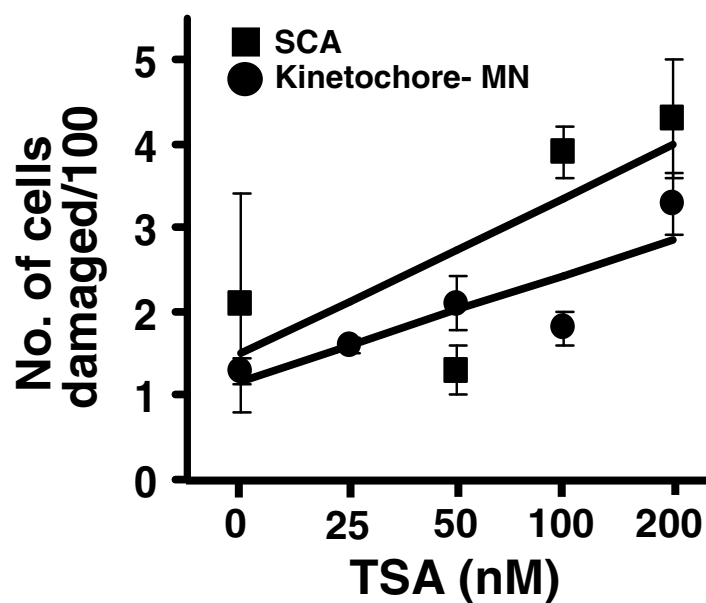
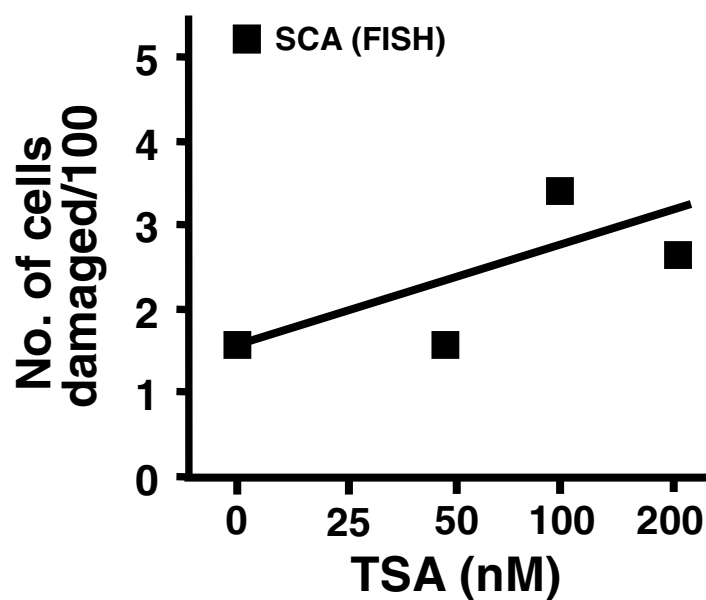
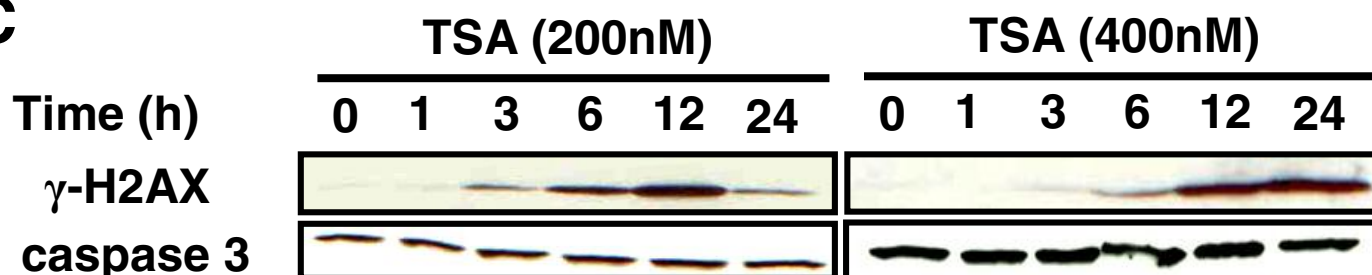
FIGURE 2**A****B****C**

FIGURE 3

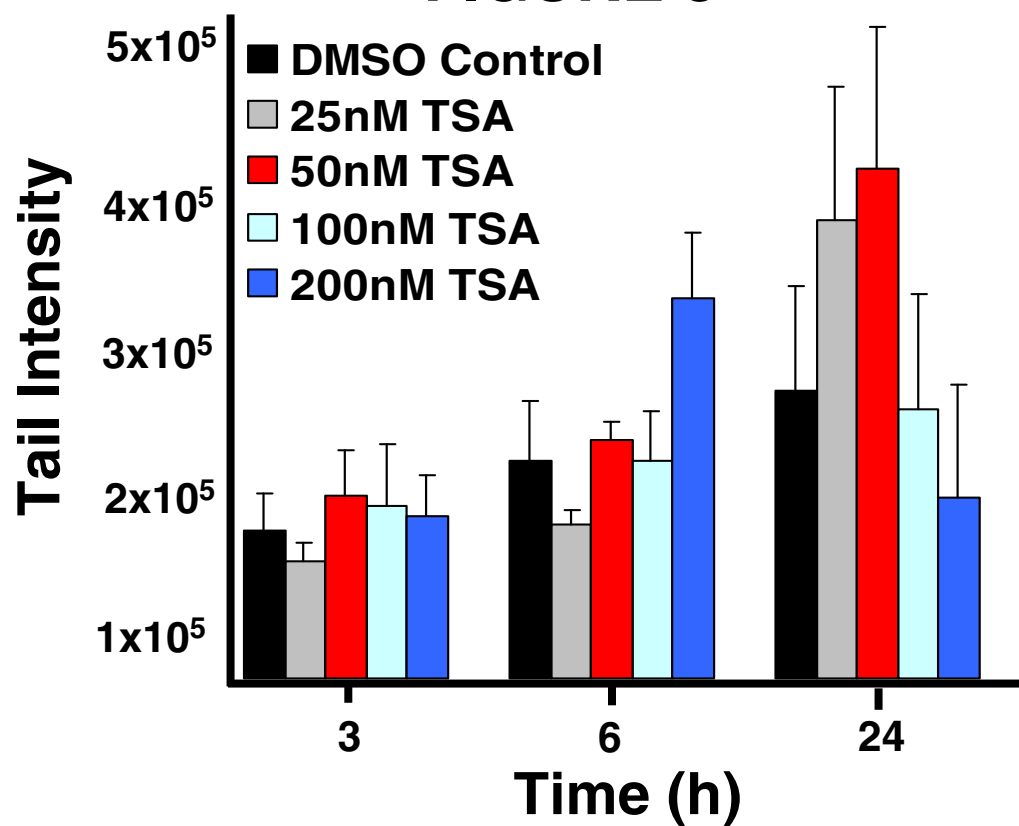
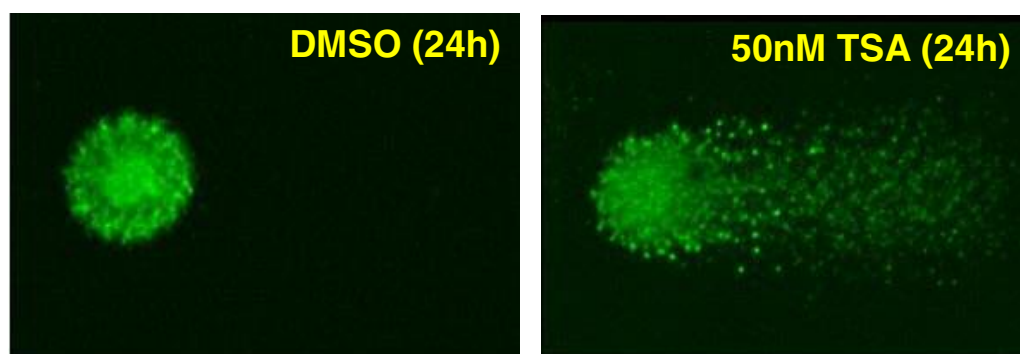
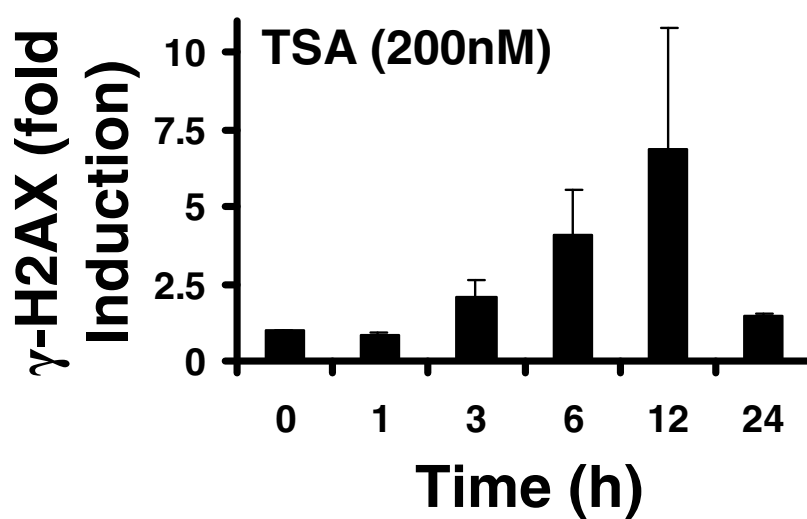
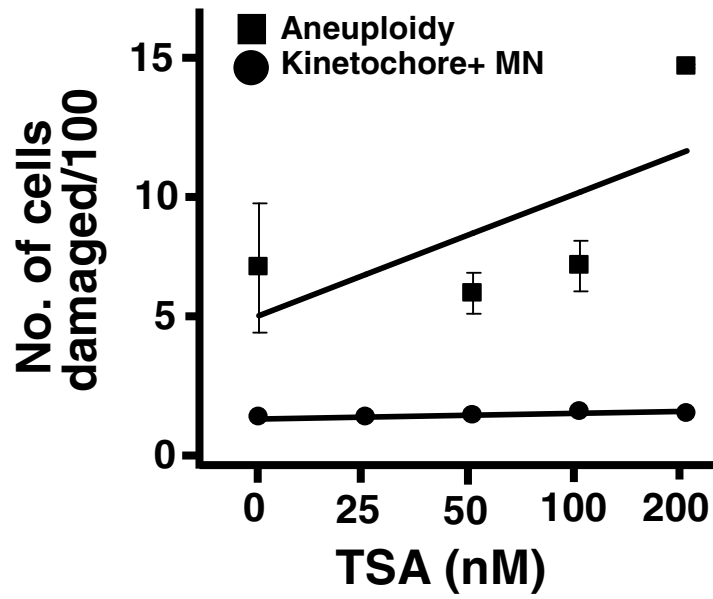
A**B****C**

FIGURE 4

A



B

