# The Role of Aneuploidy in the Malignant Transformation of Human Bladder Epithelial Cells

By: Jeannie Marie Camarillo

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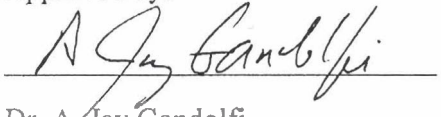

Dr. A. Jay Gandolfi
Department of Pharmacology and Toxicology

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**Abstract**

Much effort has gone into the study of the metabolites of arsenic, the most toxic to humans being monomethylarsonous acid [MMA(III)]. Previous research has shown that an immortalized human urothelial cells line, UROtsa, is malignantly transformed following 12 weeks of 50 nM exposure to MMA(III). While the timeline for malignant transformation has been determined, it has yet to be understood the mechanism for malignant transformation. Aneuploidy is present in a variety of human cancers, including bladder cancer. Induction of aneuploidy by a carcinogen can destabilize the number and structure of chromosomes and lead to dysregulation of regulatory proteins, DNA synthesis, and DNA repair. Cell cycle analysis was performed to determine the percentage of aneuploid cells. Following 8 weeks of MMA(III) exposure, UROtsa cells have a higher population of tetraploid cells. Two potential mechanisms behind the observed tetraploidy: mitotic slippage through ATM dysregulation and centrosome amplification resulting in malsegregation of chromosomes. Investigation into the mechanism behind the tetraploidy revealed that MMA(III) has no effect on ATM activity or on centrosome numbers. Further work must be done to determine the mechanism by which MMA(III) can induce tetraploidy and if that tetraploidy is a required step in the malignant transformation process.

## Introduction

Arsenic is an element commonly found in the earth's crust and present in groundwater across the world. The guideline value for the appropriate level of arsenic that can be safely consumed from drinking water is 10 µg/l (WHO, 2003). Despite guidelines set by the World Health Organization, areas such as Bangladesh, Vietnam, Myanmar, Nepal, Cambodia, Laos, and India face serious problems in regard to contamination of drinking water. These locations commonly exhibit levels of arsenic five times higher than the recommended value (IARC, 2004). Chronic exposure to arsenic causes cancers of the liver, lung, bladder, and kidneys (Smith et al., 1992).

Once arsenic enters the body, it undergoes a series of biotransformations that produce metabolites that are potentially more toxic. Inorganic arsenic is transformed into monomethylarsonous acid (MMA) then dimethylarsinous acid (DMA) and in some cases trimethyl arsenic acid (TMA), all of which bear different oxidation states, either trivalent (III) or pentavalent (V) (National Research Council, 2001 update). The trivalent forms have been recognized as the most toxic (Styblo et al., 2000; Cohen et al., 2002) and monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)] are more cytotoxic than the inorganic arsenicals arsenate [As(V)] and arsenite [As(III)] (Petrick et al., 2000; Styblo et al., 2002). Despite the known toxicity of the metabolites of arsenic, research has yet to determine which arsenical causes cancer.

The bladder is constantly in contact with all forms of arsenic. Due to bioconcentration of urine by the kidneys, the bladder is exposed to much higher concentrations of arsenic than other organs (Chen et al., 1988). It has been previously proposed that arsenic causes co-mutagenicity through inhibition of DNA repair (Okui and

Fujiwara, 1986). Ding et al. (2009) demonstrated an interaction between arsenite and a PARP-1 zinc finger resulting in a decrease in PARP-1 activity and increases in oxidative DNA damage. Recently, Wnek et al. (2011) showed that MMA(III) can also lead to a decrease in PARP-1 activity through interaction with the zinc finger, but activity can be restored through zinc supplementation. DNA damage in the urothelium can therefore cause decreased DNA repair in the presence of arsenicals, leading to an increased susceptibility to DNA mutations that could eventually result in cancer.

Previous work had demonstrated malignant transformation in an immortalized human urothelial cells line (UROtsa) following chronic, low level MMA(III) exposure. Work by Bredfeldt et al. (2006) was able to demonstrate that following 52 weeks of exposure to 50 nM MMA(III), UROtsa cells were able to form colonies in soft agar and tumor growth in SCID mice, showing that these cells were malignantly transformed. More recently, Wnek et al. (2010) shortened this timeframe of transformation, showing that at 12 weeks of 50 nM MMA(III) exposure UROtsa cells had the capability for growth in soft agar and tumor growth in SCID mice. Despite an understanding of the time period required for malignant transformation, the mechanism of development is still not fully understood.

One potential mechanism for malignant transformation includes the development of aneuploidy. Aneuploidy is present in a variety of human cancers, including breast, bladder, brain, bone, liver, lung, colon, prostate, pancreatic, ovarian, testicular, and cervical (Iarmarcovai et al., 2006). Induction of aneuploidy by a carcinogen can destabilize the number and structure of chromosomes and lead to dysregulation of

regulatory proteins, DNA synthesis, and DNA repair (Wise and Wise, 2010). It remains to be elucidated as to whether aneuploidy is a causative event in cancer or a consequence.

Aneuploidy can arise through a variety of mechanisms. Fenech (2002) identified five factors that can lead to abnormal chromosome number, including abnormal centrosome number leading to multipolar spindles, chromosome loss at anaphase as a result of kinetochore defects, malsegregation of chromosomes at anaphase as a result of defects in the separation of chromatids, mitotic slippage caused by inhibition of mitosis, and failure of cytokinesis following nuclear replication as a result of defects in microfilament assembly.

Abnormal centrosome number is believed to lead to genomic instability. Multiple centrosomes can lead to chromosome loss or gain by causing abnormal spindle and abnormal chromosome segregation (Hollander et al., 2002). It has been previously shown that arsenicals are able to induce centrosome abnormalities. Both As(III) and DMA(III) are able to induce centrosome abnormalities and can cause tetraploidy and malignant transformation (Liao et al., 2007; Liao et al., 2010; Ochi et al., 2003). Irregular centrosome numbers are a possible cause of tetraploidization, which is a possible mechanism of malignant transformation.

Defects in ataxia telangiectasia and Rad3 related (ATR) or ataxia telangiectasia mutated (ATM) phosphorylation can contribute to loss of proper cell cycle checkpoint regulation (Florl and Schulz, 2008). ATR and ATM are activated during DNA double strand breaks (DSBs), with ATR activated in response to DSBs at the replication fork and ATM activated at other DSBs that can also occur in nonreplicating cells (Cuadrado et al., 2006). Defects in DNA damage repair through ATM/ATR can lead to genomic instability

and malignant transformation (Liang et al., 2009). Tetraploidy resulting from ATM/ATR defects can arise through mitotic slippage and endoreduplication. Persistent DNA damage signaling results in ATM/ATR activation for both DNA repair and cell cycle regulation; however, lack of entry into mitosis can result in a switch to a $G_1$-like state, from which the cells can re-enter S phase thereby becoming tetraploid (Davoli et al., 2010). Because MMA(III) is known to cause DNA single and double strand breaks (Wnek et al., 2009 and unpublished data), it is possible that mitotic slippage and endoreduplication is a mechanism of tetraploidy.

Previous research has not investigated MMA(III)-induced aneuploidy in either acute or chronic exposure. Determination of whether MMA(III) can induce aneuploidy is essential in fully understanding the transformation process. Once it can be shown that chronic, low level MMA(III) exposure is able to induce aneuploidy, the underlying mechanism can be solved. This study will investigate two potential mechanisms of aneuploidy, ATM dysregulation that can result in mitotic slippage and centrosome amplification which can lead to inappropriate chromosomal segregation. And understanding of the mechanism to aneuploidy would help to elucidate the pathway of arsenic carcinogenesis.

## Materials and Methods

*Reagents*. Dulbecco's Modified Eagle Medium, fetal bovine serum, antibiotic-antimycotic, and 1x trypsin-EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, $CH_3AsI_2$) was prepared by the Synthetic Chemistry Facility Core (Southwestern Environmental Health

Sciences Center, Tucson, AZ) using the method of Millar et al. (1960). Etoposide was obtained from Trevigen, Inc. (Gaithersburg, MD). Water used in studies was distilled and de-ionized.

*Dosing Solutions.* Preparation of doing solution and procedures were derived from Bredfeldt et al. (2006). Pure MMA(III) was stored in ampules at 4 °C. Fresh stock solutions of 25 mM MMA(III) were made and diluted to a final concentration of 5 µM prior to dosing (1:100 dilution) to obtain a final concentration of 50 nM MMA(III). All dosing solutions were sterile filtered with a 0.2-nm acrodisc and stored in sealed, sterile tubes at 4 °C that were opened only for dosing in a sterile cell culture hood. MMA(III) solutions in distilled, de-ionized at 4 °C were stable for approximately 4 months (Gong et al., 2001).

*Cell Culture*. UROtsa cells were a gift from Drs. Donald and Mary Ann Sens (Univeristy of North Dakota). Cell culture was adapted from Wnek et al. (2009). Briefly, UROtsa cells were cultured in DMEM growth medium with 5% v/v FBS and 1% antibiotic-antimycotic. Culture medium was changed every 2-3 days. Cells were stored in an incubator with an atmosphere of 5% $CO_2$:95% air at 37 °C. When cells reached confluency, they were removed with trypsin-EDTA (0.25%) and subcultured at a ratio of 1:4. MMA(III) treated cells were continuously exposed to 50 nM MMA(III) in the enriched culture medium and dosed every 2-3 days.

*Cell Cycle Analysis.* UROtsa, URO-MSC4, URO-MSC8, URO-MSC12, and URO-MSC16, cells were plated in six-well plates (Greiner Bio One) at a density of $4 \times 10^5$ cells per well. When cells were 80-90% confluent, the media was removed and transferred to a centrifuge tube, the cells were washed twice with 1x PBS and transferred

to the tube, and the cells were trypsinized. Once all cells detached the trypsin was deactivated with 5% FBS/media and cell suspension was transferred to tube containing conditioned media and PBS washings and centrifuged at 1500rpm for 10 minutes. The media was removed and cells were slowly resuspended in 1 ml ice-cold 70% ethanol while vortexing and stored at -20 °C overnight. Cells were centrifuged at 2000rpm for 15 minutes, ethanol was removed, and cells were resuspended in 1 ml cold PBS and transferred to 5 ml round bottom tubes (BD Falcon, Franklin Lakes, NJ). 50 µl of 10 mg/ml RNase A in TE buffer was added followed by 25 µl of 1.6 mg/ml propidium iodide in dd$H_2O$. Tubes were covered, incubated at 37 °C for 30 minutes, and placed on ice until analysis. Samples were analyzed at the Arizona Cancer Center Cytometry Core Facility using a FACScan cytometer (BD Biosciences, San Jose, CA). Cells were analyzed via excitation/emission wavelengths at 488/650 nm.

*Western Blots.* UROtsa, URO-MSC4, URO-MSC8, URO-MSC12, and URO-MSC16, cells were plated in six-well plates (Greiner Bio One) at a density of $4 \times 10^5$ cells per well. After 24 hours, cells were washed twice in ice-cold PBS and cells were then scraped in radioimmunoprecipitation lysis buffer containing 50 mM Tris-HCl (pH 8.6), 1% nonyl phenoxylpolyethoxylethanol, 0.25% $C_{24}H_{39}NaO_4$, 150 mM NaCl, 1mM PMSF, l g/ml aprotinin, l g/ml leupeptin, 1 mM NaF, 1 mM $Na_3VO_4$, 1 mM EDTA, and l g/ml protease inhibitor cocktail. The lysates were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. Supernatant protein concentrations were determined by the Bicinchoninic Acid Kit for protein determination (Sigma-Aldrich). Fifty micrograms of each sample was loaded onto 3-8% NuPAGE Novex Tris-Acetate gels (Invitrogen, Carlsbad, CA). Samples were separated via SDS-PAGE with Xcell SureLock Mini-Cell (Invitrogen) and

transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Inc./GE Healthcare, Piscataway, NJ) with the Mini-Proteon II (Bio-Rad, Hercules, CA) and blocked for one hour at room temperature with 5% nonfat dry milk in Tris-buffered saline Tween-20. Blots were incubated overnight at 4°C with primary antibodies for phospho-ATM and β-actin (Cell Signaling Technology, Danvers, MA) at manufacturer's recommended dilution. The appropriate secondary antibody linked to horseradish peroxidase was used for detection of the primary antibody. Chemiluminescent detection was performed with enhanced chemiluminescence Western blotting substrate (Pierce Biotechnology, Inc., Rockford, IL). Images were scanned with a Scanjet 5370C (Hewlett Packard, Palo, Alto, CA) at maximum resolution and prepared in Adobe Photoshop 3.0 (San Jose, CA). Data shown were representative of a total of four experiments ($n = 3$).

*Immunofluorescence.* Samples for immunofluorescence were grown on Lab-Tek II chamber slide system (Nalge Nunc International, Rochester, NY) at a density of $5 \times 10^4$ cells per well. Cells were fixed in ice-cold methanol for 10 minutes, washed twice with 1x PBS, permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, and washed three times with PBS. Cells were blocked with 1% BSA in PBST for 60 minutes and incubated with anti-γ-tubulin mouse monoclonal antibody (1:200; Sigma, St. Louis, MO) in 1% BSA in PBST overnight at 4 °C. Cells were washed three times for 5 minutes each and incubated with the AlexaFluor 488 goat anti-mouse secondary antibody (1:1000; Invitrogen) in 1% BSA in PBST for one hour at room temperature in the dark. Cells were washed three times for 5 minutes each and stained with DAPI (1µg/ml; Roche, Germany) for one minute and rinsed with PBS. Cells were mounted with Prolong Gold Antifade (Invitrogen) and stored in the dark until viewing. Images were taken using an Olympus

IX70 microscope with a 20X objective. In each sample, 200 cells were counted after staining and cells with more than 2 centrosomes were classified as abnormal.

## Results

*Cell Cycle Analysis*

Cell cycle analysis was performed to determine the cell cycle phase at various time points of MMA(III) exposure as well as determine the number of chromosomes (Fig. 1). Cells stained with propidium iodide showed that there is an increasing population of $G_1$ tetraploid cells. There is a significant decrease in the number of $G_1$ diploid cells following 8 weeks of exposure to MMA(III) (Fig. 2a). The significant decrease in diploid cells resulted in a subsequent increase in $G_1$ tetraploid cells at that same time point. By 16 weeks of exposure, 93.14% of the cells were tetraploid (Fig. 2b).

*Western Blot*

Because ATM has a significant role in both DNA damage repair and cell cycle regulation, it is important to determine if MMA(III) has any impact on ATM and the development of tetraploidy. ATM is present in the cell in an inactive dimer, but with DNA damage it undergoes an autophosphorylation, resulting in dissociation of the dimer complex and an active phosphylated ATM (pATM). Following its activation, it is recruited to the DNA damage site (Derheimer and Kastan, 2010). Western blot for pATM did not show any changes in levels of pATM between the untreated UROtsa control and the 50 nM treated UROtsa variants (Fig. 3.).

*Immunofluorescence*

Centrosome amplification has previously been shown to lead to chromosomal instability (Laio et al., 2007; Laio et al., 2010). To determine the effects of chronic arsenic exposure

on centrosome number in UROtsa cells, cells were incubated with cell culture media with or without 50 nM MMA(III) for 24 hours before fixation, permeation, and immunostaining. DNA was stained with DAPI and centrosomes were stained with anti-γ-tubulin. In the study, cells with greater than two centrosomes were considered abnormal. All cells, UROtsa, URO-MSC4, URO-MSC8, and URO-MSC12, had a low percentage of cells with abnormal centrosomes (Fig. 4). These data demonstrate that chronic MMA(III) exposure does not induce abnormal chromosome number.

## Discussion

It has been previously shown that chronic, low level exposure to arsenicals can cause malignant transformation (Bredfeldt et al., 2006; Wnek et al., 2010; Tokar et al., 2010; Sens et al., 2004). While it has been determined that UROtsa cells chronically exposed to 50 nM MMA(III) for 12 weeks are malignantly transformed (Wnek et al., 2010), the mechanism of malignant transformation remains to be determined.

Aneuploidy is an early event in metal carcinogenesis and is a factor in several types of human cancers. Metals such as arsenic, cadmium, chromium, cobalt and nickel all show evidence of aneuploidy in human cell lines, however, the mechanism of each metal to induce aneuploidy is not fully understood and widely debated. For arsenic induction of aneuploidy specifically, four possible routes have been studied thus far: 1) disruption of microtubule assembly dynamics, 2) bypass of the spindle assembly checkpoint leading to premature anaphase and tetraploidy 3) centrosome amplification and 4) tetraploidy resulting in further destabilization to hyperdiploid cells causing gene expression imbalances (Wise and Wise, 2010). While these potential mechanisms have

studies supporting them, many also have evidence against them, so future work must be done to further elucidate the pathway.

This study investigated two potential mechanisms of MMA(III)-induced tetraploidy. Chronic MMA(III) exposure does not show any alteration in level of pATM, showing the dysregulation or inactivation of ATM is not playing a role in tetraploidization through mitotic slippage and endoreduplication. However, mitotic slippage and endoreduplication could be a potential mechanism that is independent of ATM. Improper functioning of many cell cycle regulators can compromise cell cycle checkpoints and eventually result in tetraploidy.

Abnormal centrosome numbers have been observed following acute exposure to arsenicals (Ochi et al., 2003; Liao et al., 2007; Liao et al., 2010), however studies have not yet addressed centrosome abnormalities following chronic arsenic exposure. The main route to centrosome amplification involves centrosome duplication becoming uncoupled with cell divisions and duplication occurring when the cell cycle is stalled, which can occur during DNA damage (Holmes and Wise, 2010). Arsenicals, including MMA(III), can cause DNA damage indirectly through generation of reactive oxygen species (ROS) (Eblin et al., 2006), making this mechanism of centrosome amplification the possible route to tetraploidy. However, in this study, there were a low percentage of cells with abnormal centrosome numbers, indicating that chronic MMA(III) is not inducing centrosome amplification.

Further work must be done to determine the mechanism of tetraploidy. While it does not appear the ATM activity or centrosome abnormalities play a role in tetraploidization, it remains to be determined if the other mechanisms of aneuploidy play

of role in MMA(III)-induced tetraploidization. Mitotic slippage can result despite proper ATM function through dysregulation of other cell cycle regulators. Cytokinesis failure can also be a contributing factor in the development of tetraploidy through the development of binucleate cells and can also contribute to increased centrosome numbers (Sargona et al., 2010). Microtubule disassembly may also play a role in tetraploidization. Previous studies have noted the ability for arsenicals, including sodium arsenite and MMA(III) to interact with sulfhydryl-containing residues (Ramirez et al., 1997; Wnek et al., 2011). Since α- and β-tubulin contain cysteine residues that are thought to be critical to their function (Mellon and Rebhum, 1976), interaction of MMA(III) with these residues could cause disruption of microtubule formation and therefore affect microtubule binding to chromosomes and chromosome segregation and spindle assembly (Ramirez et al., 1997). Once the mechanism of tetraploidy can be determined, it would be beneficial to determine if the tetraploidy is the sole determinant of malignant transformation or if it is just a step in the process.

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## Abbreviations

As(III), arsenite; As(V), arsenate; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related, DMA(III), dimethylarsinous acid; DSB, DNA double strand break; MMA(III), monomethylarsonous acid.

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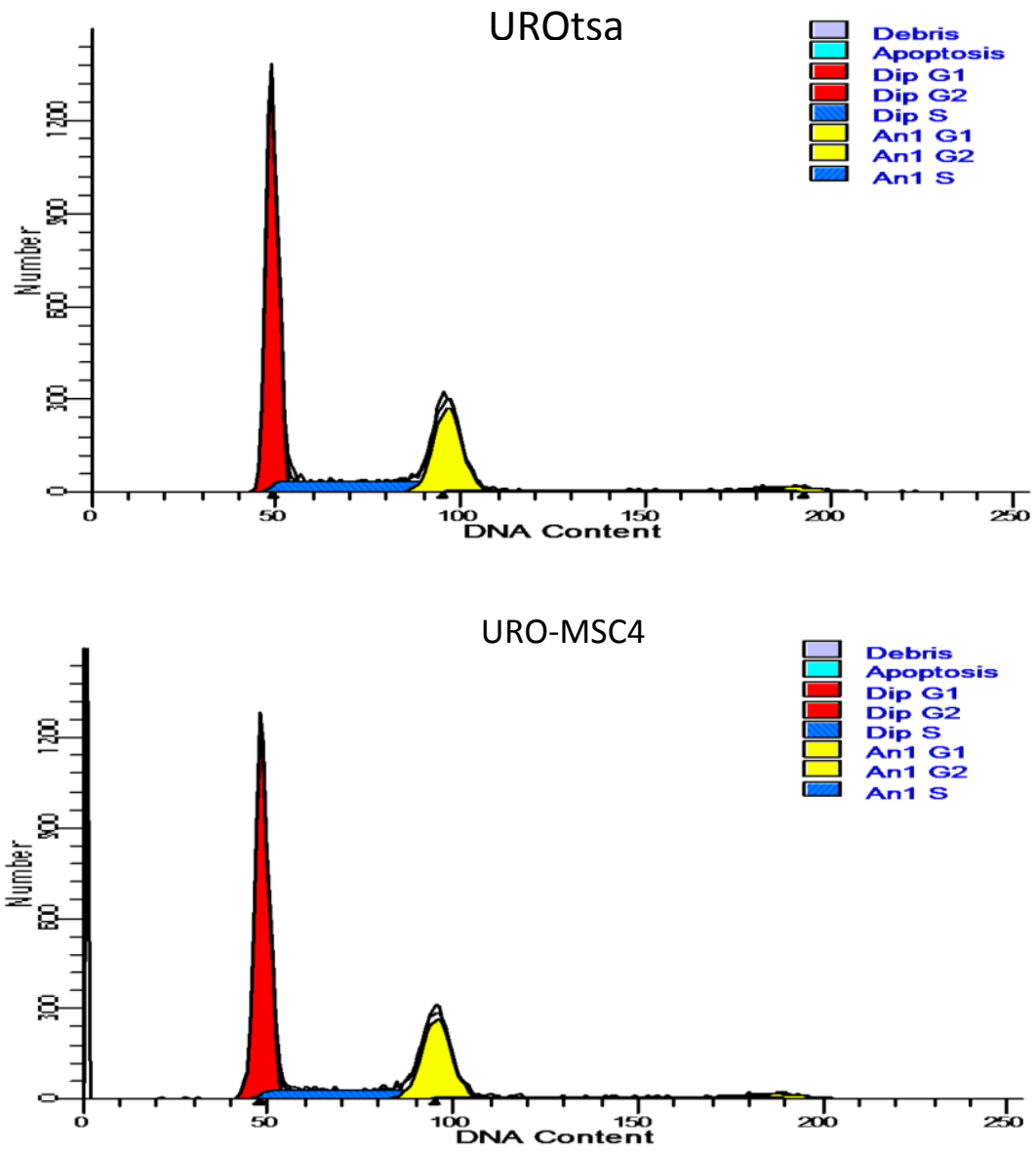
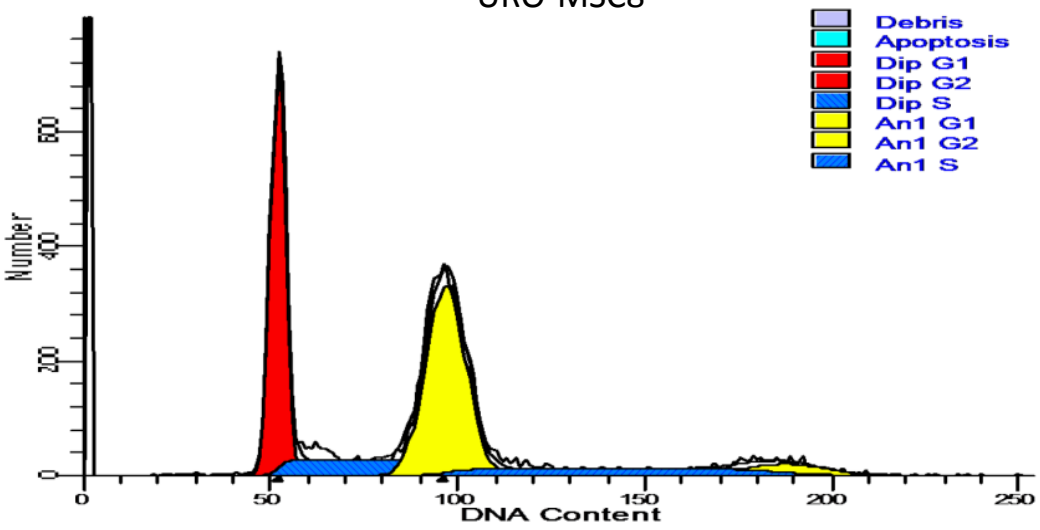
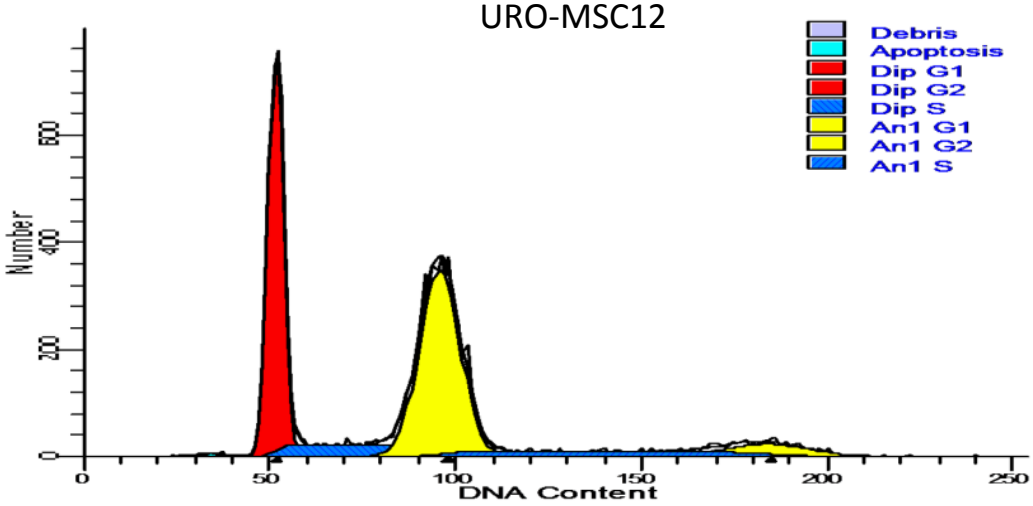
Fig. 1. Cell cycle analysis of cells following exposure to MMA(III). Cells were stained with propidium iodide and cell cycle was analyzed with flow cytometry. Similar results were obtained from other experiments. The percentage of tetraploid cells for UROtsa, URO-MSC4, URO-MSC8, URO-MSC12, and URO-MSC16 was 26.38%, 26.63%, 47.98%, 48.78%, and 93.14%, respectively (*n*=3).



URO-MSC8




URO-MSC12



URO-MSC16

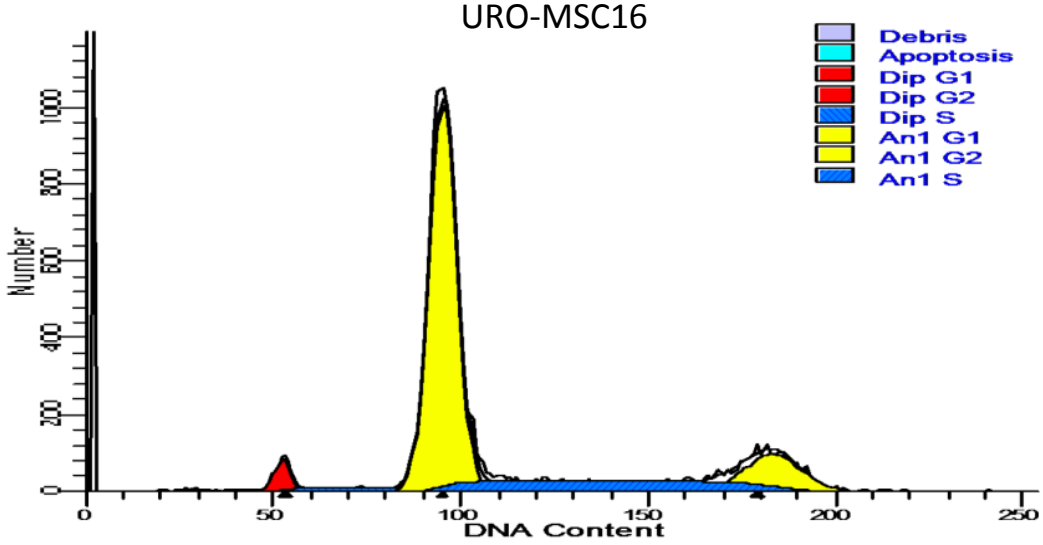
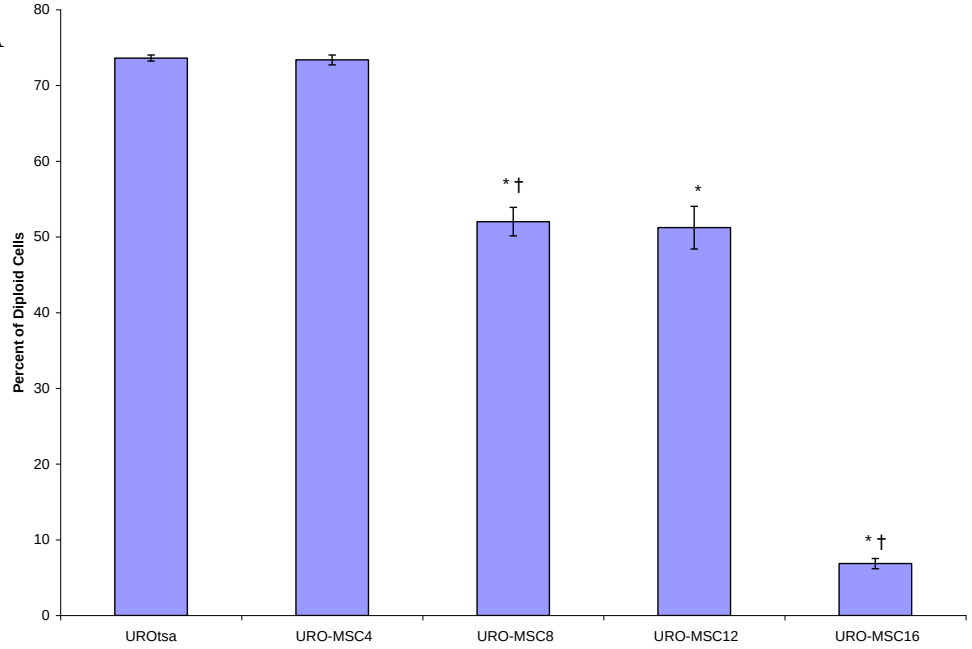

Debris
Apoptosis
Dip G1
Dip G2
Dip S
An1 G1
An1 G2
An1 S
Number
0
200
400
600
800
1000
0
50
100
150
200
250
DNA Content

Fig.2. Percentage of diploid and tetraploid cells following exposure to MMA(III). (a) Percentage of diploid cells for untreated UROtsa control and UROtsa cells exposed to 50 nM MMA(III) for 4, 8, 12, and 16 weeks (URO-MSC4, URO-MSC8, URO-MSC12, URO-MSC16, respectively). (b) Percentage of tetraploid cells for untreated UROtsa control and UROtsa cells exposed to 50 nM MMA(III) for 4, 8, 12, and 16 weeks (URO-MSC4, URO-MSC8, URO-MSC12, URO-MSC16, respectively). "*" Marks significantly significant difference ($p \leq 0.001$) between MMA(III)-exposed UROtsa variants (URO-MSC#) and untreated UROtsa control. "†" Marks a significant ($p \leq 0.001$) (a) downward or (b) upward trend. Significant differences were determined using the Student's *t*-test and error bars represent the standard deviation ($n$=3).

A




B

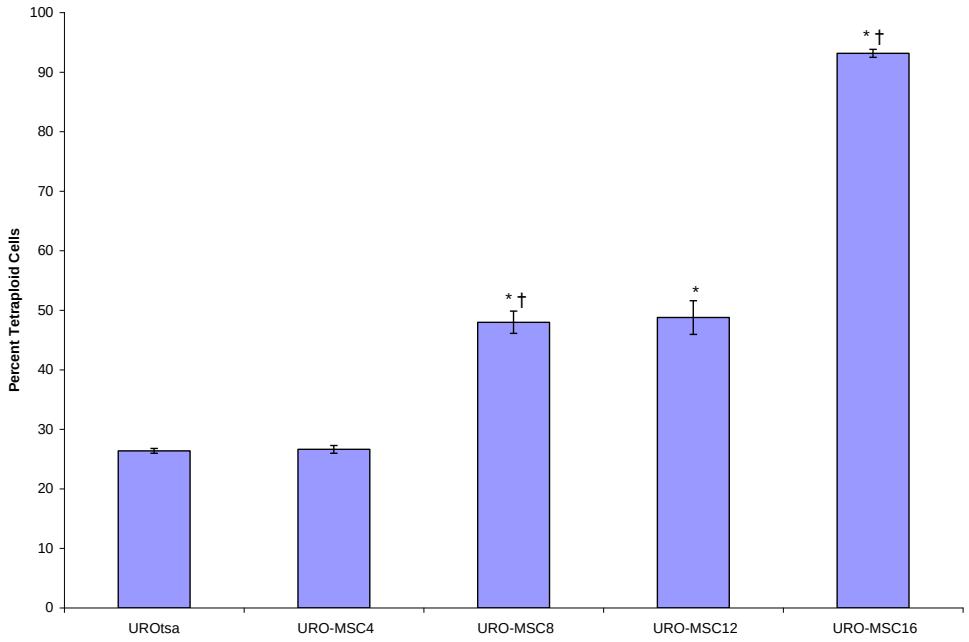


Fig. 3. Effects of MMA(III) on pATM expression. Levels of pATM were analyzed for untreated UROtsa control and the 50 nM MMA(III) exposed URO-MSC4, URO-MSC8, URO-MSC12, and URO-MSC16. UROtsa cells treated with 60μM etoposide for 8 hrs was used as a positive control. There were no changes in the levels of pATM for any of the URO-MSC variants compared to the control (*n*=5).

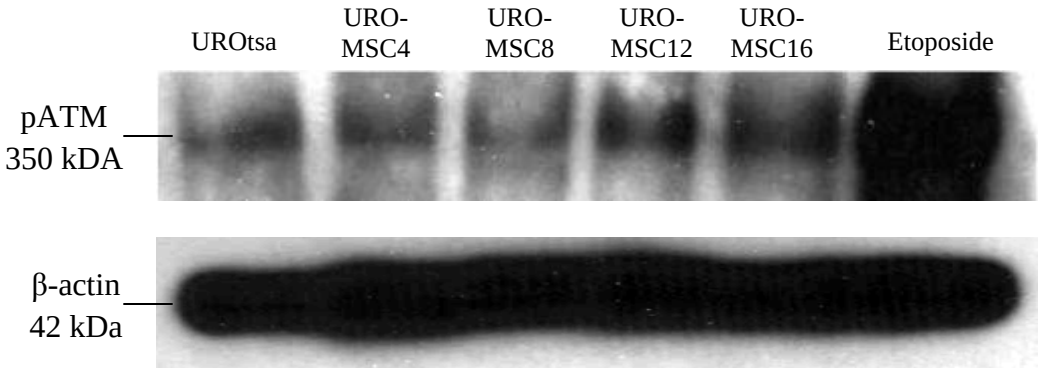


Fig. 4. Immunofluorescence for abnormal centrosome numbers. Untreated UROtsa control, URO-MSC4, URO-MSC8, and URO-MSC12 were stained with γ-tubulin for observing centrosomes. There were no significant differences in the number of centrosomes between the untreated control and the UROtsa variants.