

Manganese Inhibits Viability of Prostate Cancer Cells

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Abstract. *Background/Aim: Androgen deprivation therapy is usually in the initial phase a successful treatment for prostate cancer but eventually most patients develop androgen-independent metastatic disease. This study investigated if manganese (Mn) reduces viability of prostate cancer via induction of apoptosis. Materials and Methods: The prostate cancer cell lines PC3, DU145 and LNCaP underwent dose- and time-dependent screening of viability, analyzed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Flow cytometry was used for the cell-cycle and apoptosis analyses. Intracellular Mn concentration was measured using inductively coupled plasma-mass spectrometry. Results: At Mn concentrations of 200-1000 μ M, the effect on viability was most pronounced in PC3 followed by LNCaP cells. These cell lines also showed higher intracellular concentration of Mn compared to DU145. In all cell lines, Mn increased the proportion of cells arrested in the G₀/G₁ phase and induced apoptosis. Conclusion: To our knowledge, this is the first report demonstrating Mn as a potential agent in prostate cancer therapy.*

Prostate cancer was the second most common cancer in men worldwide in 2012, with a rate of 94.9 and 85 per 100,000 men (age-standardized) in Western and Northern Europe (1). It can progress from a clinically localized androgen-sensitive primary tumor to androgen-independent metastatic disease. Androgen-sensitive tumors initially respond to hormonal therapy. However, within 1-3 years of treatment, most patients develop castration-resistant tumors due to

amplification of the androgen receptor or alteration of signal transduction pathways. At this androgen-independent stage, use of chemotherapeutic agents such as the mitotic spindle inhibitor docetaxel have contributed to a significant increase in survival rate (2). Successful chemotherapy of patients with late-stage metastatic prostate cancer is dependent on cells being able to maintain their ability to undergo apoptosis. However, tumor cells inherit the ability to avoid apoptosis, and this is an underlying molecular reason contributing to disease progression and chemotherapy resistance (3). Development of therapies that focus on these mechanisms is thus essential.

Manganese (Mn) is a highly abundant metal in nature, mostly present in inorganic forms. As a trace metal, it plays essential roles for human, animals and plants, and takes part in many metabolic functions, cellular protection, bone and skeleton mineralization processes and reproductive mechanisms (4). However, it is known that humans exposed to high doses of bioavailable Mn, e.g. miners and welders inhaling Mn dust, may suffer neuronal injury, resembling Parkinson's disease (5, 6). Studies on PC12 cells derived from the adrenal gland in rats, showed that Mn induces DNA fragmentation, a characteristic marker of apoptosis (7). Deng *et al.* showed that Mn exposure of cultured astrocytes from rats inhibits cell viability, elevates the level of lactate dehydrogenase (LDH) leakage, and induces G₀/G₁ phase arrest and apoptosis (8). Studies on the arthropod *Nephrops norvegicus* have demonstrated that an excess of Mn inhibits proliferation of hematopoietic stem cells and induces apoptosis of these precursor cells (9). The literature is sparse on how overexposure to this trace metal affects human cells but some toxicological studies have been conducted. Pascal and Tessier showed that Mn is cytotoxic to human lung epithelial cells (10) and Zhao *et al.* reported that Mn induced G₀/G₁ and S phase arrest in a human lung adenocarcinoma cell line (11). It has also been shown that Mn induces apoptosis of HeLa and NIH3T3 cells through the activation of the caspase-12 pathway (12, 13) and *via* caspase-8 pathway in human lymphoma B-cells (14).

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As reviewed by Desoize (15), metal ions have been used for a considerable time as anticancer agents, *e.g.* platinum [in the form of cis-diamminedichloridoplatinum(II)] but the results of treatments are not yet satisfactory. To our knowledge, there have been no studies of the effect of Mn on prostate cancer cells. Here, we aimed to take advantage of the acquired knowledge of the ability of Mn to induce apoptosis in order to explore its potential of being used as tumor suppressor. This could open up future development of a more sustainable treatment of prostate cancer. Analyses of cell viability, morphology, cell-cycle progression and apoptosis after Mn exposure were conducted to evaluate dose and time response.

Materials and Methods

Cell culture and reagents. Three human prostate cell lines (PC3, DU145 and LNCaP obtained from the American Type Culture Collection, Manassas, VA, USA) were used in this study. PC3 cells derived from bone metastasis and DU145 cells derived from brain metastasis were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA). The cell line LNCaP, derived from metastasis of lymph nodes, were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640; Life Technologies) medium. For maintaining cells, they were cultured in T-75 culturing flasks (Sarstedt, Nümbrecht, Germany) in media supplemented with 10% fetal calf serum (FCS; Life Technologies) and 1% penicillin-streptomycin (PenStrep; 100 U/ml; Sigma-Aldrich, St. Louis, MO, USA). The cell cultures were kept in a humidified atmosphere with 5% CO₂ at 37°C and media were changed twice a week and cells were passaged once a week before reaching confluence.

For the experiments, cells were washed with Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies), trypsinized, counted microscopically in a Bürker chamber and seeded into plates. Appropriate cell density was reached in culture media without PenStrep following incubation in a humidified atmosphere of 5% CO₂ at 37°C over night to allow them to attach. MnCl₂ was dissolved in Hepes buffer (10 mM Hepes) and 0.14 M NaCl; pH 7.6 (Sigma-Aldrich) before starting exposure to Mn as described below for the different experiments.

Cell viability assay. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Roche Diagnostics GmbH, Germany, Penzberg, Germany). The MTT assay is based on the reduction of the substrate tetrazolium salt, to the reaction product formazan, by the succinate – tetrazolium reductase system located in the respiratory chain of the mitochondria. Since the reaction is only active in metabolically intact cells, the assay is commonly utilized to estimate cellular viability following exposure to drugs as the number of viable cells is proportional to the production of formazan (16, 17). The assay was carried out according to the manufacturer's instruction. Cells seeded into 96-well plates at a cell density of 5,000 cells/well were treated with 0 (control), 1, 10, 50, 200, 500, 1,000, 2,000 or 5,000 µM Mn, and after 24, 48, 72 and 96 h, 0.5 mg/ml MTT was added and the optical density was measured in a microplate reader after solubilization (Victor™ X4, # 2030 Multilabel Reader; Perkin Elmer Inc., Akron, OH, USA) at wavelength of 595 nm. Viability was calculated in relation to that of control cells (%).

Morphological observations. Cells were plated in 24-well plates and after reaching 60% confluency they were treated with 0, 10, 50, 200 or 1,000 µM Mn for 24, 48, 72 and 96 h. After exposure, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics) for 45 min to 1 h and the morphology was observed and documented using Zoe™ Fluorescent Cell Imager (BioRad Laboratories, Inc., Hercules, CA, USA).

Cell cycle analysis. Propidium iodide (PI) was used for analyzing DNA content in order to determine cell-cycle progression. Cells were plated in 6 cm plates and after reaching 60% confluency they were treated with 0, 50, 200 or 1,000 µM Mn for 24 or 48 h. Detached cells were collected with the medium and attached cells were collected by trypsinization, washed with PBS and fixed with 70% ice-cold ethanol overnight at 4°C. Cells were again washed with PBS and treated with 50 mg/ml RNase A (Roche) and 50 mg/ml PI (Sigma-Aldrich) for 30 min in the dark at room temperature. The cells were then subjected to flow cytometry (Novocyte™; ACEA Bioscience, San Diego, CA, USA) and aspirated at a low flow rate (14 µl/min) to minimize analysis of cell doublets which may interfere the determination of DNA content. Data of 10,000 events were collected for each sample. Data acquisition and cell-cycle distribution (Watson model) were carried out using NovoExpress (Novocyte™; ACEA). A coefficient of variation of less than 5% was considered as an acceptable analysis.

Apoptosis analysis. Annexin V Apoptosis Detection Kit Fluorescein Isothiocyanate (FITC) (eBioscience, San Diego, CA, USA) was used to determine phosphatidyl serine externalization and membrane integrity according to manufacture's instructions. Cells were plated and exposed to Mn as described above before being washed in D-PBS, resuspended in 195 µl of 1x binding buffer and stained with 5 µl annexin V-FITC. After 10-min incubation at room temperature in the dark, the cells were washed with 1x binding buffer and resuspended in 190 µl 1x binding buffer and then 10 µl PI was added. The cells were analyzed by flow cytometry (Novocyte™; ACEA). Data acquisition was carried out using NovoExpress (Novocyte™; ACEA). FITC-positive cells were designated as early-stage apoptotic cells, PI-positive cells were designated as necrotic cells and PI/FITC-positive cells were designated as late-stage apoptotic cells.

Intracellular manganese. Cells were seeded in 10 cm plates and after reaching 80% of confluency were exposed to Mn (0, 10, 50, 200 and 1000 µM) for 48 h. Detached and attached cells were harvested and pooled from three culture flasks of each cell line (n=2) and washed by centrifugation three times in PBS. Cell pellets were resuspended in approximately 500 µl PBS and digested in 20% HNO₃ at 120°C at 1.2 bar for 20 min. Dissolved Mn was measured with inductively coupled plasma-mass spectrometry (NexION 350D; Perkin Elmer Inc., Shelton, CT, USA). Samples were diluted 1/99 v/v in 1% nitric acid solution before measurement of Mn in standard mode using ULTRA Scientific (Kingstown, RI, USA) 1,000 ppm standard diluted in 1% HNO₃ to 0.01, 0.1, 1, and 10 ppb. No elemental corrections for polyatomic interferences were deemed necessary. Blanks and calibration standards were prepared in solutions containing experimental blanks (cells not exposed to manganese treatment) diluted 1/99 v/v in 1% nitric acid solution.

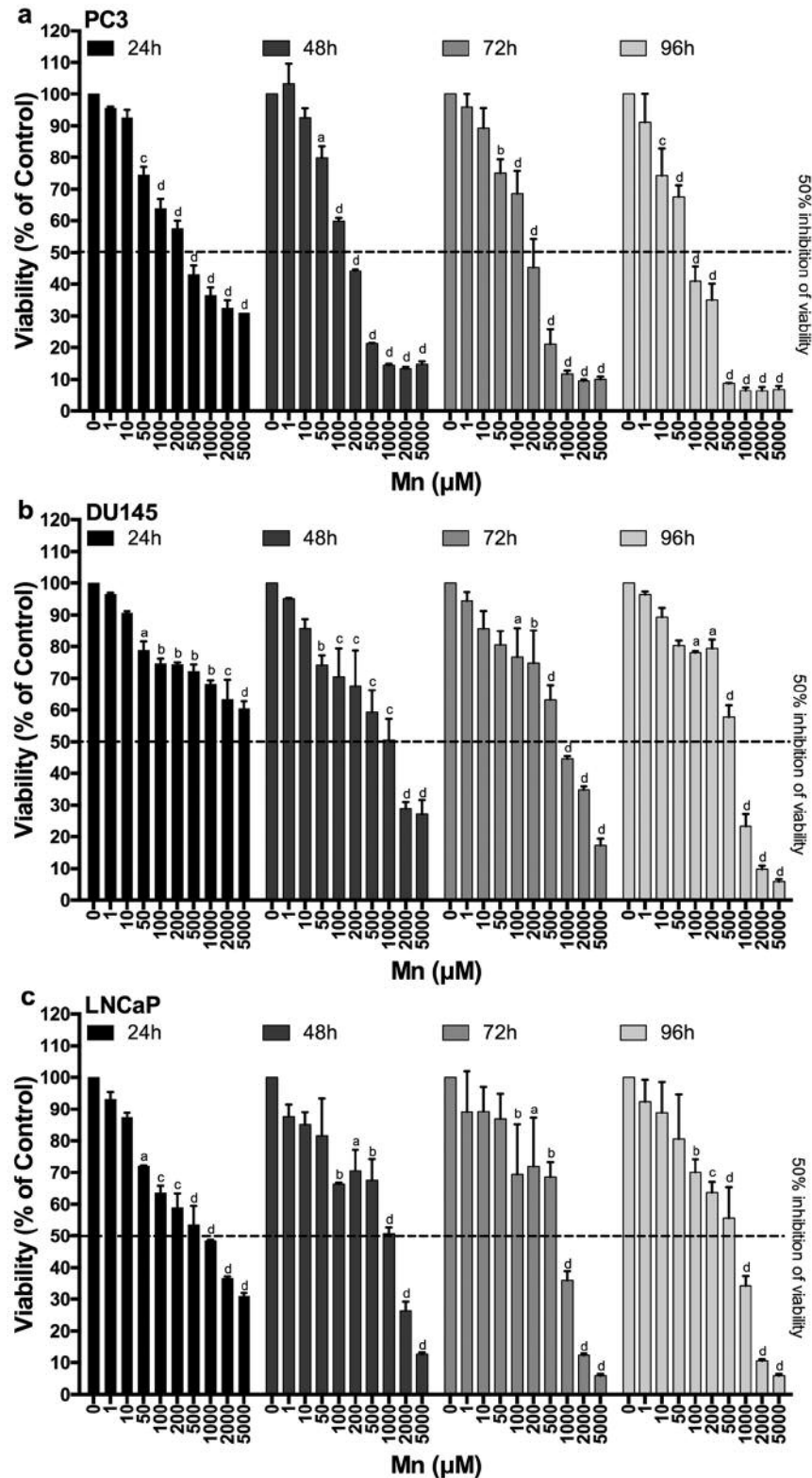


Figure 1. Effect of Mn on cell viability of PC3 cells (a), DU145 (b) and LNCaP (c) prostate cancer cells. Cells were treated with different concentrations of Mn for 24, 48, 72 and 96 h and the viability was analyzed by dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide. Data are expressed as mean \pm SEM of two independent experiments performed in triplicates. Letters indicate significant difference at: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ and ^d $p < 0.0001$ relative to the control (0).

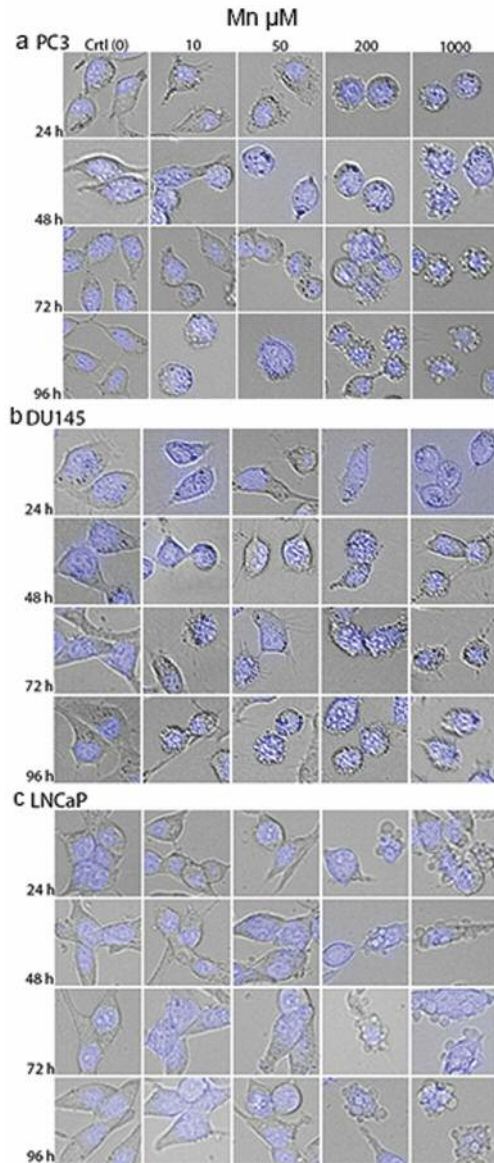


Figure 2. Morphology of PC3 (a), DU145 (b) and LNCaP (c) prostate cancer cells exposed to different concentrations of Mn for 24, 48, 72 and 96 h. Ctrl: Control. Images magnified $\times 20$ with digital zoom.

Statistical data analysis. All data presented are the mean \pm SEM from two independent experiments and for MTT analysis, the two experiments were performed with three replicates. The data were analyzed using two-way analysis of variance (ANOVA; factors: Mn concentration and exposure time) and Tukey test for multiple comparison. Pearson's correlation analysis was used for exploring the potential relationship between cell content of Mn and cell viability. The statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA) and a *p*-value of less than 0.05 was considered to be statistically significant.

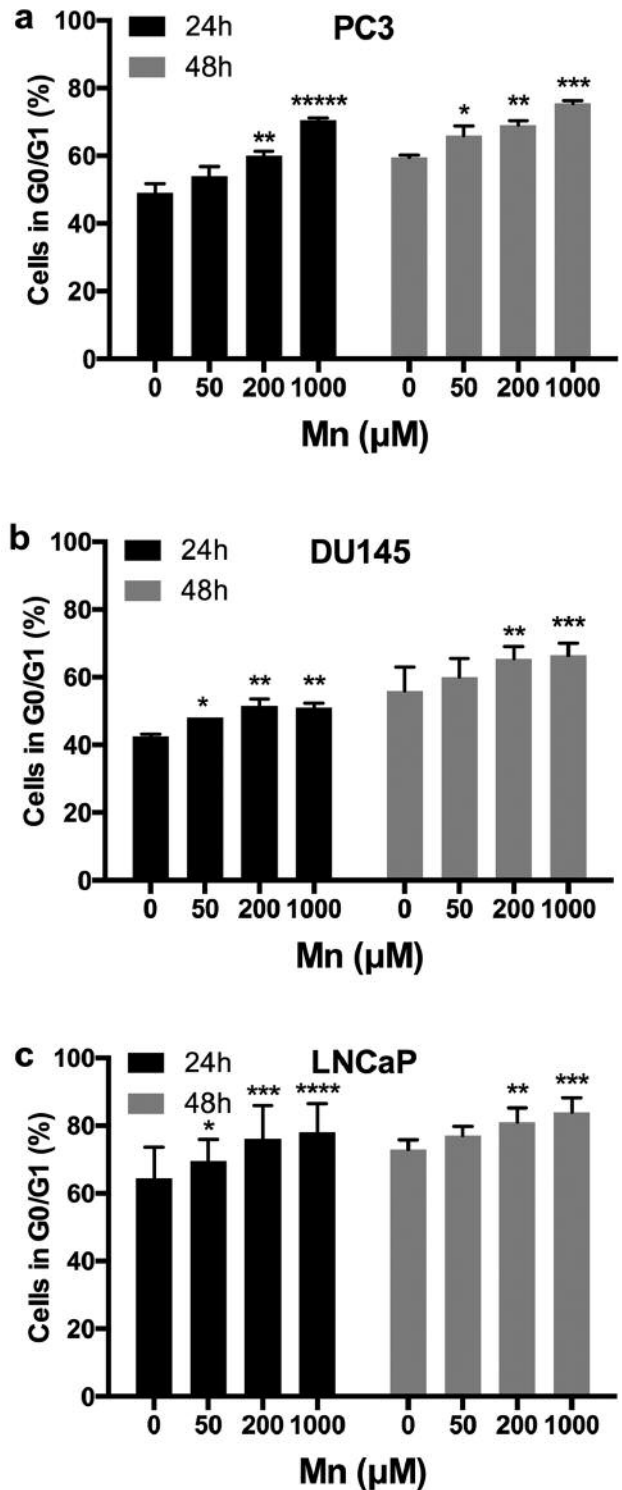


Figure 3. Cell-cycle analysis of cells in G₀/G₁ after Mn treatment in PC3 (a), DU145 (b) and LNCaP (c) presented as a percentage of that of the control cells (not exposed to Mn). Data are expressed as mean \pm SEM of two independent experiments. Statistically significantly different from the control (0 μ M Mn) at: **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

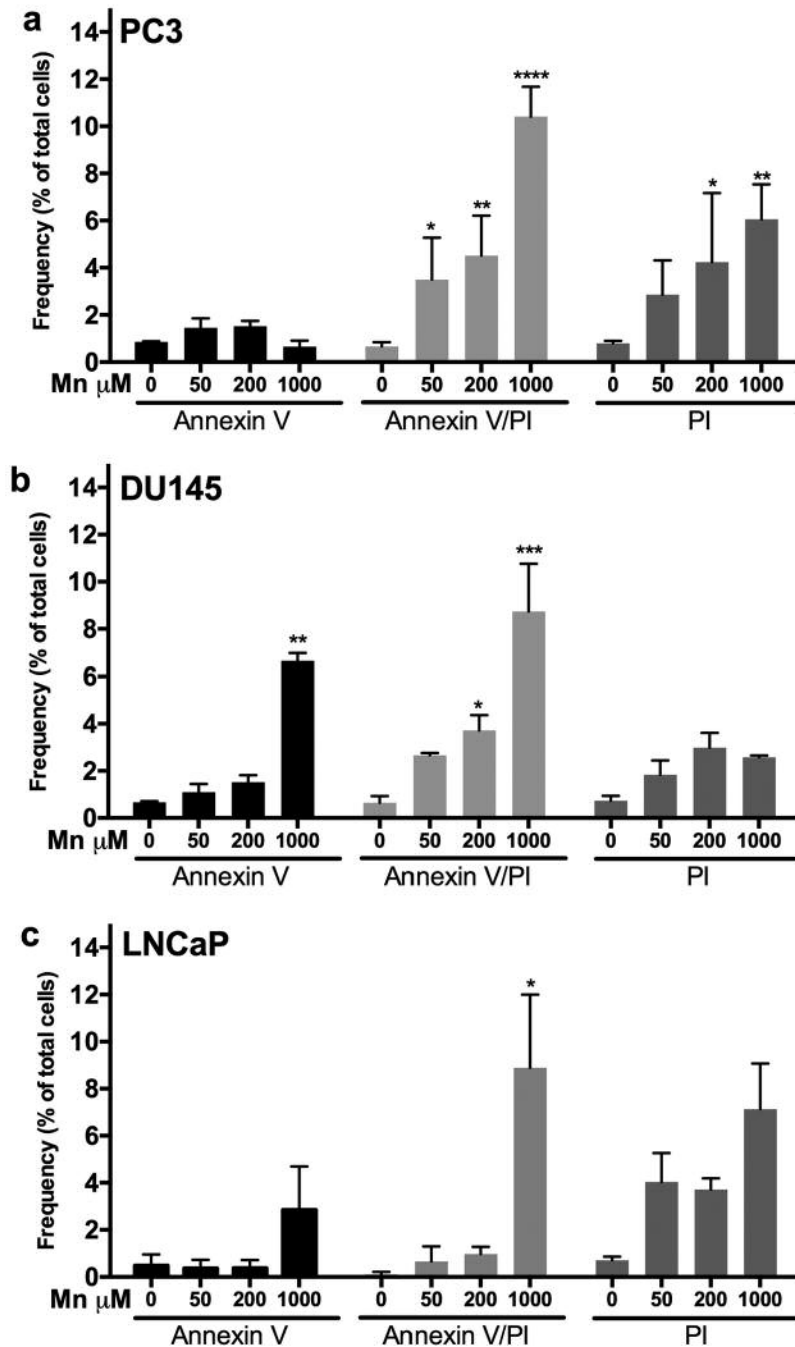


Figure 4. Analysis of apoptosis by flow cytometry after annexinV-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. PC3 (a), DU145 (b) and LNCaP (c) cells were treated with different concentrations of Mn for 48 h. Annexin V indicates early apoptosis, AnnexinV/PI indicates late apoptosis and PI indicates necrotic cells. Data are expressed as mean \pm SEM of two independent experiments. Statistically significantly different from the control (0 μ M Mn) at: * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001.

Results

Cell viability. When the prostate cancer cell lines PC3, DU145 and LNCaP were exposed to different concentrations

of Mn for different lengths of time, it was shown that Mn inhibited the viability of all cell lines in a concentration-dependent manner (Figure 1). The viability of PC3 cells was significantly reduced at all time points after exposure

Table I. Significant differences (*p*-values) in viability between prostate cancer cell lines.

Treatment duration (h)	Cell line comparison	Mn concentration (μM)				
		200	500	1000	2000	5000
24	PC3 vs. DU145	<0.01	<0.0001	<0.0001	<0.0001	<0.0001
	PC3 vs. LNCaP	n.s.	<0.05	<0.01	n.s.	n.s.
	DU145 vs. LNCaP	<0.05	<0.01	<0.01	<0.0001	<0.0001
48	PC3 vs. DU145	<0.01	<0.0001	<0.0001	<0.05	n.s.
	PC3 vs. LNCaP	<0.0001	<0.0001	<0.0001	n.s.	n.s.
	DU145 vs. LNCaP	n.s.	<0.05	n.s.	n.s.	<0.05
72	PC3 vs. DU145	<0.05	<0.001	<0.01	<0.05	n.s.
	PC3 vs. LNCaP	<0.05	<0.0001	<0.05	n.s.	n.s.
	DU145 vs. LNCaP	n.s.	n.s.	n.s.	n.s.	n.s.
96	PC3 vs. DU145	<0.0001	<0.0001	ns	n.s.	n.s.
	PC3 vs. LNCaP	<0.001	<0.0001	<0.01	n.s.	n.s.
	DU145 vs. LNCaP	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: Not significantly different ($p \geq 0.05$).

to 50 μM Mn and after 96 h even at 10 μM (Figure 1a). Neither DU145 cells (Figure 1b) nor LNCaP cells (Figure 1c) showed a significant decrease at 10 μM Mn concentration at any time point. However, after exposure to 100 μM Mn, viability of both LNCaP and DU145 cells significantly decreased.

There was no difference at any time point in the viability between the cell lines after exposure to Mn at concentrations <200 μM. With the exception of the highest concentration, the viability of PC3-cells compared to the DU145 cells was significantly lower at 200-5000 μM after 24, 48 and 72 h (Table I). After 96 h, a significant difference between these cell lines was shown only at 200 and 500 μM. Moreover, viability of LNCaP cells was lower after 24 h compared to that of DU145 but at longer exposure times, there was almost no difference. At 200-1000 μM Mn, at all time points PC3 cells generally had lower viability compared to that of LNCaP cells (for details see Table I).

Morphological observations. Microscopic observations of the different cell lines after exposure to different concentrations of Mn for 24-72 h revealed that PC3 cells were most affected. These cells showed morphological features characteristic of apoptosis such as cell shrinkage, cell wall deformation and gradually lower cell density after exposure to 200 μM for 24 h, 50 μM for 48 h and 72 h, and 10 μM for 96 h. Typical apoptotic bodies were seen after 48 h exposure to 1000 μM and to 200 μM after 72 and 96 h (Figure 2a). DU145 cells showed no indication of apoptosis after exposure to the different concentrations of Mn for 24 h. However after 48 h exposure to 200 μM, cell shrinkage and cell wall deformation were apparent (Figure 2b). LNCaP cells showed no indication of apoptosis after exposure to 10

or 50 μM Mn at any time point. However, morphological changes occurred after the cells had been exposed to 200 and 1000 μM Mn, with obvious apoptotic cells after 72 h exposure (Figure 2c).

Cell cycle distribution. The effect of Mn on cell-cycle progression was analyzed by observing cells in G₀/G₁ phase after exposure to different concentrations of Mn for 48 and 72 h (Figure 3). Compared to the untreated cells, the percentage of cells in G₀/G₁ increased after exposure to 200 μM Mn in all cell lines (Figure 3).

Apoptosis. Apoptosis was analyzed by staining externalized phosphatidylserine with annexin V and internalization of PI after exposure to Mn for 48 h. There were no early apoptotic stages in PC3 and LNCaP cells (Figure 4), however, in DU145 cells, early apoptotic stages were observed after exposure to 1,000 μM Mn (Figure 4). In PC3 cells, late apoptotic stages were shown after exposure to all concentrations (50-1,000 μM) and in DU145 cells, late stages were recognized at 200 and 1,000 μM Mn. LNCaP cells showed late apoptosis only after exposure to the highest concentration of Mn. There was also an increase in necrosis of PC3 cells exposed to 200 and 1,000 μM Mn.

Intracellular Mn concentration. Intracellular concentrations of Mn of unexposed cells were similar (~1.1 μM) between the different cell lines PC3, DU145 and LNCaP (Figure 5). After 48 h of exposure to different concentrations of Mn, the two-way ANOVA showed significant difference between the intracellular Mn concentration of the cell lines ($p=0.003$) but it differed only after exposure to the highest concentration (1,000 μM Mn). At this exposure, the intracellular Mn concentrations of PC3 (38.2±14.3 μM) and LNCaP (34.6±0.7 μM) cells were

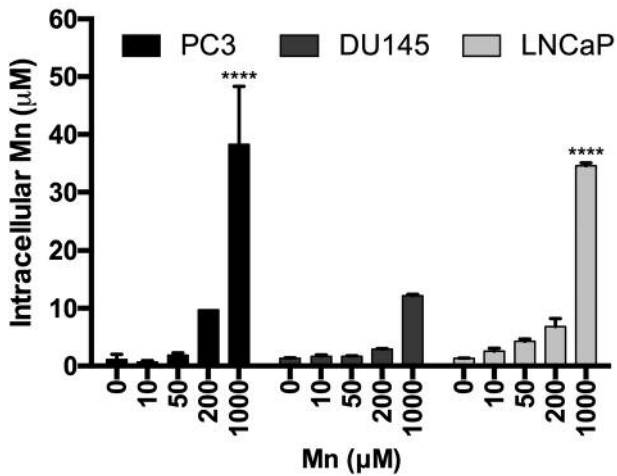


Figure 5. Manganese concentration (μM) in PC3, LNCaP and DU145 cells after exposure to different concentration of Mn for 48 h. Significantly different at **** $p < 0.0001$.

significantly higher compared to those of DU145 cells ($12.2 \pm 0.4 \mu\text{M}$; $p < 0.001$). The Mn concentration in DU145 cells did not significantly increase with increasing exposure to Mn. In both PC3 and LNCaP cells, the concentrations of intracellular Mn gradually increased after exposure to 50 μM and were significantly raised at 1,000 μM ($p < 0.001$). Pearson correlation analysis showed that there was a significant increase of intracellular Mn in both the PC3 and LNCaP-cells with decreasing cell viability (correlation coefficient: -0.929 , $p < 0.05$ and -0.966 , $p < 0.05$, respectively). For DU145 cells, the correlation coefficient was -0.816 , $p = 0.09$. The relationships between cell viability and intracellular concentrations of Mn in the different cell lines are illustrated in Figure 6.

Discussion

In this *in vitro* study, it was shown that exposure to Mn of prostate cancer cell lines PC3, DU145 and LNCaP significantly affected the viability of the cells. The measured MTT reductase activity reflected the decrease in number of metabolic active cells, which were affected both by dose and time. After exposure to Mn within the concentration range of 200-1000 μM , the viability also differed between cell lines. PC3 cells were more sensitive compared to LNCaP and DU145 cells. When comparing the required dose and time needed to reduce the viability by 50%, it was shown that after 24 h, PC3 cells reached this point at a concentration corresponding to $\sim 350 \mu\text{M}$ Mn, while 1,000 μM was needed for LNCaP cells. None of the concentrations led to 50% reduction in viability of DU145 cells after 24 h but after 48 h of exposure to 1,000 μM such an affect was achieved. In

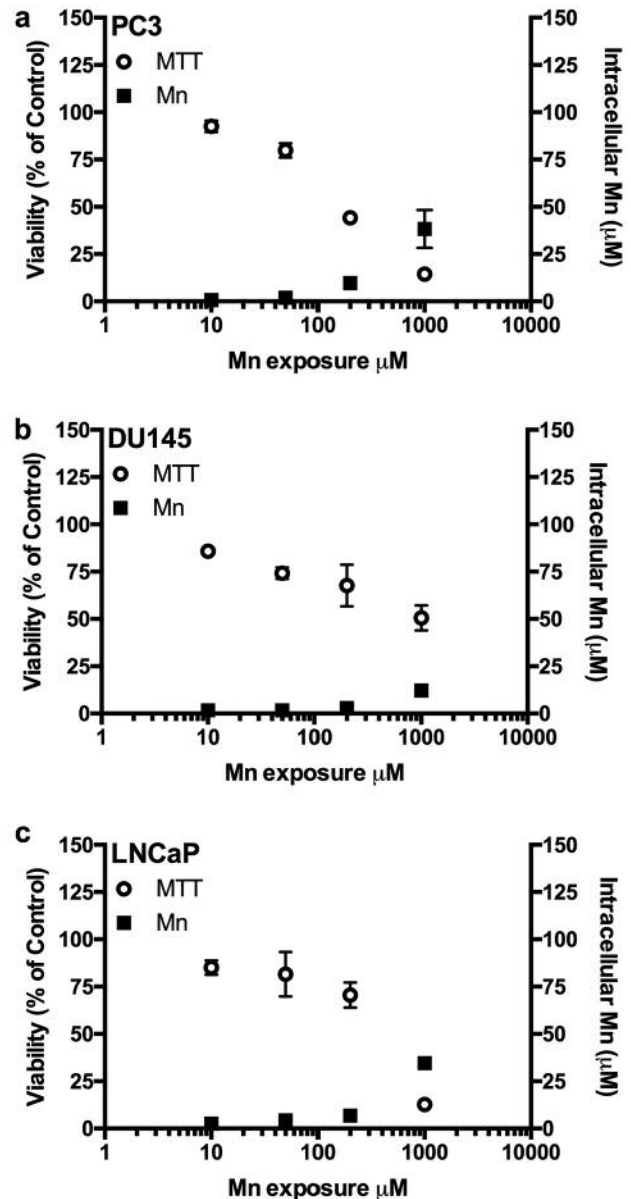


Figure 6. Illustration of the relationship between cell viability (MTT%) and intracellular concentration of Mn (μM) after exposing PC3 (a), DU145 (b) and LNCaP (c) cells to different concentrations of Mn for 48 h. x-Axis shows the inverse logarithm of Mn concentration.

contrast, at 48 h, 50% reduction was registered for PC3 cells at $\sim 150 \mu\text{M}$. It was demonstrated that after exposure to 2-200 μM of Mn for 48 h, the viability of lung epithelial cells was significantly reduced (10) and the Mn concentration giving 50% reduction was comparable to that for the PC3 cells in our study. Similarly, viability of neural stem cells (C17-cells) was significantly reduced after exposure to 50-250 μM Mn for 24 h, reaching 50% reduction at $\sim 100 \mu\text{M}$ (18).

Zhao *et al.* reported that Mn induced G₀/G₁ and S phase arrest in a human lung adenocarcinoma cell line (11). Our results also showed that PC3, DU145 and LNCaP cells enter G₀/G₁ arrest after 24-h exposure to 200 µM Mn. This indicates that the viability of the cells are affected by Mn, but does not verify if this arrest induces cell death. However, it has been reported that Mn also induces apoptosis of other type of human cells, such as HeLa cells (13) and lymphoma B-cells (14). Most attention regarding Mn toxicity has been directed towards nerve cells, as reviewed by Gerber *et al.* (19). The upstream molecular mechanisms involved are not yet fully known but the induced toxicity is known to be associated with oxidative stress (20-22). Formation of reactive oxygen species causes DNA damage (23, 24), arrests cell proliferation and activates transcription factors triggering apoptosis (25).

Activation of the executioners of apoptosis, the caspases, can be mediated either *via* the extrinsic pathway through the plasma membrane by the so-called death receptors, or through the intrinsic pathway by mitochondrial proteins (26, 27). However, in both HeLa and NIH3T3 cell lines, Mn has been shown to induce apoptosis independently of mitochondria, *via* a caspase-12 dependent pathway (12, 13). The capacity to respond by repairing damaged DNA relies on the level of redox imbalance and is likely to determine whether the cell will undergo apoptosis, necrosis or survive.

It has been reported that neural rat cells exposed to Mn respond with down-regulation of p53-induced phosphatase 1 (*Wip1*) which is believed to affect p53 signaling and neuronal apoptosis (28). This indicates that p53 expression may have a protective role against Mn toxicity. The cell lines used in the present study, PC3, DU145 and LNCaP, have different p53 expression (29). Both LNCaP and DU145 cells have two 17p alleles and are able to express p53 protein, however, LNCaP is considered to have wild-type *p53*, while DU145 expresses a mutated form. PC3 cells only have one 17p allele (30) and are unable to express p53 (*p53*-null). This may explain the different success in maintaining viability that was registered after exposure to 200-1,000 µM of Mn.

At the higher concentrations, 2000 and 5,000 µM, the differences between the cell lines were less pronounced, particularly after the longer exposure times, 72 and 96 h. Then viability was in general below 10% and there were morphological signs of cell damage due to apoptosis and necrosis, indicating that Mn at these concentrations was beyond any therapeutic value. Therefore, flow cytometry using annexin V and PI to discriminate between apoptotic and necrotic cell death was conducted after exposure to 0-1,000 µM of Mn for 48 h. Only PC3 cells showed a significantly increase in PI-positive cells after 200 and 1000 µM exposure, which indicates that the cells underwent necrosis. Even at 50 µM, there was an increased proportion of doubly stained cells, indicating cells at a late stage of apoptosis. Early-stage apoptosis was only found in DU145 cells exposed to 1,000 µM, while late-stage

apoptosis was found at this concentration in both DU145 and LNCaP cells. Thus it was concluded that Mn induced apoptosis of all the cell lines but some of the more sensitive PC3 cells were even forced into necrosis. Seemingly, a concentration below 200 µM, and a shorter exposure time should be appropriate for inducing apoptosis of PC3 cells while at the same time avoiding necrosis.

Conclusion

Recent cancer research has recognized apoptosis as being central to the regulation of tumor formation and therapy. Here we can conclude that a cost-effective and easily accessible metal, Mn, has potential as a therapeutic option to inhibit growth of prostate cancer cells. The cytotoxicity of Mn was seemingly reflected by the intracellular amount of the metal. The highest intracellular concentrations of Mn after 48 h of exposure to 200-1,000 µM were found in PC3 and LNCaP cells and it was also these cell lines which were most affected in viability and morphology. On the other hand, the flow cytometric analyses revealed that Mn increased cell-cycle arrest and apoptosis in all three cell lines, of which PC3 cells were the most sensitive and even reached a degree of necrosis. These findings will enable further studies of relevant doses of Mn in the exploration of apoptotic pathways and the possibility to combine Mn treatment with other chemotherapeutic agents.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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