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Arsenicum album Induces Cell Cycle Arrest and Apoptosis, and Inhibits Epithelial–Mesenchymal Transition in Hormone-Dependent MCF7 Breast Cancer Cells

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Abstract

Background Arsenic trioxide (As_2O_3) has been in therapeutic use since the 18th century for various types of cancers including skin and breast; however, it gained popularity following FDA approval for its use against acute promyelocytic leukemia. This present work was designed to evaluate the anti-cancer potential of a homeopathic potency of arsenic trioxide (*Arsenicum album* 6C) in hormone-dependent breast cancer.
Methods Breast cancer cells (MCF7) were treated with *Arsenicum album* (*Ars* 6C) to evaluate its anti-proliferative and apoptotic potential. We examined the effect of *Ars* 6C on the cell cycle, wound healing, reactive oxygen species (ROS) generation, and modulation of expression of key genes which are aberrant in cancer.
Results Treating breast cancer cells with *Ars* 6C halted the cell cycle at the sub-G0 and G2/M phases, which could be attributed to DNA damage induced by the generation of ROS. Apoptotic induction was associated with upregulation of Bax expression, with concurrent downregulation of the Bcl-2 gene. *Ars* 6C was also seen to reverse epithelial to mesenchymal transition and reduce the migration of breast cancer cells.
Conclusion The findings suggest that *Ars* has significant anti-proliferative and apoptotic potential against breast cancer cells. Further studies are required to elucidate the mechanism by which *Ars* exerts its effect in the *in vivo* setting.

Keywords
- breast cancer
- homeopathy
- arsenic trioxide
- *Arsenicum album*
- apoptosis
- cell cycle
- migration
- ROS generation
- hallmarks of cancer

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Background

Arsenic trioxide, As_2O_3 (ATO), has been used as a therapeutic agent for over 2000 years.[1] Some pharmaceutical texts from the 1800s have reported the use of arsenic pastes for breast and skin cancers.[2] ATO has also been indicated for leucocythemia (increased white blood cell count) in studies carried out in the late 19th century.[3] Studies from China reported the usage of ATO for acute promyelocytic leukemia (APL).[4] Several clinical trials conducted in the United States showed the efficacy of ATO in APL, resulting in a better clinical outcome, which led to FDA approval of ATO for relapsed APL in 2000.[5]

Arsenic in its inorganic form is poisonous and its toxicological effects environmentally have led to serious public health issues in several parts of the world, including various parts of West Bengal, Bihar, Jharkhand and Uttar Pradesh in India.[6] Interestingly, a study conducted in Chile to assess the proportion of deaths due to cancers during high exposure to inorganic arsenic in city water found that there was reduced mortality in breast cancer patients during the same period. Smith et al thus concluded in their study that the major reduction in breast

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cancer deaths could be attributed to the concomitantly high levels of exposure to inorganic arsenic during the same time period. They presented plausible data from various breast cancer cell lines that pointed to the therapeutic potential of inorganic arsenic against breast cancer.[7]

"Poisons in small doses are the best medicines; and the best medicines in too large doses are poisonous": this was the argument of William Withering, the 18th century founder of digitalis. He was a strong proponent for the use of arsenic for therapy.[2] Furthermore, the Arnold (German pharmacologist) – Schutz (psychiatrist) Law proposed that "minimum dose stimulates, medium inhibits and maximum destroys".[8] As per homeopathic philosophy, the concept of minimum dose was introduced by Dr. Christian Friedrich Samuel Hahnemann, "the Father of Homeopathy", after observations of aggravation in several patients.[9]

In line with these theories and the law of minimum dose of medicines in homeopathy, we investigated the role of a homeopathic potency of ATO, commonly known as *Arsenicum album* (*Ars*), in breast cancer. We examined the effect of *Ars* on the key hallmarks of cancer: namely evasion of apoptosis, alterations of the cell cycle, enhanced cell migration, and increased reactive oxygen species (ROS) generation. The levels of several apoptotic, cell cycle and epithelial-to-mesenchymal transition (EMT) genes in MCF7 hormone-dependent breast cancer cell line were assessed to throw light on the possible mechanism of action of *Ars*.

Materials and Methods

Cell Culture Conditions

MCF7 (hormone-dependent breast cancer) cells were obtained from the National Centre for Cell Science, Pune, India. The cells were maintained in complete medium which included Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Life Technology Ltd., Paisley, UK) and 1% Penicillin-Streptomycin (Gibco, Life Technology Ltd., Paisley, UK). MCF10A (normal epithelial breast) cells were obtained from ATCC, USA. These cells were maintained in complete medium which included Dulbecco's Modified Eagle's Medium/Nutrient mixture F12, DMEM/F12 (Sigma-Aldrich, St. Louis, USA) supplemented with 5% horse serum (Gibco, Life Technology Ltd., Paisley, UK), 0.5 µg/mL hydrocortisone (Invitrogen, Bangalore, India), 10 µg/mL insulin (Sigma-Aldrich, St. Louis, USA), 20 ng/mL epidermal growth factor (Peprotech, USA), 100 ng/mL cholera-toxin (Sigma-Aldrich, St. Louis, USA) and 1% penicillin-streptomycin (Gibco, Life Technology Ltd., Paisley, UK) and kept at 37°C in an incubator with 5% CO_2.

Treatments

Arsenicum album in 6C potency (10^{-12} dilution with succussion) was procured from Dr. Willmar Schwabe India Pvt Ltd., manufactured as per the Homoeopathic Pharmacopoeia of India. Since 90% alcohol was used as vehicle in the medicines, 90% v/v alcohol was therefore employed as solvent control (SC) in all the experiments.

Crystal Violet Assay for Cell Viability

Crystal violet (HiMedia, Mumbai, India) staining was done to assess the attached live cells on the culture plates. Briefly, 1×10^4 cells were seeded in 96 well plates and treated with SC or *Ars* 6C for 48 hours. The cells were washed with Dulbecco's phosphate buffered saline (HiMedia, Mumbai, India) and stained with 0.5% crystal violet; absorbance was measured at 570 nm. Each experiment was conducted three times.

Flowcytometric Analysis for Apoptosis

Annexin V-FITC/propidium iodide (PI) staining was carried out to analyze the mode of cell death using flowcytometry. Annexin V-FITC detects apoptotic cells and PI stains the late apoptotic or necrotic cells. Briefly, 2×10^5 cells were seeded in 60 mm culture dishes and treated for 96 hours with either SC or *Ars* 6C. The cells were then washed with ice cold PBS and counted. 1×10^6 cells were resuspended in 1X binding buffer and 5 µL Annexin V-FITC and 5 µL PI added to the samples and incubated for 10 minutes as per the manufacturer's protocol (BD Annexin V-FITC Apoptosis Detection Kit, BD Biosciences, Mountain View, California, USA). The samples were analyzed by Accuri C6 flow cytometer (BD Biosciences, USA); three independent experiments were carried out.

Microscopic Analysis of Apoptosis

Ethidium bromide (EtBr) (M.P. Biomedicals, Kaysersberg, France) and acridine orange (AO) (M.P. Biomedicals, Kaysersberg, France) dual staining was done to visualize morphological changes in cancer cells using fluorescence microscopy. Approximately 2×10^4 cells were plated in six well plates and incubated with *Ars* 6C or SC for 96 hours. The wells were gently washed with PBS. A staining solution was prepared by mixing EtBr (100 µg/mL) and AO (100 µg/mL) and added to each well. The wells were photographed after 5 minutes using fluorescence microscopy (Nikon Eclipse Ti, New York, USA). Multiple fields were analyzed.

Flowcytometric Analysis of Cell Cycle

Cell cycle arrest of breast cancer cells was assessed at separate phases of the cycle (G0/G1, S, G2/M). Briefly, 2×10^5 cells were seeded in 60 mm dishes and treated with *Ars* 6C and SC for 96 hours. The cells were washed with PBS and fixed with ice cold 70% ethanol and made RNA free using RNAse A (100 µg/mL; Thermo Scientific, Life Technologies, California, USA). 5×10^5 cells were counted and stained with PI (50 µg/mL; Invitrogen, Life Technologies, Parsley, USA) and incubated in the dark at room temperature for 15 minutes, followed by analysis using an Accuri C6 flow cytometer (BD Biosciences, USA).

ROS Generation Detection

To detect the level of ROS generation, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, USA) was used. Briefly, 2×10^5 cells were seeded in 60 mm dishes and dosed with *Ars* 6C or SC for 96 hours. They were washed with PBS and incubated with 1 µmol/L DCFH-DA at 37°C for 10 minutes in the dark. The fluorescence intensity of the samples was analyzed by FACS (Aria III, BD Biosciences, USA).

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In vitro Wound-Healing (Scratch) Assay

The wound healing (scratch) assay is done to assess the migration of adherent cells *in vitro*. The method measures the distance of the migrated cells to the cell free area. Approximately 1×10^5 cells were seeded in a six well plate. A scratch (cell-free area) was made by a 200 µL pipette tip when 90% confluency was attained. The dishes were then treated with *Ars* 6C and SC. The gap was photographed under a phase-contrast microscope at 0 and 48 hours. The images were analyzed quantitatively with computer software ImageJ. The distance between the wound was calculated (µm) and compared to the distance for the SC treated dishes.

Real-Time Polymerization Chain Reaction (RT-PCR) Analysis

Gene expression analysis following treatment reflects the changes in the various biological processes and to study this process we isolated the RNA from the cells. For total RNA extraction, 2×10^5 cells were seeded in 60 mm dishes and treated with *Ars* 6C or SC for 96 hours. The cells were harvested by TRIzol (Invitrogen, California, USA) and precipitated by chloroform (Merck, Mumbai, India) and isopropanol (Emsure, Darmstadt, Germany). DNA contamination was removed by DNase treatment as per the manufacturer's instructions (Qiagen, Germany) and the purity was checked using Nanodrop (Thermo Fisher Scientific, USA). A Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Life Technologies, California, USA) was used as per the manufacturer's protocol to synthesize cDNA by reverse transcription. Quantification of relative mRNA expression of the selected genes was done using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Life Technologies, California, USA), and real-time qPCR was performed by the Step One Plus Real-Time PCR System (Applied Bioscience, USA). Primers were designed with the help of the National Centre of Biotechnology Information database (National Library of Medicine, USA) (►**Table 1**). GAPDH served as the reference housekeeping gene. Real time PCR was carried out for 40 cycles under the following conditions: initial denaturation at 95°C for 3 minutes, denaturing at 95°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and the melt curve was recorded at 55-95°C (in 0.5°C increments). The $2^{-\Delta\Delta\Delta\Delta Ct}$ method was used to calculate the relative fold change.

Statistical Significance

Three independent experiments were performed, and the results presented as mean ± standard deviation. The statistically distinct groups were analysed with Student's *t*-test and two-way analysis of variance, and multiple comparisons done with Tukey's multiple comparison tests using GraphPad Prism 8 software. A p-value <0.05 was considered as statistically significant.

Results

Arsenicum album displays differential cytotoxicity

Crystal violet assay was carried out to evaluate the cell viability of MCF10A (normal breast epithelial cells) in comparison to MCF7 (hormone-dependent breast cancer cells) following treatment with SC or *Ars* 6C for 48 hours. The graph shows that there was 91% ± 5.6 viability in MCF10A cells, compared to 72% ± 6.6 viability in MCF7 cells after treatment with *Ars* 6C (►**Fig. 1**).

Arsenicum album Induces Apoptosis in MCF7 Cells

Flowcytometric Analysis

Double staining using Annexin V-FITC/PI of *Ars* 6C and SC treated cells showed induction of apoptosis, which was quantified through flowcytometric analysis. Cell membrane disintegration was revealed by uptake of PI, whereas intact membranes prevented the entry of PI and hence the results could differentiate between apoptotic and live cells following treatment. The dot plot graph displayed live cells (lower left quadrant), early apoptotic cells (lower right quadrant), late apoptotic (upper

Table 1 Primer sequences of Bax, Bcl-2, p53, p21, Cyclin D1, Survivin, Caspase 8, E-cadherin, N-cadherin, Vimentin and GAPDH genes

Gene	Forward primer	Reverse primer
Bax	5′TCTACTTTGCCAGCAAACTGGTGC3′	5′TGTCCAGCCCATGATGGTTCTGAT3′
Bcl-2	5′GTGGCCTTCTTTGAGTTCGGT3′	5′GTGCCGGTTCAGGTACTCAGT3′
p53	5′CTCTCCCTGTTGGTCGGTGG3′	5′TCAGACAGGGTTTGGCTGGG3′
p21	5′TGGAGACTCTCAGGGTCGAAA3′	5′GGCGTTTGGAGTGGTAGAAATC3′
Cyclin D1	5′CCGTCCATGCGGAAGATC3′	5′GAAGACCTCCTCCTCGCACT3′
Survivin	5′GTTGCGCTTTCTGTC3′	5′CTTTCTCCGCAGTTTCCTCA3′
Caspase 8	5′CCAGAGACTCCAGGAAAAGAGA3′	5′ATCCAGCAGGTTCTGTAGGCA3′
E-cadherin	5′GAACAGCACGTACACAGCCCT3′	5′GCAGAAGTGTCCCTGTTCCAG3′
N-cadherin	5′GACGGTTCGCCATCCAGAC3′	5′TCGATTGGTTTGACCACGG3′
Vimentin	5′CGGGAGAAATTGCAGGAGGA3′	5′AAGGTCAAGACGTGCCAGAG3′
GAPDH	5′AGCCACATCGCTCAGACA3′	5′TGGACTCCACGACGTACT3′

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Fig. 1 *Arsenicum album* displays differential cytotoxicity. Crystal violet assay to assess cell viability of MCF10A (normal breast epithelial cells) and MCF7 (hormone-dependent breast cancer cells) following treatment with SC or *Ars* 6C for 48 hours. Data are presented as mean ± standard deviation ($n = 3$). ****$p \leq 0.0001$, indicates significant difference in percentage of cell viability of MCF10A with respect to MCF7 cells.

right quadrant) and necrotic cells (upper left quadrant) (►**Fig. 2A**). The cell population was represented as percentages that were obtained from three sets of independent experiments. The breast cancer (MCF7) cells when treated with *Ars* 6C led to increased early apoptosis of 38.1% ± 11.8 compared to 9.5% ± 3 early apoptosis in SC treated cells. The percentage of late apoptosis also increased in MCF7 when treated with *Ars* 6C and SC and revealed 17.1% ± 6.6 and 12.6% ± 0.1 cell populations respectively. ►**Fig. 2A** is a representative image of the experiment.

Microscopic Analysis

Morphological changes in the cancer cells when treated with *Ars* 6C or SC were visualized through fluorescent microscopy by staining with EtBr/AO. AO can enter viable cells by proton trapping, whereas EtBr cannot pass through the membrane unless damaged. In ►**Fig. 2B**, *Ars*-treated cells showed signs of apoptosis, with compromised cell membranes, leading to the uptake of EtBr fluorescent dye. Early apoptosis was typically marked by flattened cells, shrunken nuclei and granular yellowish-green staining (marked with arrows in the enlarged inset image), whereas late-phase apoptosis was seen by orange-green staining of the cells. The SC-treated cells had intact structure that retained the AO dye uniformly, with no uptake of EtBr.

Gene Expression Analysis

Upregulation of the pro-apoptotic gene Bax and downregulation of the anti-apoptotic gene Bcl-2 are suggestive of apoptosis. In our study we witnessed an increasing trend in Bax expression (1.03 ± 0.01) and significant downregulation of Bcl-2 (0.73 ± 0.01) following treatment with *Ars* 6C, compared with SC treatment. Increase in the Bax/Bcl-2 ratio

Fig. 2 *Arsenicum album induces apoptosis in MCF7 cells.* **(A)** *Flowcytometric assessment of apoptosis by Annexin V-FITC/PI staining assay*. LL, lower left quadrant (live cells); LR, lower right quadrant (early apoptotic cells); UR, upper right quadrant (late apoptotic cells); UL, upper left quadrant (necrotic cells). **(B)** *Microscopic visualization of apoptosis by EtBr/AO dual staining.* The figure compares morphological features of cancer cells treated with SC and *Ars* 6C. The inset shows the magnified view of the field. The arrows show apoptotic cells which appear to be flattened in nature after *Ars* 6C treatment. Magnification: x20; scale bar: 100 μM. The images are representative of three independent experiments. (For interpretation of the references to color in figure legend 1B, the reader is referred to the online version of this article). **(C, D, E, and F)** *Gene expression analysis of Bax, Bcl-2, Caspase 8, Survivin and p53 respectively, in MCF7 cells*. *GAPDH* was used as house-keeping gene in all experiments. The $2^{-\Delta\Delta\Delta\Delta Ct}$ method was used to calculate the relative fold change. Data are presented as mean ± standard deviation ($n = 3$). *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$, indicate significant difference in percentage of gene expression with respect to solvent control.

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is an indicator of apoptosis which was reflected in our study: we found a significant increase ($p \leq 0.0001$) in Bax/Bcl-2 ratio (1.41 ± 0.03) after treatment with *Ars* 6C, in comparison to SC treated cells (►**Fig. 2C**).

Caspases act as primary effectors to execute the various features of programmed cell death. These caspases are present in the cell as zymogens and are activated through proteolytic cleavage of the inactive pro-caspases to the active caspases, which then initiate apoptosis. Caspase 8 typically gets activated by extracellular signals acting through death receptors and executes the extrinsic cell-death pathway. In our current study there was significant upregulation (2.06 ± 0.31) of Caspase 8 following *Ars* 6C treatment, as compared to SC treated cells ($p \leq 0.01$) (►**Fig. 2D**).

Survivin is a member of the "inhibitor of apoptosis" protein family that modifies caspase activation and blocks cell death. It also plays a critical role in the cell cycle and is expressed during G2/M phase arrest, when there is DNA damage. Survivin is over-expressed in various human cancers, particularly breast. Our results showed downregulation of survivin ($p \leq 0.05$) after *Ars* 6C treatment (0.61 ± 0.18) as compared to SC treated cells (►**Fig. 2E**).

p53 is a critical tumor suppressor gene that activates various responses in cancer such as cell cycle arrest and apoptosis. It senses DNA damage and halts the cell cycle for DNA repair or, in the case of irreparable DNA damage, may lead to apoptosis. In our study, we saw downregulation of p53 (0.50 ± 0.11), $p \leq 0.01$ (►**Fig. 2F**).

Arsenicum album Inhibits Cell Proliferation and Induces Cell Cycle Arrest in MCF7 Cells

Flowcytometric Analysis

Treatment with *Ars* 6C led to cell cycle arrest in the sub-G0 and G2/M phases. The results shown through flowcytometric analysis of cells stained with PI are clearly indicative of this. After SC treatment, there were $1.2 \pm 0.1\%$ and $12 \pm 0.2\%$ cells arrested in the sub-G0 and G2/M phase respectively, as compared to *Ars* 6C treatment which arrested $2.5 \pm 0.2\%$ and $18 \pm 0.4\%$ of cells in the sub-G0 and G2/M phase respectively. ►**Fig. 3A** is a representative image of the experiment.

Gene Expression Analysis

To validate the flowcytometry results, we performed gene expression analysis for key genes of the cell cycle, namely p21 and Cyclin D1. There was a significant increase ($p \leq 0.01$) in p21 expression (1.53 ± 0.14) when treated with *Ars* 6C, as compared to SC (►**Fig. 3B**). Conversely, as shown in ►**Fig. 3C**, a downregulation (0.34 ± 0.13) of Cyclin D1 in *Ars* 6C treated samples ($p \leq 0.001$) was observed.

Arsenicum album Induces Apoptosis via ROS Generation in MCF7 Cells

To assess the contribution of oxidative stress in the induction of cell death via apoptosis, levels of ROS were determined. For this we used a DCFH-DA probe to analyze ROS generation. SC treated cells showed $63.7 \pm 1.6\%$ ROS generation whereas *Ars* 6C treated cancer cells displayed $75.7 \pm 6\%$ after 96 hours of treatment. ►**Fig. 4** is a representative image of the experiment and illustrates changes in intra-cellular ROS generation using flowcytometry-based assay.

Arsenicum album Inhibits Migration in MCF7 cells

Wound Healing (Scratch) Assay

Cancer cell migration or malignant transformation leads to spread to distant sites, leading to poor prognosis of the disease. The interaction of cells with the extracellular matrix is studied with the scratch assay and we have shown that *Ars* 6C inhibited migration of cancer cells into the cell-free area by $73.7 \pm 1.5\%$ ($p \leq 0.0001$) as compared to SC (►**Fig. 5A**).

Gene Expression Analysis

EMT occurs in cancer cells, leading to phenotypical changes and consequent migration of cancer cells to distant sites. Upregulation of E-cadherin (epithelial marker) and downregulation of N-cadherin and Vimentin (mesenchymal marker) are indicators of inhibition of the EMT characteristic of cancer cells. Gene expression analysis for E-cadherin, N-cadherin and Vimentin to verify our findings from the wound healing assay revealed interesting results. *Ars* 6C treatment retained the epithelial character of cancer cells as it led to a significant ($p \leq 0.001$) upregulation of E-cadherin (1.5 ± 0.08), with a concomitant downregulation of N-cadherin (0.46 ± 0.18; $p \leq 0.01$), and Vimentin (0.42 ± 0.13; $p \leq 0.01$), as shown in ►**Fig. 5B**.

Discussion

As per the International Agency for Research on Cancer, cancer of the breast is one of the most diagnosed in the world and appeared at the top of the list in 2020.[10] The treatment modalities vary depending on the molecular subtypes.[11] Regardless of surgical removal of the primary tumor, there may be relapse within a few months to years.[12] Therapeutic strategies such as cytotoxic drugs kill the cancer cells; however collateral effects lead to the killing of normal healthy cells. These non-specific effects of the drugs are a major reason for acute or long-term toxicities.[13]

ATO is an inorganic compound associated with major toxic hazards. It is carcinogenic in nature and has been reported to be a ground water contaminant in several parts of India.[14]

Homeopathy is a branch of science that treats patients holistically with highly diluted and succussed medicines that have minimal adverse effects.[9] ATO in high dilutions (*Arsenicum album)* is used as a homeopathic medicine to treat various disease conditions involving the gastrointestinal, skin, respiratory and neuro-vascular systems.[15] Due to its similarity in symptomatology with arsenic poisoning, *Ars* is a valuable remedy for ATO-induced ground water pollution toxicity. In the study of Ive et al, it was found that *Ars* 6C, 30C and 200C had therapeutic effect on arsenic-induced toxicity in leucocytes.[16] Similarly, Kundu et al reported the efficacy of *Ars* 30C in reducing liver cytotoxicity and restoring the

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Fig. 3 *Arsenicum album inhibits cell cycle progression in MCF7 cells.* **(A)** *Flowcytometric assessment of cell cycle distribution of MCF7 cells.* x- and y-axes denote DNA content and cell number respectively. **(B and C)** *Gene expression analysis of p21 and Cyclin D1 respectively in MCF7 cells. GAPDH* was used as house-keeping gene in all experiments. The $2^{-\Delta\Delta\Delta Ct}$ method was used to calculate the relative fold change. Data are presented as mean ± standard deviation ($n = 3$). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$, indicate significant difference in percentage of gene expression with respect to solvent control.

Fig. 4 *Arsenicum album initiates ROS generation.* Flowcytometric analysis of ROS generation in MCF7 cells treated with solvent control or *Ars 6C* for 96 hours. x and y axes denote ROS and cell number respectively. ROS, reactive oxygen species.

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Fig. 5 *Arsenicum album inhibits migration of MCF7 cells*. **(A)** *Represents the migration of MCF7 cells visualized under microscope in the wound healing assay*. The distance between the outer lines depicts the initial scratch made in the confluent dishes. The distance between the inner lines shows the filling up of gap after 48 hours. Quantification of the results by subtracting the distance between initial wound and the gap at the end of treatment period and normalized to solvent control. Magnification: x10; scale bar: 100 μM. $^{****}p \leq 0.0001$ indicates significant difference in percentage of migration with respect to solvent control. **(B)** *RT-PCR analysis of E-cadherin, N-cadherin, and Vimentin expression in MCF7 cells*. Transcript levels of all the genes were determined ($n = 3$). GAPDH was used as house-keeping gene. The $2^{-\Delta\Delta\Delta\Delta Ct}$ method was used to calculate the relative fold change. Data are presented as mean ± standard deviation ($n = 3$). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ indicate significant difference in percentage of gene expression with respect to solvent control.

damage caused by ATO in a mouse model.[17] In another study by Banerjee *et al*, *Ars* 200C had a hepatoprotective effect against repeated injections of ATO.[18] Thus, these reports suggest that *Ars* high dilutions are non-toxic and can prevent and treat arsenic poisoning, which could be a major causative factor for various malignancies. A marked increase in mortality due to lung, bladder and kidney cancers was seen in several regions of Chile, due to higher levels of exposure to inorganic arsenic present in the drinking water. Surprisingly, breast cancer mortality rate was reduced during the same period. To find out the biological plausibility for this contradictory finding, *in vitro* studies were carried out to explore the therapeutic potential of ATO.[7] With regards to this interesting finding that ATO may in fact have a therapeutic effect against breast cancer, we decided to study the anticancer potential of *Ars* on a breast cancer model *in vitro*. ATO is known to be toxic to humans.[13] Therefore, firstly we ruled out the possibility of toxicity of *Ars* 6C to normal breast cells. Our study showed that though *Ars* 6C was cytotoxic to MCF7 cells it had minimal effect on the viability of normal breast epithelial MCF10A cells (►**Fig. 1**).

Apoptosis renders a physiologically protective mechanism to eliminate genetically damaged cells.[19] Several studies have demonstrated chemoprevention and chemotherapy by initiating or stimulating apoptosis to arrest cancer growth in cell and animal models, suggesting that apoptosis is a crucial process to suppress carcinogenesis.[20] *Conium maculatum*,[21] *Lycopodium clavatum*,[22] *Sulphur*,[23] *Calcarea carbonica*[24] and *Thuja occidentalis*[25,26] are examples of homeopathic drugs that have been shown to induce apoptosis in various cancers, both *in vitro* and *in vivo*. In the present study, we demonstrated that *Ars* 6C induced apoptosis in MCF7 cells after 96 hours of treatment. This finding is supported by a body of evidence in our study: (1) flowcytometric analysis with Annexin V-FITC/PI dual-stained samples demonstrated early and late apoptosis in *Ars*-treated cells as

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compared to SC treated cells (►**Fig. 2A**); (2) microscopy visualized cancer cells undergoing apoptosis following treatment (►**Fig. 2B**). The process of apoptosis can be initiated either by an intrinsic or an extrinsic pathway. In the case of the intrinsic pathway there is an alteration in the expressions of the Bcl-2 family of proteins, which leads to permeabilization of the mitochondrial outer membrane and release of cytochrome C to form apoptosomes. This then turns on the cascade of caspase activation, leading to apoptosis.[19] We demonstrated an increase in the Bax/Bcl-2 ratio by gene expression analysis, thereby confirming initiation of the intrinsic pathway (►**Fig. 2C**). On the other hand, the extrinsic pathway is activated via death receptors at the cell surface which bind to ligands and activate the initiator caspase 8 for further initiation of apoptosis.[27] In our study, we found there was an upregulation of caspase 8, confirming also the involvement of the extrinsic pathway for apoptosis (►**Fig. 2D**). Our results indicate that *Ars* was playing a dual role in the induction of apoptosis.

We further demonstrated that *Ars* could arrest the cell cycle at the sub-G0 and G2/M phases (►**Fig. 3A**). Many studies have reported that DNA damage is one of the major molecular events that is associated with cell cycle arrest and apoptosis. Lower levels of DNA damage may trigger DNA repair mechanism, leading to cell cycle arrest in the G2/M phase. On the other hand, when there is irreparable DNA damage, cells are eliminated by apoptosis.[28,29] In our study, we showed cell cycle arrest of cancer cells treated with *Ars* using flowcytometry. This result was validated by an upregulation of p21 (cyclin-dependent kinase inhibitor) and simultaneous downregulation of Cyclin D1 at the RNA level (►**Fig. 3B and C**). Hence, we may conclude that there is a high degree of DNA damage in cancer cells following *Ars* treatment, causing the cells to enter apoptosis. In addition, we observed that some of the cells that suffered DNA damage were arrested in the G2/M phase of the cell cycle. At this stage, the underlying mechanism is unexplored and requires further studies.

To determine other factors responsible for apoptosis and cell cycle progression, we analyzed the role of survivin. Survivin is a member of the "inhibitor of apoptosis" family that has multiple functions in cancer biology, namely (1) survival, (2) aggressiveness, (3) relapse and (4) resistance to treatment. It has been reported to inhibit the initiator caspase 9, as well as terminal effectors caspase 3 and caspase 7, in the apoptotic protease cascades. Interestingly, survivin had no effect on caspase 8 action.[30,31] Additionally, survivin modulated cell division and cell cycle progression and was maximally expressed at the G2/M phase.[32] Due to these pleiotropic effects on apoptosis and cell cycle progression, survivin is becoming an attractive target in cancer treatment. In breast cancer, survivin is believed to be overexpressed, thereby protecting cancer cells against apoptosis.[33] Remarkably in our study, we saw downregulation of survivin when MCF7 cells were treated with *Ars* (►**Fig. 2E**). Our result suggests that the anti-cancer property of *Ars* might partly be due to the downregulation of survivin, leading to the induction of apoptosis.

p53 is a tumor suppressor gene that is also termed the "guardian of the genome".[34] It senses DNA damage and can induce p53-dependent or p53-independent pathways to block cancer cells at the G2/M phase of the cell cycle.[35] We have reported downregulation of p53 gene expression (►**Fig. 2F**), suggesting a p53-independent pathway of G2/M arrest in cells treated with *Ars*.

To ascertain the cause of DNA damage we studied the production of ROS generation. ROS are involved in processes that lead to mitochondrial outer membrane permeabilization and the subsequent release of cytochrome C, which further initiates the activation of programmed cell death effectors.[36] We measured ROS levels in MCF7 cells following *Ars* 6C treatment by staining with DCFH-DA and assessed the levels using flowcytometry. We observed significant differences between *Ars* 6C and SC treated cells, suggesting that *Ars*-induced DNA damage and cytotoxicity might be associated with the generation of ROS in MCF7 cells (►**Fig. 4**).

We further investigated the role of *Ars* on cell migration. Cancer cell motility is a critical feature leading to metastasis. Our results from the wound healing assay revealed a disruption in the migration ability of MCF7 cells when treated with *Ars* as compared to the SC treated cells (►**Fig. 5A**). EMT is the primary trigger that initiates this migratory process in cancer cells. The hallmark of EMT is upregulation of N-cadherin and downregulation of E-cadherin cell adhesion molecules. Therefore, this switch between N-cadherin and E-cadherin is a potential target that can lead to the inhibition of EMT.[37,38] Vimentin is another mesenchymal biomarker that is being explored.[39] We therefore studied the mRNA expression of E-cadherin, N-cadherin and Vimentin in this context and found that there was a significant upregulation of epithelial marker E-cadherin and downregulation of the mesenchymal markers N-cadherin and Vimentin (►**Fig. 5B**). These results provide valuable insights into the potential of *Ars* 6C to inhibit the migration ability of MCF7 cells by acting on components of the EMT pathway.

Conclusion

We have presented the first direct evidence that the inhibitory effect of *Ars* 6C on breast cancer at the cellular level was primarily reflected by the inhibition of cell cycle progression at the sub-G0 and G2/M phase which was a result of DNA damage brought upon by the generation of ROS. Furthermore, treatment with *Ars* 6C induced apoptosis in MCF7 cells by modulating both the intrinsic and the extrinsic pathways. *Ars* 6C also reversed EMT and attenuated the migratory abilities of MCF7 cells. Moreover, *Ars* 6C revealed no significant cytotoxicity to normal breast epithelial cells. Our study highlights the anti-cancer potential of homeopathic ATO against hormone-dependent breast cancer

Highlights

- *Arsenicum album* (*Ars*), in 6C potency, induces apoptosis in hormone-dependent breast cancer (MCF7) cells.
- Apoptosis is mediated via ROS generation following treatment.

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- *Ars* inhibits cell proliferation and induces cell cycle arrest in MCF7 cells.
- *Ars* in 6C potency has the potential to inhibit migration in MCF7 cells.

Authors' Contributions

N.B. conceptualized the study, performed experimental investigation and formal analysis, and was a major contributor in writing the manuscript. M.G. performed formal analysis. C.T. performed formal analysis. B.C.D. performed formal analysis. S.T. conceptualized the study and designed its methods, supervised the work and edited the manuscript.

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Conflict of Interest

None declared.

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