Modulatory effects of Silibinin on Arsenic-mediated Biological and Molecular events in Prostate Cancer

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CERTIFICATE

The research work embodied in this thesis entitled **"Modulatory effects of Silibinin on arsenic-mediated biological and molecular events in prostate cancer"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full, for award of any degree or diploma of any university.

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TO MY

FAMILY AND

FRIENDS

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ABBREVIATIONS

AICR	American Institute for Cancer Research
APL	Acute Promeyolocytic Leukemia
APS	Ammonium persulphate
AR	Androgen receptor
As	Arsenic
ATCC	American type cell culture
ATM-Chk1/2	Ataxia telangiectasia-mutated—Check-point kinase1/2
BSA	Bovine Serum Antigen
CDK	Cyclin dependent kinase
CSIR	Council of Scientific and Industrial Reaserch
CSFs	Colony stimulating factors
DMA III	Dimethyl mono arsenite (III)
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent assay
eNOS	endothelial Nitric Oxide Synthase
FACS	Fluorescent Activated Cell Sorter

FBS	Fetal Bovine Serum
IARC	International agency for research on cancer
JNKs	c- Jun N-Terminal Kinase
MAPKs	Mitogen Activated Protein Kinase
MMA III	Monomethyl arsenite (III)
MTT	3-(4, 5-Dimethylthiozal-2-yl)-2,5-DiphenyltetrazoliumBromide
NRC	National Research Council
PBS	Phosphate Buffered Saline
PCa	Prostate cancer
PI	Propidium Iodide
PKA	Protein Kinase A
PKC	Protein Kinase C
ROS	Reactive oxygen species
Rb	Retinoblastoma
RPMI 1640	Roswell Park Memorial Institute-1640
SB	Silibinin
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
WCRF	World Cancer Research Fund

INTRODUCTION

INTRODUCTION

Cancer is a group of multiple disorders characterized by uncontrolled proliferation of cells as a result of alteration in the genetic information of the cells. Although cancer is a disease of altered gene expression, majority of the cancers are not heritable. It is a multifactorial and a multistagic disorder. A number of factors, both endogenous and exogenous are known causes of cancer. Exogenous factors include tobacco smoking and its use, infectious agents, medication, industrial chemicals, radiation and carcinogenic agents such as arsenic in food and drinking water as established by the International Agency for Research on Cancer (IARC). Consequently, cancer chemotherapy and cancer chemoprevention have become focus of the research community.

Cancer chemotherapy involves the use of anticancer drugs that may be obtained from natural or synthetic sources that kill cancer cells. Mustard gas, used as a chemical agent during World War I, was found to reduce the white blood cell count in leukemia since then, nitrogen mustard and its derivatives had been in use to treat neoplasia.

Chemotherapeutic drugs can be grouped based on their mechanism of action, chemical structure or their sources etc. Following is the classification of commonly used chemotherapeutic drugs based on their mode of action:

1) Alkylating agents
2) Antimetabolites
3) Natural products
4) Hormones and antagonists
5) Biological response modifiers
6) Differentiating agents
7) Proteasome inhibitors
8) Substituted urea
9) Tyrosine kinase inhibitors
10) Monoclonal antibodies

However, majority of malignant tumors are chemoresistant and therefore there is an urgent need to look for adjuvant therapies. The estimates of WCRF/AICR reports suggest that cancer is 30 to

40 percent preventable through appropriate nutrition and physical activity. Therefore, our focus was to study the chemopreventive modulation of arsenic- induced biological events in cancer cells using phytochemicals such as, silibinin.

REVIEW OF LITERATURE

Arsenic

Arsenic is a metalloid that is found ubiquitously in the environment. It is the twentieth most abundant element on the earth's crust and is known to be the component of more than 245 minerals. It occurs in the form of pyrites of the trivalent species arsenite and that of pentavalent species arsenate, in the natural deposits which dissolves in water at specific conditions of pH and temperature and reaches the aquifer. There, it forms salts of monovalent and divalent metals such as sodium, calcium etc. which are responsible for the ground water contamination. Further, ground water contamination due to arsenic can be due to dissolution of arsenical pesticides or disposition of industrial effluents containing arsenical chemicals. Thus, the ground water contamination due to arsenic can be either due to natural or anthropogenic causes. Humans are exposed to arsenicals mainly from air, water and food. However, groundwater contamination due to arsenic is the major concern since elevated levels of its salts in ground water lead to arsenic toxicity.

Reports of arsenic toxicity due to contamination of water are available from more than 30 countries. The major regions of Indian subcontinent affected by the ground water contamination due to arsenic are river basin of Ganga, Brahmaputra and Meghna and Bangladesh. Some cases for liver fibrosis due to drinking water were reported from Chandigarh in early 1984, and skin lesions from Kolkata, West Bengal in 1984. In addition, arsenic contamination has been found in the states of Bihar, Uttar Pradesh, Jharkhand, Assam, Chhattisgarh and Andhra Pradesh (WHO 2001).

Chronic arsenic toxicity also known as arsenicosis adversely affects multi-organ systems of human body and leads to systemic toxicity. The symptoms due to arsenicosis are insidious initially since its manifestation is dependent on the concentration of the dose and the duration of exposure. The uptake of arsenic leads to biotransformation of the cells leading to arsenic toxicity.

Toxicity by arsenic can be carcinogenic or non-carcinogenic. The drinking water standard for arsenic is ~0.133μM or 10 μg/ml (IARC 2004), above which if taken leads to adverse effects like dermal lesions, hypertension, ischemic heart disease, liver disease, arteriosclerosis, diabetes, neuropathy and cancer (NRC 1999, 2001). Arsenicosis is due to the biotransformation of cells which is due to both the inorganic and organic forms of the element. Initially, it was thought that

metabolism of arsenic was a process to eliminate arsenic, however, later it was confirmed that this process led to the formation of arsenic species that leads to systemic arsenic toxicity.

Table1. Summary of epidemiologic studies on high level As exposure (≥100µg/l) & cancer risk

S. No.	Study design	Population source (number of participants)	Exposure	Comparison	Risk estimates
1.	Case- control	Taiwan (444)	Years of water consumption	>40 vs. 0 years	OR= 3.01
2.	Case- control	Chile (571)	Water –As concentration from 1950 to 1994 life time residential exposure	200 to 400 vs. 0 to 10µg/l	OR cancer controls= 9.5 Or non-cancer controls= 8.5
3.	Cohort	Taiwan (2556)	Cumulative exposure to arsenic in drinking water	20 + 0 mg/l x years	RR= 4.01
4.	Cohort	United States (4058)	Drinking water	General population	SMR men=0.57 SMR women=0.44
5.	Cohort	Bangladesh (65876)	Drinking water	≥599 vs. < 50µg/l	RR men= 4.22 RR women=9.0

6.	Case- control	Taiwan (444)	Years of water consumption	>40 vs. 0 years	OR= 4.10
7.	Case- control	Argentina (128)	Drinking water	>200 vs. 0 to 50µg/l years of well water use 61 to 70 years	OR= 0.6 OR non smokers= 1.28 OR smokers=2.54
8.	Cohort	Taiwan (2556)	Cumulative exposure to arsenic in drinking water	20 + 0 mg/l x years	RR= 3.58
9.	Case- control	Finland (336)	Cumulative exposure to arsenic in drinking water	>20mg vs.< .05mg	RR short latency=1.50 RR long latency=0.53
10.	Cohort	United States (4058)	Drinking water	General population	SMR men=0.42 SMR women=0.81
11.	Cohort	Bangladesh (65876)	Drinking water	≥599 vs. < 50µg/l	RR men= 16.87 RRwomen=25.79
12.	Cohort	Taiwan (4074)	Drinking water	Arseniasis-endemic vs. general population	SIR = 1.96

13.	Cohort	United States (4058)	Drinking water	General population	SMR men=0.88 SMR women=0.72
14.	Cohort	United States (4058)	Drinking water	General population	SMR men=1.45

AR= attributed risk

SMR= standardized mortality ratio

SIR= standardized incidence ratio

RR= relative risk

OR= other risk estimates

Table 2. Summary of epidemiologic studies on low level As exposure (<100μg/l) & cancer risk

S. No.	**Study design**	**Population source (number of participants)**	**Exposure**	**Comparison**	**Risk estimates**
1.	Nested Case- control	USA (892)	Toenail	0.139 vs. <0.059μg/g	OR= 1.12
2.	Case- control	UK (1778)	Job records	Exposed vs. unexposed	OR= 1.1
3.	Case- control	Sweden (636)	Job and residential history	Exposed vs. unexposed	OR residents=2 OR miners=4.1 OR workers=3
4.	Cohort	China (12011)	Cumulative work	General population	AR= 15.8% As alone

			exposure vs. work records		AR= 11% As + radon AR= 8.7% combined As, radon and tobacco
5.	Cohort	USA (1393)	Job records	General population	SMR= 1.6
6.	Case- control	Utah, USA (231)	Drinking water	≥75 vs. <19 mg ≥74 vs 33mg/l x years	OR= 1.00
7.	Case- control	Western USA (509)	Drinking water	>80 vs. ≤80µg/d	OR= 0.94 OR exposure> 40 years ago among smokers= 3.67
8.	Nested case- control	Finland (373)	Toenail	>0.161 vs. < 0.05 µg/g	OR= 1.13

Uptake of arsenic by cells:

Quantum chemical studies indicate that arsenite in solution is solvated by 3 water molecules. At neutral pH, $As(OH)_3$ is the most stable structure existing in the solution (Ramírez-Solís *et al.* 2004). $As(OH)_3$ is known to be the active form of the metalloid that is subject to cellular uptake. Its transport seems not to involve ionic form or a multinuclear arsenic form by the phosphate transporters of the cells.

Arsenite can enter cells via transport proteins. Mammalian aquaglyceroporin (AQP9) has been reported to transport arsenite into cells when expressed in yeast (Liu *et al.* 2002) and lack of AQP9 expression in mice led to increased toxicity, suggesting that arsenite export from cells is also mediated by AQP9 (Carbrey *et al.* 2009). The latter study re-emphasizes that arsenite uptake also occurs through AQP9-independent mechanisms although that has not been characterized as yet. It was shown that trivalent arsenicals were much more readily taken up by cells than were their pentavalent counterparts, and were thus much more cytotoxic and genotoxic (Dopp *et al.* 2004, 2005).

For occupational inhalation, humans can be exposed to slightly soluble arsenic trioxide, arsenic trisulfide, and calcium arsenate, whereas exposure from drinking water includes sodium arsenate and sodium arsenite. Arsenite is taken into cells by passive diffusion, while arsenate competes with phosphate for uptake. Arsenite is an extremely thiol-reactive. It can affect enzyme activities by binding to critical vicinal cysteinyl residues, such as those in the lipoamide of pyruvate- dehydrogenase, tyrosine phosphatases, and enzymes involved in protein ubiquination (Gorin *et al.* 1997, Cavigelli *et al.* 1996, Klemperer *et al.* 1989). It is thought that arsenite is a sulfhydryl reagent having a high affinity mainly for vicinyl dithiols and also thiols located near hydroxyls. In contrast, arsenate is similar in structure to phosphate and may interfere with oxidative phosphorylation by forming an unstable arsenate ester (Gorin *et al.* 1997, Cavigelli *et al.*1989). Thus, arsenate affects phosphotransfer reactions, which are required for ATP generation. Furthermore, arsenate is excreted more rapidly than arsenite from the body (Bertolero *et al.* 1995).

Major determinants of the mode of action of arsenicals are the valence states, degree of methylation, charge at physiological pH and electrostatic attraction and repulsion to active sites of important macromolecules like DNA, RNA and protein. Trivalent species of arsenic are considered to be more toxic than their pentavalent counterparts. This is attributed to the fact that trivalent cation of arsenic possesses an unpaired 4s electron which incurs it with the property of biological interactions. Out of all the known arsenic compounds dimethylarsenite is the most active arsenical (Ahmad *et al.* 2000) and releases maximum iron than any other arsenical in oxidative DNA damage process.

$OH^{\circ} + DNA + Fe^{3+} \rightarrow$ oxidative DNA damage (Haber- Weiss type process)

Taken together, these studies suggest that trivalent methylated arsenicals are potent clastogens and because they can also disrupt the spindle, cause chromosome breakage and loss or gain of whole chromosomes, their uptake can be important driving forces in cancer induction.

Metabolism of arsenic:

Inorganic arsenic is considered to be highly toxic form of the element and is well absorbed from the gastrointestinal tract and distributed throughout the body. Some cells methylate inorganic arsenic very poorly or not at all, for example, keratinocytes or prostate epithelial cells (Benbrahim-Tallaa *et al.* 2007). Arsenic biotransformation is mainly due to trivalent species like dimethyl arsenite (DMA III) and monomethyl arsenite (MMA III), with mechanisms of actions like inhibition of DNA replicating, DNA repair enzymes, interference with tissue respiration and oxidative stress. Arsenate is first reduced to arsenite which is further biotransformed through methylation. Reduction of arsenate ($As5^+$) to arsenite ($As3^+$) is necessary before the occurrence of methylation. Arsenate is rapidly reduced to arsenite by glutathione *S*-transferase and/or arsenate reductase (Benbrahim-Tallaa *et al.* 2007). Arsenite is then methylated in the form of monomethylarsonate ($MMA5^+$) and dimethylarsinic acid ($DMA5^+$) by arsenic methyltransferase using *S*-adenosylmethionine (SAM) as the methyl donor. The intermediate metabolites methylarsonous acid ($MMA3^+$) and dimethylarsinous acid ($DMA3^+$) are generated during this process. The process also involves the formation of free radicals such as dimethyl arsenic peroxy free radical and dimethyl arsenic free radicals which are responsible for the generation of oxidative stress in the cellular environment.

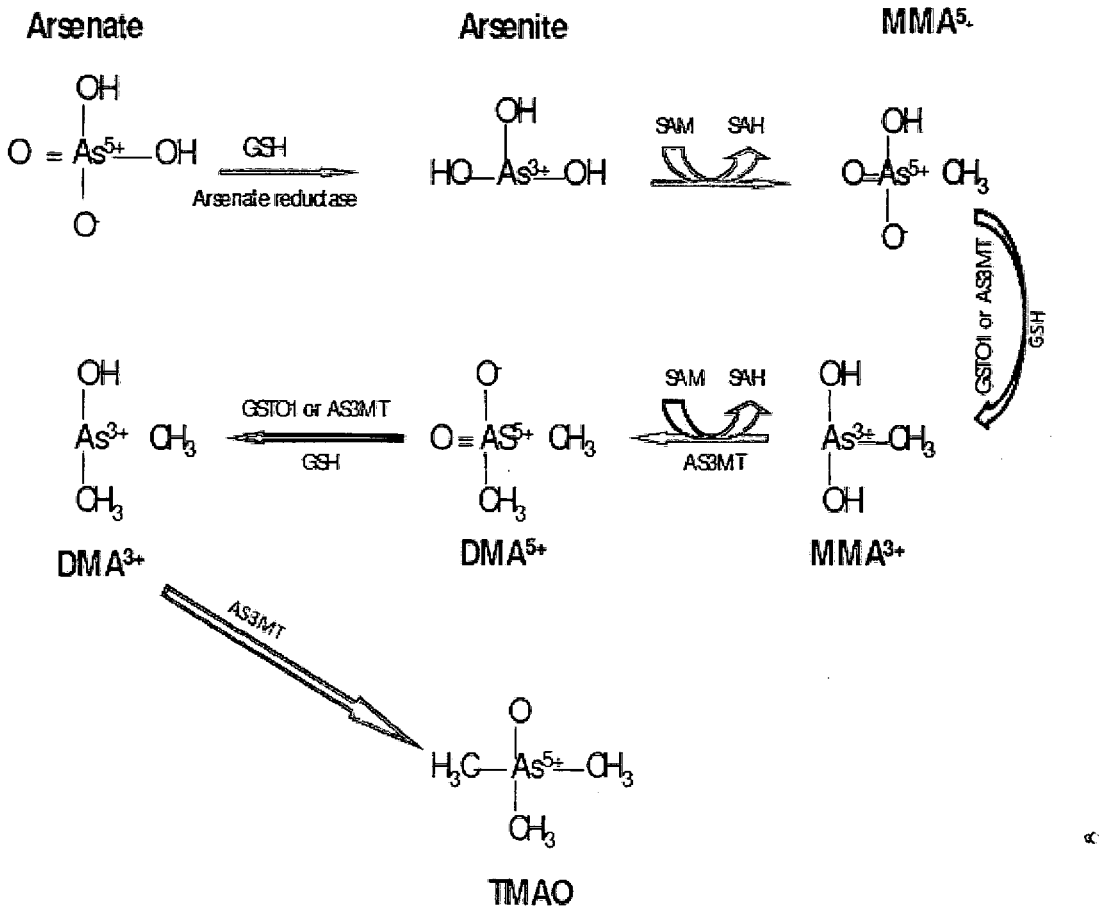


Figure 1. Arsenic metabolism showing arsenate reduction to arsenite and methyaltion to pentavalent (MMA^{5+}, DMA^{5+}) and trivalent (MMA^{3+}, DMA^{3+}) forms.

GSH, reduced glutathione; GSTO1, glutathione S-transferase omega-1; SAM, S- adenosylmethione; SAH, S-adenosylhomocysteine; AS3MT, arsenic methyltransferase Cyt 19); MMA^{5+}, mono methylarsonous acid; MMA^{3+}, mono methylarsonous acid; DMA^{5+}, dimethyl arsenic acid; DMA^{3+}, dimethyl arsonous acid; TMAO, trimethyl arsenic oxide.

(Adapted from Klaassen, 2008)

Arsenic-induced carcinogenicity

The carcinogenic potential of arsenic was first recognized by Hutchinson, a British Physician who observed skin cancers occurring in patients treated with medicinal arsenicals. It has been observed that people consuming arsenic contaminated water develop various kinds of

cancer. Arsenic is an atypical carcinogen because it is classified neither in the initiator nor the in promoter categories of carcinogenic agents. Thus, arsenic probably does not act as a classical carcinogen, but rather enhances the carcinogenic action of other carcinogens.

Arsenic induced carcinogenicity is the result of oxidative stress due to arsenic exposure. Oxidative stress is an imbalance between free radical generation and its scavenging by the antioxidant system. A decrease in the antioxidant levels in plasma from individuals exposed to arsenic in Taiwan has been reported (Wu *et al.* 2001) which showed that there was a significant inverse correlation between plasma antioxidant capacity and arsenic concentration in whole blood. Several papers have reported decreased levels of antioxidant enzymes after exposure to arsenic. Based on these studies, the following conclusions can be drawn: (i) antioxidant enzymes play an important role in maintaining cellular redox status and their levels are considered a significant marker of oxidative stress, (ii) level of antioxidant enzymes are highly correlated with the cellular redox status induced by arsenic. There are three pathways by which arsenic can decrease cellular levels of antioxidant enzymes. In the first pathway enzymes possibly act as an electron donor for the reduction of pentavalent arsenicals to trivalent arsenicals. Secondly, arsenite has high affinity to the antioxidant enzymes possessing vicinal sulfhydryl groups. The third pathway involves oxidation of antioxidant enzymes by arsenic-induced generation of free radicals. On the whole, it can be concluded that exposure to arsenite is likely to cause depletion of antioxidant enzyme level.

Generation of free radicals

Most of the adverse effects, particularly, the imbalance in the antioxidant system due to arsenic exposure are attributed to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Electron paramagnetic resonance (EPR) spectroscopic studies have revealed that arsenic enhances oxygen consumption and superoxide ($O^{2\cdot-}$) formation (Barchowsky *et al.* 1999). Reactive oxygen species produced due to arsenic exposure include superoxide ($O^{2\cdot-}$), singlet oxygen species, peroxy radical (ROO°), Nitric oxide (NO°) •) (Pi *et al.* 2003), hydrogen peroxide (H_2O_2), dimethylarsinic peroxyl radicals ([$(CH_3)_2AsOO\bullet$]) and also dimethylarsinic radical [$(CH_3)_2As\bullet$] (Rin *et al.* 1995). The exact mechanism responsible for the

generation of all these reactive species is not yet clear, but some studies proposed the formation of intermediary arsine species (Yamanaka *et al.* 2001).

However, the route to produce H_2O_2 was explained as a process of the oxidation of As (III) to As (V) which, under physiological conditions, results in the formation of H_2O_2.

$H_3AsO_3 + H_2O + O_2 \rightarrow H_3AsO_4 + H_2O_2$ ($\Delta G = -40.82$kcal/mol) (McKie *et al.* and Barrow *et al.* 2000).

The above reaction is spontaneous and exergonic with an estimated standard reaction free energy change for H_2O_2 formation of –40.82 kcal/mol (–170.87 J/mol).

H_3AsO_3 (arsenious acid) has the ability to generate a deoxygenated complex because it is a Lewis acid that may accept unpaired electrons that could originate either from water or from oxygen.

Further, it was observed that arsenite at concentrations greater than 20 μM produced concentration- and time-dependent increase in nitrite levels in the medium of arsenite-treated CHO-K1 cells. The increase in nitrite levels was related to increased NO• production in these cells. It was further concluded that the stimulation of NO• production by arsenite was through the activation of endothelial NO synthase (eNOS) by calcium. In another report, nitric oxide was observed to be involved in arsenite induced DNA damage and pyrimidine excision inhibition (Liu *et al.* 2000). This led to the conclusion that arsenite induced formation of DNA adducts through calcium mediated production of peroxynitrite, hypochlorous acid and hydroxyl radicals.

Oxidative DNA damage due to Arsenic exposure:

Increased levels of 8-Hydroxyl-2-deoxyguanosine (8-OHdG) which is considered to be a good biomarker of oxidative stress to DNA had been observed in animal models, cellular and human tissues exposed to arsenic.

It was found that MMAIII (monomethyl arsenite) and DMAIII (dimethyl arsenite) were the most potent DNA-damaging agents among all the arsenic species including NaAsIII (sodium arsenite), MMAIII, DMAIII, and DMAV(dimethyl arsenate), and also that most of the DNA damage induced by NaAsIII, MMAIII, DMAIII, and DMAV are Fpgsensitive sites (Schwerdtle *et al.* 2003). These sites are indicative of oxidative damage. Further, it was found that arsenic

induces strand breaks in a concentration-dependent manner in murine keratinocyte cell line JB6. These observations indicate that a large proportion of arsenite- induced DNA strand breaks come from excision of oxidative DNA adducts and DNA-protein cross- links, confirming that arsenite is able to induce a high level of oxidative DNA adduct formation and DNA-protein cross-links. (Bode *et al.* 2002).

Effect of Arsenic on lipids and proteins of the cell membrane

The effect of arsenite, arsenate on human erythrocyte membrane proteins and lipids has been assessed (Zhang *et al.* 2000). It was shown that the arsenite species are bound to the membrane from cytosol. In contrast, arsenate was found to bind rapidly from the outside, followed by releasing and re-binding. The binding to the membrane *via* sulfhydryl was indicated by the decrease of the sulfhydryl level of membrane proteins. The binding of arsenite and arsenate to the membrane induced changes in the fluidity of membrane lipids and left a negative charge density on the outer surface of the membrane thereby adversely affecting the membrane fluidity.

Role of Arsenic in cell proliferation and apoptosis signal cascades

It has been reported that arsenite is a potent stimulator of proto-oncogene *c-fos* and *c-jun* expression and AP-1 transactivational activity (Cavigelli *et al.* 1996). ROS induced by low concentrations of arsenic (< 5 μM) have been shown to increase the transcriptional activity of the activator protein 1 (AP-1) and the nuclear factor kappa B (NFκB) (Barchowsky *et al.* 1996, Corsini *et al.* 1999, Kaltreider *et al.* 1999 and Wijeweera *et al.* 2001), which results in cell signaling, transcription factor binding to DNA, and subsequent stimulation of cell proliferation (Germolec *et al.* 1996). There was also enhanced expression of certain genes, including MDM2 protein (Hamadeh *et al.* 1999), a key regulator of the tumor suppressor gene p53.

The induction of AP-1 activity by arsenic appears to be mediated by activation of protein kinase C (PKC) and MAPKs family member (Huang *et al.* 2001). ERKs typically transduce the signaling that leads to cell differentiation or proliferation and cell transformation, while JNKs

and p38 kinase usually respond to stress stimulation, which results in cell growth arrest and/or cell apoptosis. Arsenic has been found to induce the activation of JNKs, p38 kinase and ERKs (Huang *et al.* 1999, 2001) but differentially.

These findings suggest that ERKs activation may contribute to the carcinogenic effects of arsenic, whereas JNKs activation is associated with apoptosis and results in the anti-carcinogenic effects of arsenic (Huang *et al.* 1999). Although the mechanism by which JNKs mediate cell apoptosis in response to arsenite is not clear, several studies have shown that JNKs translocation to mitochondria is important for interactions with Bcl-xL and Bcl-2 (Kharbanda *et al.* 2000, Yamamoto *et al.* 1999). JNKs phosphorylate and inactivate Bcl-2, which plays a central role in regulation of apoptosis and the release of cytochrome *c* and apoptotic proteases (Kharbanda *et al.* 2000, Yamamoto *et al.* 1999). However, it was also demonstrated that apoptotic control mechanisms are disrupted as cells become transformed through arsenic exposure (Qu W *et al.* 2002). This apoptotic disruption is presumed to damage cells to inappropriately escape apoptosis, and potentially proliferate, and further provide initiating events in carcinogenic development. Thus, this may lead to accumulation of genetically damaged cells that have a potential to become malignant.

Models for study of Arsenic-induced prostate cancer

Mice are resistant to the induction of prostatic tumors by chemical carcinogens (Shirai *et al.* 2000). Rats are considered to be a better rodent model since prostate lesions can be chemically induced and early stages are androgen-dependent. However, Arsenic biokinetics in rats is quite dissimilar to mice and humans and rats are considered to be a poor model for human arsenic toxicology (Aposhian and Aposbian 2000). At present whole rodent prostate model of inorganic arsenic carcinogenesis are not available.

Arsenic and prostate cancer

The carcinogenic effect of arsenic on various cancer types has been studied but its potential as carcinogen in prostate cancer has gained attention recently. Arsenic exposure in drinking water from artesian wells in Taiwan was associated with prostate cancer mortality in humans (Chen *et al.* 1988). It has been shown that an excess mortality from prostate cancer was found in South- West Taiwan where drinking water contained dissolved arsenic (IRAC 2004). Similarly, when looking at the role of arsenic in drinking water and prostate cancer in the US (Lewis *et al.* 1999), it was concluded that there was a significant elevation of prostate cancer mortality associated with arsenic in drinking water.

It was found that chronic arsenite exposure (5 μM) for 30 weeks to a non-tumorigenic, human prostate epithelial cell line, RWPE-1, results in malignant transformation (Benbrahim-Tallaa *et al.* 2005). The cells were observed to overexpress K-Ras. Although changes in cell proliferation were not reported, increased ERK activity was reported and correlated with K-Ras expression suggesting that chronic arsenite exposure might increase cell proliferation in prostate epithelial cells (Benbrahim-Tallaa *et al.* 2007). In addition, arsenic-transformed RWPE-1 cells overexpressed prostate-specific antigen (PSA) in the absence of androgen, suggesting that chronic arsenite exposure could exacerbate prostate cancer progression to more aggressive forms of the disease. The p53 binding protein, Mdm2 (mouse double minute 2), was observed to be up-regulated in response to arsenite in 53-null MEFs via the ERK MAP kinases (Huang *et al.* 2008). Mdm2 is an ubiquitin E3 ligase that targets p53 for ubiquitination and degradation by the 26S proteasome or nuclear export. In cells pre-exposed to 1 μM arsenite for 24 h, the p53 response to genotoxic stress was lost, presumably resulted in the cytoplasmic localization of p53. Once in the cytoplasm, p53 loses the ability to function as a transcription factor and is unable to induce an apoptotic response to DNA damaging agents. Consequently, by incapacitating the p53 response arsenite promoted malignant cellular transformation through the accumulation of mutations and genomic instability.

Although epidemiological studies have shown that an increase in prostate cancer incidence occurs due to enhance exposure to arsenic in drinking water, it is difficult to know from these studies whether gestational, adolescent, adult, or a cumulative lifetime of arsenic exposure contributes to the development of prostate cancer. In addition to cell type specificity to arsenic

exposure, there is also individual variation to arsenic exposure. For example, in a study of a Mexican population that was exposed to arsenic in the drinking water, there were variations in arsenic methylation efficiency among individuals (Gomez *et al.* 2009). In another study of Bangladeshi adults whose diets were deficient in folate, it was found that variability in nutritional status may be a risk factor for arsenic toxicity (Gamble *et al.* 2005). Folate is needed for the biochemical pathway of one-carbon metabolism which is essential for methylation of arsenic metabolites as well as DNA. The need of the hour is to explore as many pathways as possible, associated with arsenicals to better understand arsenic biology and toxicology that will help in shaping future regulatory, and possibly, dietary standards for human arsenic exposures worldwide.

Clinical trials using Arsenic

Waxman was one of the first Western physicians to see promising results in inducing long-term remission in APL patients by intravenous doses of arsenic trioxide in a series of small studies in Chinese medical journals. Arsenic is regarded as a paradox since it acts as a potent carcinogen and studies have also displayed its role as a valuable therapeutic in cancer treatment. Using leukemia cells with genetic alterations in mitochondrial DNA and biochemical approaches, it was demonstrated (Pelicano *et al.* 2003) that As_2O_3 was a clinically active antileukemia agent, inhibited mitochondrial respiratory function, increased free radical generation, and enhanced the activity of superoxide generating agents against cultured leukemia cells and primary leukemia cells isolated from patients. This novel mechanism of action provided a biochemical basis for developing new drug combination strategies using As_2O_3 to enhance the activity of anticancer agents by promoting generation of free radicals.

Arsenic trioxide has been shown to possess a role in clinical activity for patients with multiple myeloma (Munshi *et al.* 2002, Hussein *et al.* 2004, Rousselot *et al.* 2004) and is now being studied in combination for patients with multiple myeloma (Bahlis *et al.* 2002, Wu K *et al.* 2005, Berenson *et al.* 2006, 2007). Its combination with ascorbic acid and other drugs has been evaluated in several clinical trials. In a phase I /II study As_2O_3, bortezomba and ascorbic acid were given in combination to patients with relapsed and refractory multiple myeloma (Berenson *et al.* 2007). The response obtained was similar to that observed when the drugs were given

alone. This led to the conclusion that As_2O_3 has additive effect with other agents. In multicentre phase II clinical trials melphalan, As_2O_3 and ascorbic acid were given to 65 patients with relapsed or refractory multiple myeloma. The result of this combination therapy showed 48% improvement. Moreover patients resistant to melphalan also showed a positive response to this combination (Berenson *et al.* 2006). This showed that arsenic in combination with other drugs can have a synergistic effect.

Similar positive results were obtained in phase III clinical trial where As_2O_3 was used to treat rare form of leukemia. This study affirmed that arsenic effectively maintained remission in acute promyelocytic leukemia (APL). Arsenite is now approved for the treatment of APL because it induces an apoptotic response (Chen *et al.* 1997, Sun *et al.* 1992). This apoptotic response may be due to a caspase-dependent loss of Akt activation (Mann *et al.* 2008).

Similarly, arsenite induces an apoptotic response in melanoma cells, but is not approved for the treatment of melanoma. Arsenite was used in combination with menadione to induce cell death exclusively in melanoma cells as a result of enhanced ROS generation, p38 signaling and p53 activation (Chowdhury *et al.* 2009).

Arsenic's medicinal prowess in APL led the clinicians and scientists to test its efficacy in a variety of other cancers. This resulted in around 17 ongoing clinical trials in United States to treat various forms of leukemia and cancers of central nervous system and brain under the aegis of National Institutes of Health (NIH).

Silibinin

Naturally occurring polyphenolic antioxidants are recognized as one of the most effective classes of cancer preventive agents as they exhibit little or no systemic toxicity and because oxidative stress is a known contributor to carcinogenesis.

Silibum marianum has been used as a hepatoprotectant against Amainta mushroom poisoning and oxidative damage by xenobiotics. Interest in its anticancer efficacy was brought about by a report in 1994 showing its effectivity against tumor progression in a rodent skin cancer model. From thence, silibinin and silymarin extracted from *Silibum marianum* have been shown significant antiproliferative and antiangiogenic activities *in vivo* in a number of rodent and human cancer models and synergy with cytotoxic chemotherapeutics *in vitro* and in xenografts of human prostate and ovarian cancer.

Silymarin is an active extract from the seeds of the plant milk thistle (*Silybum marianum* (L.) Gaertn. (Asterceae), and contains approximately 65–80% silymarin flavonolignans (silymarin complex) with small amounts of flavonoids and approximately 20–35% fatty acids and other polyphenolic compounds. Silibinin is a polyphenolic flavonoid isolated from milk thistle with anti- neoplastic activity in several in- vitro and in- vivo models of cancer, including prostate cancer. Silibinin is known as silybin or silibin and is the main component of silymarin. Seven different compounds or flavolignans have been isolated from silymarin extract. Two pairs of diastereomers have been isolated from silymarin which include silybin A and silybin B, and isosilybin A and isosilybin B, the latter two are regioisomers of silybin A and silybin B, respectively, from the silymarin mixture (Lee *et al.* 2003).

The chemical name of silibinin 2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl)benzodioxin and is a 1:1 mixture of silybin A and silybin B. Its molecular formula is $C_{25}H_{22}O_{10}$ and has a molecular weight of 482.44

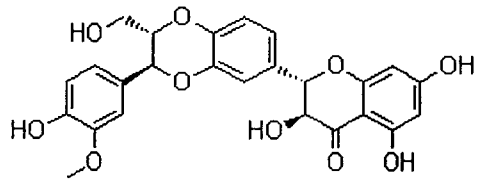


Figure 2. Structural formula of silibinin

Silibinin in *in vitro* studies

Silibinin is the most active anti-hepatotoxic agent in silymarin mixture and contains 1,4-dioxane ring in addition to flavonoid moiety. Silymarin was further (Davis-Searles *et al.* 2005) identified to be composed of seven distinct flavonolignan compound and a flavonoid. Among these, four compounds namely silybin A, silybin B, isosilybin A, and isosilybin B, showed most consistent antiproliferative effects in three different human prostate carcinoma LNCaP, DU145, and PC3 cell lines. On further expanding these preliminary observations it was shown that isosilybin B and isosilybin A exerted growth inhibition and cell death together with a strong G1 arrest and apoptosis in human prostate carcinoma LNCaP and 22Rv1 cell (Deep *et al.* 2007).

Silibinin is particularly effective in inhibiting epidermal growth factor receptor (EGFR) signaling with suppression of cyclin-dependent kinase (CDK) expression and up-regulation of the CDK-inhibitors p21(CIP1) and p27(KIP1), with concomitant increase in their binding to CDKs. Silibinin induces growth arrest at the G1 and G2 checkpoints. Silibinin, in lower doses induces the growth arrest through extracellular signal-regulated kinases (ERK1/2) inhibition and at higher doses leads to apoptosis through mitogen activated protein kinase (MAPK)/c-Jun N-terminal kinase (JNK) pathway (Agarwal *et al.* 2006, Singh *et al.* 2004, 2006). Studies have shown that silibinin inhibits both constitutively active and transforming growth factor (TGF)-a-mediated tyrosine phosphorylation of EGFR in advanced human prostate cancer DU145 cells (X. Zi *et al.* 1998). Studies have shown that silymarin and silibinin down-regulate EGFR signaling via the inhibition in the expression and secretion of growth factors, and by inhibiting growth factor binding to and activation of EGFR. This subsequently leads to impairment of downstream mitogenic events thereby leading to an anti-cancer efficacy in tumor cell lines (Deep *et al.* 2007).

Silymarin has been reported to suppress the proliferation of tumor cells in various cancers including prostate (Singh *et al.* 2006, 2004, Zi *et al.* 1998 and Tyagi *et al.* 2002), ovarian, breast (Zi *et al.* 1998), lung (Sharma *et al.* 2003), skin (Deep *et al.* 2007) and bladder (Tyagi *et al.* 2007, 2004). Numerous reports indicate that silymarin inhibits proliferation of cells by inhibiting cell cycle progression at different stages of the cell cycle.

Silymarin and silibinin treatment has been shown to inhibit the growth of androgen dependent (LNCaP) and androgen independent (PC3 and DU145) prostate cancer cells (Davis-Searles *et al.* 2005). Silymarin treatment induced binding of Cip1/p21 with CDK2 and CDK6 paralleled a significant decrease in CDK2-, CDK6-, cyclin D1-, and cyclin E-associated kinase activities, along with a decrease in cyclins D1 and E. Further, it was observed that silymarin and silibinin modulated G1 phase cyclins–CDKs–CDKIs for G1 arrest, and the Chk2–Cdc25C Cdc2/cyclin B1 pathway for G2-M arrest, together with an altered subcellular localization of critical cell cycle regulators (Deep *et al.* 2007). Studies have reported that silymarin exerts its anti-cancer effects by causing cell cycle arrest and inducing apoptosis in different type of cancers.

Studies have shown that diastereoisomers from silymarin induce apoptosis by causing the cleavage of PARP, caspase 9 and caspase 3 and decreasing survivin levels in human prostate cancer LNCaP and 22Rv1 cells (Deep *et al.* 2007). Similar results have also been seen in DU145 cells with inhibition of active Stat3. Silibinin synergies the growth-inhibitory effect of mitoxantrone, a topoisomerase II inhibitor in prostate carcinoma PC-3 cells by reducing cell viability with increased apoptosis (Flaig *et al.* 2007).

Anti-angiogenic activity is one of the fundamental ways of the cancer treatment. The anti-angiogenic potential of silibinin has been demonstrated in various cancers. It has been demonstrated that silibinin inhibits the growth and survival of human umbilical vein endothelial cells (HUVECs) by inhibiting capillary tube formation, and induction of cell cycle arrest and apoptosis together with a reduction in invasion and migration. These findings suggest that by suppressing the cancer cells invasion through the specific inhibition of AP-1-dependent MMP-9 gene expression, silibinin represents a potential anti-metastatic agent. Together, the anti-invasive as well as anti-metastatic potential of silibinin could be of a great value in the development of a potential cancer therapy.

Silibinin in *in vivo* studies

Studies have shown that dietary administration of silibinin inhibited the advanced human prostate tumor xenograft growth in athymic nude mice by exhibiting anti-proliferative, pro-apoptotic, and anti-angiogenic efficacy against prostate tumor (Agarwal *et al.* 2006, Singh *et al.* 2006). It was also shown that dietary silibinin inhibited prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice by modulating the expression of CDKs, CDKIs, and insulin like growth factor (IGF)-1 and IGF binding protein (IGFBP)-3 (Raina *et al.* 2007). In other studies reported in literature, it was demonstrated that administration of silibinin significantly inhibited Nbutyl- N-(4-hydroxybutyl) nitrosamine induced urinary bladder carcinogenesis in male ICR mice by causing cell cycle arrest and induction of apoptosis (Tyagi *et al.* 2007).

Silibinin and prostate cancer

Studies aiming at testing *in vivo* efficacy of silymarin/silibinin revealed that dietary feeding of silibinin to athymic nude mice bearing DU145 prostate tumor xenograft significantly inhibited tumor growth and increased secreted levels of insulin-like growth factor binding protein 3 (IGFBP-3) in plasma without any toxicity symptoms in the animals fed with the agent (Singh *et al.* 2002a., 2002b). These findings were in concurrence with *in vitro* studies with PC-3 cell lines, it was observed that silibinin upregulated the expression of IGFBP-3, thereby resulting in an increased level of secreted IGFBP-3 in conditioned medium (Zi *et al.* 2000).

These observations have strong implications, as mitogenic and cell survival signaling mediated by IGFBP-insulin like growth factor-1 (IGF-1)/IGF-1R (insulin like growth factor-1 receptor) is often constitutively upregulated in human prostate carcinoma cell lines and is often implicated in advanced and androgen independent stage of prostate cancer (Stattin *et al.* 2000). In yet another study (Kohno *et al.* 2005), dietary administration of silymarin at 100 or 500 ppm dose level for 40 weeks showed a significant reduction in the incidence of prostatic adenocarcinoma in 3,2-dimethyl-4-aminobiphenyl (DMAB)- induced prostate carcinogenesis in male F344 rats. Importantly, it was also reported that no toxicity symptoms were observed in the

animals fed with silymarin-supplemented diet for 40 weeks. Together, these observations convincingly supported conducting clinical trials with silibinin in prostate cancer patients.

Silibinin in clinical trial

Phase I clinical trial with silibinin in prostate cancer patients was accomplished using silibinin phytosome (SiliphosR), a commercial preparation of silibinin, at a dose of 13 g, divided in 3 daily doses, in patients with advanced prostate cancer. Phase II clinical trial was also carried out to assess the effect of silibinin administration on prostate cancer progression using surrogate biomarkers as endpoints. Silymarin was used (along with soy, lycopene and anti-oxidants) in a phase III clinical trials to delay prostate specific antigen progression after prostatectomy and radiotherapy in prostate cancer patients (Schro¨der *et al.* 2005). Silibinin has now entered the phase II clinical trials with respect to prostate cancer.

Combination chemotherapy

Most of the anticancer chemotherapeutic drugs do not discriminate between the normal dividing and cancer cells and therefore are toxic to the normal dividing cells. These are therefore referred to as cytotoxic drugs. Further, they affect various parts of the body thereby resulting in the systemic toxicity and cause a number of side effects such as nausea, vomiting, hepatotoxicity, cardiotoxicity, immunosuppression, neurotoxicity etc. therefore, to address this issue there is an urgent need to look for combined modalities. A number of different strategies are being practiced in combination with chemotherapy to combat this deadly disease. Some of them are mentioned below:

1) combined modality chemotherapy that involves the use of drugs with other cancer treatments, like surgery and radiation therapy
2) neo adjuvant chemotherapy or preoperative treatment where preceding surgery or radiation therapy, chemotherapeutic drugs are given to reduce
3) adjuvant chemotherapy or postoperative treatment, which involves continued drug intake even after surgery to reduce the risk of recurrence

4) palliative chemotherapy that involves drug dosing only for decreasing the tumor load; it does not have any curative effects, but is expected to increase life expectancy slightly; and
5) combination chemotherapy, which involves treating a patient with a number of different drugs simultaneously (Raina *et al.* 2007).

Any single drug does not have an impact on cancer since cancers acquire multiple oncogenic mutations that are often functionally redundant. The cells in the regressing cancer often acquire additional mutations that render them resistant to a single drug that is effective against that cancer. This results in tumor recurrence. Therefore, targeting multiple signaling pathways that are prone to upregulation in a given type of cancer is the best strategy to achieve a long term control over that cancer. This strategy of targeting several pathways simultaneously to achieve additive or synergistic efficacy is described as multifocal signal modulation therapy.

Silibinin in combination treatment against prostate cancer

Silibinin strongly synergized the growth-inhibitory effect of doxorubicin in prostate carcinoma DU145 cells (combination index, 0.235– 0.587), which was associated with a strong G2-M arrest in cell cycle progression, showing 88% cells in G2-M phase by this combination compared with 19 and 41% of cells in silibinin and doxorubicin treatment alone, respectively. In addition, this combination resulted in strong inhibitory effect on cdc25C, cdc2/p34, and cyclin B1 protein expression and cdc2/p34 kinase activity. More importantly, this combination caused 41% apoptotic cell death compared with 15% by either agent alone (Tyagi *et al.* 2002). Silibinin and doxorubicin alone as well as in combination were also effective in inhibiting the growth of androgen-dependent prostate carcinoma LNCaP cells. (Tyagi *et al.* 2002).

A similar chemo combination study showed that silibinin sensitized the hormone refractory DU145 prostate carcinoma cells to cisplatin- and carboplatin that induced cell growth inhibition and apoptotic death (Dhanalakshmi *et al.* 2003). The *in vitro* effects of silibinin and the platinum compounds, cisplatin (2μg/mL) and carboplatin (20μg/mL), on DU145 cells were assessed either alone or in combination. The findings of these studies revealed that the 48% cell growth inhibition observed with cisplatin increased to 50%–100% when used in combination

with 50–100 μmol/L silibinin. Similarly, the growth inhibition by carboplatin increased from 68% to 80%–90% when used in combination with 50–100 μmol/L silibinin. The combination also resulted in a stronger G2–M arrest accompanied by a decrease in the expression levels of Cdc2, cyclin B1, and Cdc25C.

Combination studies of silibinin with docetaxel and mitoxantrone, a topoisomerase II inhibitor were also carried out in PC-3, DU145, and LNCaP prostate cancer cell lines (Flaig *et al.* 2007). A combination of silibinin (10–40 μmol/L) and docetaxel (2.5–5 nmol/L) exhibited little or no synergy in growth inhibitory and apoptotic effects in PC-3, DU145, and LNCaP cells, however, silibinin was able to overcome the relative resistance of PC-3 cells to mitoxantrone (25–200 nmol/L)-induced apoptosis. In this study, the combination of silibinin and mitoxantrone was also able to exhibit a synergistic action for apoptosis induction in DU145 and LNCaP cells and caused a synergistic decrease in the cell viability of all three cell lines.

AIMS

&

OBJECTIVES

AIMS AND OBJECTIVES

Having reviewed the vast literature on arsenic we realized that the risk of developing cancer by the consumption of water contaminated with arsenic was one of the major health concerns particularly in the Eastern parts of India such as West Bengal, Bihar and other neighboring countries like Bangladesh, Nepal, Taiwan etc. This prompted us to propose a strategy that could reduce the risk of tumor growth, by the use of chemopreventive agent, Silibinin, in people who consume arsenic-contaminated water and are at a high risk of developing cancer. The phytochemical, silibinin has been found to be very effective in the control of various cancers. Keeping in mind the above hypothesis we set out the following objectives to carry out laboratory studies:

Objectives:

1. To assess the effect of arsenite on cancer cell growth and death and associated molecular changes, and their modulation by silibinin.
2. To analyze the effect of arsenite on cancer cell cycle progression and associated molecular changes, and its modulation by silibinin.
3. To examine the effect of arsenite on migratory and/or invasive potential of cancer cells, and their modulation by silibinin.

MATERIALS AND METHODS

MATERIAL AND METHODS

Cell culture

DU145 - Human prostate carcinoma cell line (The cells were procured from the University of Colarado, Health sciences center, Denver, USA).

Source organ - Prostate

Disease - Carcinoma

Derived from metastatic site- Brain

Culture conditions- Grown as adherent monolayer in RPMI 1640 basal media containing 10% of heat inactivated Fetal Bovine Serum and 1% antibiotic at 37°C, 95 % humidified incubator with 5% CO_2.

Equipments

Biosafety cabinet, Phase contrast inverted microscope, CO_2 incubator, petriplates of 100mm and 60mm, pipettes of 5 and 10 ml, pipetter, micropipets, haemocytometer, falcon tubes (15ml & 50ml), micro tips, centrifuge, ampule rack, tissue paper, marker pen, sterile gloves, laboratory coat, safety visor.

Reagents

Sodium metaarsenite ($NaAsO_2$), Silibinin, RPMI 1640 (Roswell Park Memorial Institute) media (Himedia Laboratories) with L-Glutamine and Sodium bicarbonate, Trypsin-EDTA solution (Ref T001-5X100ML), Fetal bovine serum (FBS) (Ref RM 1112- 100ML), Pen-Strep Antibiotic were purchased from Himedia laboratories Pvt Ltd, Ethanol 70%, DMSO.

A. Media preparation

Cell culture media was prepared by the addition of 10% FBS and 1% antibiotic Pen-Strep of 100X concentration, of the total volume of the media, to the basal RPMI 1640 media. Fetal

bovine serum (FBS) was heat inactivated at 56°C for 30 minutes and then added to the media for the preparation of media for androgen independent prostate cancer cell line DU145.

B. Revival of the cells

Cell culture media was warmed to 37°C in a water bath. Ampule of cells was retrieved from liquid nitrogen storage and was thawed by placing them immediately in a water bath maintained at 37°C for 1-2 minutes. Vials were wiped with 70% ethanol and immediately transferred to the cell culture hood. Contents (1 ml of cell suspension) of the vial were slowly added into 2ml pre-warmed growth medium in 60mm petri plate which was then incubated at 37°C and 5% CO_2 in the CO2 incubator. Cells were regularly examined under the phase contrast microscope and were monitored for contamination. The media of plates was changed alternately, till desired confluency was achieved.

C. Splitting and maintenance of cells

Culture was examined under an inverted microscope to assess the degree of confluency and to confirm the absence of bacterial and fungal contaminants. Once desired confluency was achieved, spent media was aspirated and 1ml Trypsin-EDTA solution was introduced into the cell monolayer. Petri plates were then incubated in the CO_2 incubator for 5 minutes. Cells were then examined under an inverted microscope to ensure complete trypsinization of the cells.1ml fresh serum-containing media was added to inactivate trypsin. Cell suspension was then transferred from the plates to 15 ml falcon tubes which were subjected to centrifugation at 1500 rpm for 5 minutes at room temperature. Supernatant was removed and 10ml fresh media was added in the falcon. The cells were resuspended and transferred to new 100 mm plates containing pre-warmed media. Petri-plates were incubated. Cells were examined microscopically after 24 hours. The media of plate was changed alternately till the desired confluency was achieved.

Cell counting by Haemocytometer

After trypsinization, cell number was determined using haemocytometer. 10µl of the homogeneous cell suspension was loaded on each of the two chambers of the haemocytometer.

Haemocytometer was then examined under the phase contrast microscope at 100X magnification.

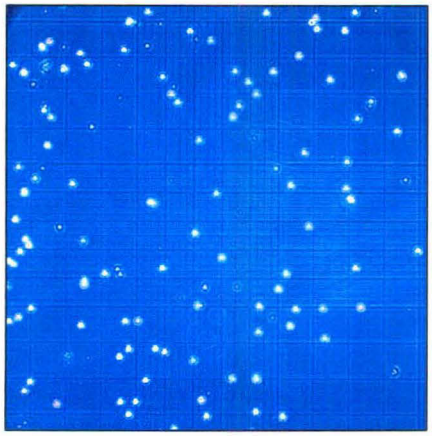


Figure 3. 100X Magnification view of counting chamber after sample injection

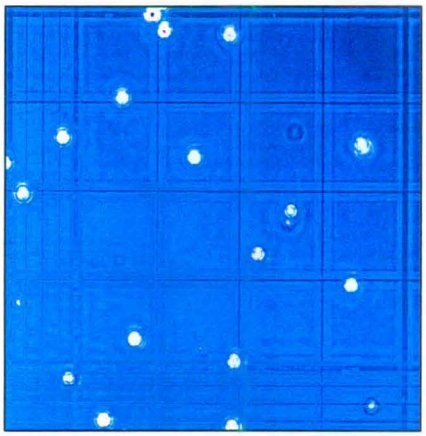


Figure 4. 200X Magnification view of counting chamber after sample injection

The cell number was determined using the formula:

Mean Average Cell number = Average total cells in each chamber × Dilution factor × 10^4cells/ ml

Optimum Dose Selection

A fixed number (1×10^5) of exponentially growing cells were seeded in several individual 60mm culture plates and allowed to grow. Several such individual cell culture experiments were setup for the determination of optimum dose.

Seeding for drug treatment:

Exponentially growing cells (sub-confluent cells) were used in the entire set of experiments. For treatment with the agents, 1 x10^5 cells were seeded in a 60 mm culture dishes and incubated at 37°C for 24 hrs. The cells were then treated with the drugs, the next day and were incubated at 37°C for 24 hrs and 48 hrs depending on the treatment time points. All the treatments were done in triplicates.

Table 3. Drug treatment for cell counting *via* trypan blue dye exclusion assay

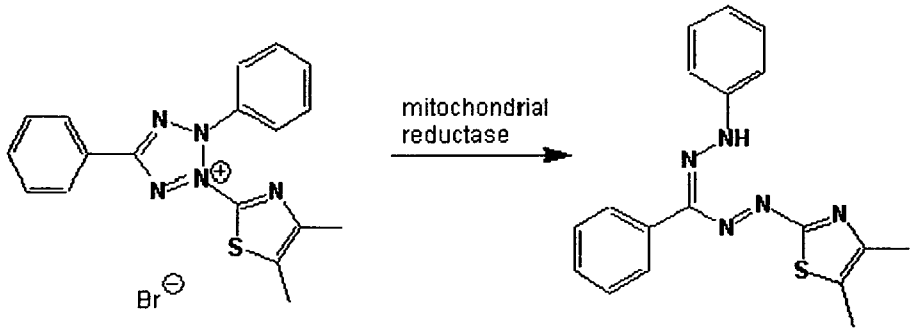
S.NO.	Group treatment	Volume of 5mM sodium meta arsenite (µl)	Volume of 100mM Silibinin (µl)	Volume of DMSO (µl)	Total volume of media (ml)
1.	Control	_	_	10	10
2.	0.5µM Arsenite	1	_	10	10
3.	5µM Arsenite	10	_	10	10
4.	100 µM Silibinin	_	10	_	10
5.	0.5µM Arsenite + 100 µM Silibinin	1	10	_	10
6.	5µM Arsenite + 100 µM Silibinin	10	10	_	10

After 24 hrs of seeding of the cells, the consumed media was removed from the plates. The cells were treated with different concentrations of the arsenic and silibinin alone and in combination (as in the table) and set for control was treated with DMSO (0.1%). Each set of treatment was done in triplicates to minimize experimental error. The Media containing different concentrations of the drug was added to each set of plate and the plates were incubated at 37°C.

1. MTT Assay (Cell viability assay)

Principle

The MTT assay is based on the ability of the mitochondrial dehydrogenase enzyme (from viable cells), to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membrane. Addition of organic solvent such as dimethyl sulphonate (DMSO) leads to solubilization of crystals so formed, thereby giving a purple color. The number of surviving cells is directly proportional to the amount of formazan product formed which in turn is directly proportional to the intensity of the colour. The results can be read on a multi-well scanning spectrophotometer (ELISA plate reader) at 570nm. This assay gives an idea about the cytotoxic effect of the compound/drug under examination.



3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Pale yellow)

Formazan (Purple)

Figure 5. Reaction representing conversion of MTT to formazan

Reagents

MTT (Sigma Aldrich), DMSO (Sigma Aldrich), RPMI -1640 (Himedia Laboratories)

Procedure

10,000 cells were seeded into each well of the 96- well plate. Exponential phase cells were used throughout the experiment. Cells were allowed to attach (for 24 hrs), and then treated with

different concentrations of drug for two time points that is 24 hrs and 48 hrs. Three wells served as control. After the drug treatment for the specified time period, the medium containing the drugs was removed and 100 µl of the MTT stock (5 mg/ml) was added to each well (concentration of MTT should be 500 µg in each well), MTT was added into three more empty wells as the control. After 3 to 4 hours of incubation at 37°C in a 5% CO_2 incubator, MTT was removed and 100 µl of DMSO was added to each well. After 15-20 minutes of further incubation, the wells were read on an ELISA plate reader at 570 nm wavelength. The data was recorded, using the software package SoftPro Max. The viability (%) was calculated as follows:-

$$\%\text{Viability} = \frac{\text{Average of test wells O.D - Average of control wells O.D}}{\text{Average of control wells O.D – Average of blank wells O.D.}} \times 100$$

(O.D. = Optical Density; Test wells = treated well)

2. Trypan Blue Dye exclusion assay

Principle: Trypan blue is a vital stain used to selectively color dead tissues or cells, blue. Live cells or tissues with intact cell membranes are not colored. Since cells are very selective in the compounds that pass through the membrane, in a viable cell, trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method.

Reagents: Trypan blue (0.4%- Himedia laboratories)

Procedure

After selcted treatments, the cells in the supernatant were collected from each plate and transferred to the corresponding 15 ml falcon tubes. The attached cells were removed after trypsinization (l ml of trypsin). Once the cells were completely detached, 1 ml of chilled 1X PBS

was added to each plate. Cells were flushed out of the plate completely and were transferred to corresponding falcon tubes.

The cell suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded slowly and pellet was resuspended in 1 ml fresh PBS. For viability assay, 100 µl volume of the homogeneous cell suspension was transferred to microcentrifuge tube to which 10 µl of 0.4% trypan blue dye was added. The suspension was mixed well and left for 5 minutes at room temperature. 10µl of stained cell suspension was then loaded on each chamber of the heamocytometer. The chambers were counted for live (unstained cells) and dead (blue stained) cells and the procedure were repeated for each set of samples. The percentage of viable cells was calculated by dividing the number of viable cells by total number of cells multiplied by 100. Remaining 900µl of sample was used for the cell cycle distribution analysis by FACS.

3. Annexin V FITC and propidium iodide assay for apoptosis

Principle

Annexin V has a high affinity in calcium dependent manner to negatively charged phospholipid phosphatidylserine, which is found on the outer cell membrane early during apoptosis. As cell membrane becomes increasingly permeable during the later stage of apoptosis, propidium iodide readily moves across the cell membrane and binds to DNA. This combination allows the differentiation of cells among three different populations using FACS:

Normal cells: annexin V negative and PI negative

Early apoptotic cells: annexin V positive and PI negative cells

Late apoptotic cells: annexin V positive and PI positive cells.

Reagents Annexin V-FITC apoptosis detection kit (Strong Biotech Corporation)

Procedure

1.5 X 10^5 cells were seeded in triplicates for each of the six group treatments in 60 mm. dishes and incubated at 37°C to adhere to the plates. Next day the cells were treated with the drugs as described for trypan blue assay and kept at 37°C. After specified periods of drug treatment time that is for 24 and 48 hrs cells were harvested by the process same as that for trypan blue assay in 15 ml falcon tubes. The cell suspension in 1X PBS was centrifuged at 1500 rpm for 5min and pellet so obtained was resuspended in staining solution and incubated for 10-15 min at 37°C. After incubation was complete 0.4 ml of binding buffer was added to each sample and then analyzed on a flowcytometer. Electronic compensation of the instrument was also done to exclude the overlapping of the emission spectra of the two dyes.

Preparation of staining solution

Staining solution for 20 assays was prepared at a time which consisted of 40µl of annexin V-FITC and 40µl of propidium iodide in 2 ml of binding buffer (as directed in Annexin V-FITC apoptosis detection kit).

4. Clonogenic assay

Reagents

Six well plates, silibinin (10 µM), sodium metaarsenite, acetic acid, methanol and crystal violet.

Procedure

Around 3000 DU145 cells were seeded in six well plates and were incubated at 37°C, 5% CO2 to attach to the plates. Cells were then treated with different group treatments as give in the table. Plates were left undisturbed at 37°C, 5% CO2 for 7 days and were observed for colony formation every alternate day. Media was then removed after a week without disturbing the colonies so formed and the plates were washed with 1X PBS. The colonies were then fixed using 2 ml. solution 12.5% acetic acid and 30% methanol in distilled water to each of the wells for 5 minutes. The fixative was removed gently and the wells were rinsed with distilled water. The cell

colonies were then stained with 2 ml. of 0.5% crystal violet into each well for 15 to 20 minutes. The stain was then rinsed off with water and cleaned till the background was clear. The colonies were then observed and counted under the microscope. Only those colonies were taken into account that had more than 50 cells and those that did not overlap with other colonies.

Table 4. Drug treatment for clonogenic assay

S.NO.	Group treatment	Volume of 5mM sodium meta arsenite (µl)	Volume of 10mM Silibinin (µl)	Volume of DMSO (µl)	Total volume of media (ml)
1.	Control	_	_	10	10
2.	0.5µM Arsenite	1	_	10	10
3.	5µM Arsenite	10	_	10	10
4.	10 µM Silibinin	_	10	_	10
5.	0.5µM Arsenite + 10 µM Silibinin	1	10	_	10
6.	5µM Arsenite + 10 µM Silibinin	10	10	_	10

5. Cell cycle distribution analysis by Fluorescence activated cell sorter (FACS)

Principle

Propidium iodide (PI) intercalates with the nucleic acid, RNaseA degrades the RNA while EDTA helps propidium iodide to intercalate with the nucleic acids. Saponin prevents the clumping of the cells and increased permeability of the cell membranes. Propidium iodide in

stained cells binds to the DNA in a stoichiometric manner such that there is a direct relationship between DNA content and Propidium Iodide (PI) fluorescence. Flowcytometer sorts a heterogeneous mixture of cells based upon the fluorescence of the DNA into different phases. A single-parameter histogram with PI area is plotted on X-axis and counts along the Y-axis to illustrate relative DNA content. This gives an idea about the distribution of the cells in various phases of the cell cycle. The cells thus fall in three categories viz., G1/G0, S and G2/M phases. In the present study, flowcytometry was used to evaluate the effect of drug on cell cycle.

Reagents

Saponin (Fluka Analyticals), Propidium iodide, EDTA (stock 0.5M) (Sigma Aldrich), RNaseA (Fermentas), 1X PBS

Preparation of Propidium Iodide cocktail

Saponin 0.3% w/v (stock 10%), Propidium iodide 25µg/ml w/v (stock 5mg/ml), 0.1mM EDTA (stock 0.5M), 10µg/ml RNase A w/v in PBS stock (10mg/ml). The above reagents were dissolved in 1X PBS and stored in dark till use.

Procedure

The cell suspension prepared from the treated plates, was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in PI cocktail solution. The procedure was carried out in dark. All tubes were incubated at 4°C for overnight in dark. The data was acquired using Cell Quest software in a FACS Calibur (Becton Dickinson, USA) for 10,000 events per sample and analyzed using MOD FIT software after appropriate gating, to determine the percentage of cells in each phase of the cell cycle.

6. Immunoblot analysis

DU145 cells were treated with sodium arsenite and silibinin (control, 0.5µM sodium arsenite, 5µM sodium arsenite, 100µM silibinin, 0.5µM sodium arsenite + 100µM silibinin and 5µM sodium arsenite + 100µM silibinin) for 48 hrs for the analysis of key molecules of different pathways. Cell lysates were prepared in nondenaturing lysis buffer (10mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 0.3mM phenyl methyl sulfonyl

fluoride, 0.2mM sodium orthovanadate, 0.5% NP-40, 5 µg/ml aprotinin). Protein concentration in lysates was determined using Bradford assay. The instruments used were Perkin elmer and Shimadzu spectrophotometer. For immunoblot analyses, 70–100 µg of protein lysate per sample, was used. Membranes were probed for desired proteins using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by ECL detection system.

(a) Whole cell lysate preparation:

Reagents

Lysis buffer

Composition of cell lysis buffer

10 Mm Tris (pH 7.0), 150mM NaCl(Merck), 1% Triton X-100 ,1 mM EDTA, 1 mM EGTA, 0.2 mM Sodium orthovanadate (Sigma Aldrich), 0.5% NP-40, 0.3 mM PMSF (Stock: 100 mM in Isopropanol), 10µg/ml Aprotinin (Stock: 2mg/ml in autoclaved DD water)

Procedure

At the end of the treatment time, 24 hrs and 48 hrs, entire media from the culture dishes was sucked out. All the dishes were washed gently with 1X PBS, 2-3 times (use 3 ml for first time and 2 ml for next two times of 1X PBS for each 100 mm dish). After pouring PBS into the plates, plates were swirled slowly and then PBS was sucked out with the pipette. The dishes were tilted for 2-3 minutes and the remaining PBS was taken out (to avoid the dilution of the lysate). 200 µl of cell lysis buffer was added in each 100 mm dish (the buffer was spread covering the whole area of the culture dish). Dishes were incubated approximately for 20 minutes on ice. Cells were scraped with a scrapper and collected in sterile micro-centrifuge tubes, these tubes were kept on ice, for an additional 20 minutes. Samples were freeze-thawed for 3-4 times with vortexing (dry ice-water bath-37^{0}C thaw-vortex) and were spun at 14000 rpm for 30 minutes at 4^{0}C. Supernatant which is the whole cell lysate was collected and pellet was discarded. Protein estimation was performed for each sample and equal amount of protein was loaded for western blotting.

(b) Protein Estimation

Reagents

5x Bradford (stock), bovine serum albumin (BSA-1mg/ml stock), cell lysate, double distilled water

Equipments

ELISA plate (96 wells plate), ELISA plate reader

Total protein estimation was carried out using Bradford's assay for the whole cell lysates prepared from DU145 cells treated with sodium metaarsenite and silibinin.

Sample preparation for protein estimation

1µl of each lysate sample was taken in sterile, labeled eppendorf tubes. 800µl double distilled water was added to each eppendorf tube. 200 µl of 5x Bradford solution was added to each eppendorf tubes.

(c) Preparation of BSA standards

Preparation of BSA working solution

100 µl BSA (1mg/ml) was dissolved in 900 µl double distilled water to make it 100 ng/µl working solution.

Double distilled water, BSA working stock, 5x Bradford stock were dissolved in following amount to make the BSA standards.

Table 5. Sample preparation for standard curve of BSA

Concentration of BSA	**BSA working stock**	**DD H_20**	**5x Bradford stock**
Blank	0	800µl	200µl
2µg	20µl	780µl	200µl
4µg	40µl	760µl	200µl
6µg	60µl	740µl	200µl
8µg	80µl	720µl	200µl
10µg	100µl	700µl	200µl

The optical density was recorded at 595 nm using Perkin elmer spectrophotometer, shimadzu and the ELISA plate was read on Elisa plate reader at 595 nm. Soft Max Pro software was used to measure the optical density of samples in case of elisa plate reader

(d) Immunoblotting Procedure

Gel composition

Table 6. 12% resolving gel composition

Components	**10 ml**	**20 ml**
D.D. Water	3.3	6.6
30% Acrylamide:Bis Mix	4.0	8.0
1.5 M Tris (pH 8.8)	2.5	5.0
10%SDS	0.1	0.2
10%APS	0.1	0.2
TEMED	0.004	0.008

Table 6. 5% Stacking Gel Composition

Components	**3 ml**	**5 ml**
D.D. Water	2.1	3.4
30% Acrylamide:Bis Mix	0.5	0.83
1.5 M Tris (pH 6.8)	0.38	0.63
10%SDS	0.03	0.05
10%APS	0.03	0.05
TEMED	0.003	0.005

(e) Sample preparation

Equal quantity of protein samples (60 to 80µg of whole cell lysate) and 2x sample buffer were mixed and vortexed. The samples were heated at 95°C for 5 minutes and were then vortexed for 5 seconds and centrifuged at 10,000 rpm for 3 minutes. Supernatant were loaded into wells carefully to avoid any debris.

(f) SDS Gel

Desired samples were loaded carefully into the wells. The gel was run for some time at 80 volts till the proteins entered the resolving gel and voltage was changed according to our protein of interest (120 volts) till dye seeped out of the resolving gel. Clamps were removed and the gel was carefully kept in the transfer buffer.

(g) Transfer procedure (Semi Dry)

Transfer tank was filled with 1x Transfer buffer. Wattman filter papers were soaked in 1x transfer buffer along with transfer pads. Made sandwich of **(-ve pole) transfer pads-two Wattman filter papers-Gel-Membrane-two Wattman filter papers-transfer pads (+ve pole)** and clamped between transfer sheets. The whole assembly was fitted in the transfer apparatus such that the gel was on the –ve pole side and the membrane on the +ve pole so that, the negatively charged proteins, could migrate towards the +ve pole. The whole assembly was run at 20 volts constant voltage for 1 hr.

(h) Making antibody solution

Desired amount of antibody was pipetted out and poured in blocking buffer. To minimize the wastage, we used plastic pouches for antibody incubation with membrane and sealed them tightly to prevent any leakage.

(i) Immunoblotting

Membrane was removed carefully from the assembly and marked at one of it's ends. The membrane was then kept in blocking buffer for 1hour at RT (room temperature) on mild shaking rocker. It was incubated with primary antibody for 1 hour at RT with full shaking and again

incubated at 4 degree O/N (overnight). Next day, the membrane was incubated at RT for 30 minutes on shaker and washed in 1x wash buffer, 3 times, for 5-10 minutes each wash.

The membrane was then incubated with secondary antibody for 1 hour at RT on full shaking rocker. The membrane was again washed in 1x wash buffer 3 times for 5-10 minutes each wash.

(j) Detection and film development (for ECL detection)

After washing the membrane, it was kept in small plastic tray and incubated with ECL-detection system till bands started glowing (bands glow in the dark). The reagent 1 and 2 of the detection system were mixed in an equal ratio to give a final volume equivalent to 0.125 ml/cm^2 membrane. The membrane was taken out, wiped with tissue paper and kept in the autoclave-bags/ sheets. All procedures after this were carried out in absolute darkness or red light (620-750 nm) for visual aid. The membrane was kept (in autoclave bag) at one corner of the cassette plate and an equal-sized X-ray film was put over it (in absolute dark; the direction of the film was marked by cutting it at the desired corner).The film was exposed for a specific time so that bands appeared on it (it depends upon the signal and protein amount).After exposure, the film was removed and dipped in the developer solution for 2 minutes and then washed in tap water for one minute. Immediately after that, it was dipped in fixer for 2 minutes and then washed again in tap water. Normal lights were switched on to see the bands. The bands were marked properly for record.

(k) Stripping of the membrane

The membrane was stripped for detection of some more different proteins for the same lysate. After development of the film, membrane was submerged in the stripping buffer and was incubated at 55°C for 30 minutes with mild shaking in water bath. The membrane was washed after draining out the stripping buffer, with 1X wash buffer for at least 4 times. For probing with next set of antibodies, the membrane was blocked again and above mentioned procedure for blotting was followed.

5. Migration assay

Reagents

Six well plates, silibinin (100 μM), sodium metaarsenite, methanol and crystal violet, cell inserts of 0.8μm, scalpel, DPX mount, glass slides.

Principle

DU145 cells are considered to possess high metastatic potential. They show chemotaxis and move towards chemo attractants. Media in the lower chamber (containing 10% FBS) of the assembly acts as chemo attractant for the cells in the upper chamber (suspended in serum free media). Thus serum in the lower chamber gives the cells stimulus to cross the PET membrane and enter the lower chamber. Total cells attached to lower surface of the membrane give the migratory ability of the cells.

Procedure

DU145 cells were seeded in duplicates in 60mm dishes from a 40 to 50 % confluent 100mm plate. Cells were then incubated for 24 hrs and then treatment done for the selected doses. Cells were then trypsinized after 24 hrs of treatment and $4x10^4$ were seeded in a serum free media in each culture insert of 0.8μm pore size. 1ml of media with 10% FBS was added into the lower chamber of the assembly. The assembly was then incubated at 37°C and 5% CO_2 for 20 hrs. Inserts were then removed and their inner walls were swabbed with a cotton swab. Inserts were then dipped in 100% methanol for 10 minutes to fix the cells, and then stained with 0.5% crystal violet stain for ten minutes. Inserts were washed twice with double distilled water to remove extra stain and were left inverted to dry. The PET membrane was cut out with a scalpel and mounted onto glass slides using DPX- mount after inverting the inserts. Slides were allowed to dry and were later visualized under microscope to count the number of cells. Five random fields were chosen under 400X magnification. The number of cells in each treatment was then counted and mean of the cells calculated and % migration was calculated using the following formula:

Percentage migration= [(mean of cells in test)/ (mean of cells in the control)] x 100

6. Matrigel invasion assay

Reagents

Six well plates, silibinin (100 μM), sodium metaarsenite, methanol, crystal violet, matrigel coated transwell cell inserts of 0.8μm (BD biosciences), scalpel, DPX mount, glass slides.

Principle

DU145 cells are considered to possess high metastatic potential. They show chemotaxis and move towards chemo attractants. Media in the lower chamber (containing 10% FBS) of the assembly acts as chemo attractant for the cells in the upper chamber (suspended in serum free media). Thus serum in the lower chamber gives the cells stimulus to invade matrigle and cross the PET membrane in order to enter the lower chamber. Total cells attached to lower surface of the membrane give the invasive potential of the cells.

Procedure

DU145 cells were seeded in duplicates in 60mm dishes from a 40 to 50 % confluent 100mm plate. Cells were then incubated for 24 hrs and then treatment done for the selected doses. Cells were then trypsinized after 24 hrs of treatment and $4x10^4$ were seeded in a serum free media in each culture insert of 0.8μm pore size of the boyden chamber (BD Biosciences). 1ml. of media with 10% FBS was added into the lower chamber of the assembly. The assembly was then incubated at 37°C and 5% CO2 for 20 hrs. Inserts were then removed and their inner walls were swabbed with a cotton swab to remove out the matrigel. Inserts were then dipped in 100% methanol to fix the cells for 10 mins, and then stained with 0.5% crystal violet stain for ten minutes. Inserts were then washed twice with double distilled water to remove extra stain and left inverted to dry. The PET membrane was cut out with a scalpel and mounted onto glass slides using DPX- mount. Slides were allowed to dry and were later visualized under microscope to count the number of cells. Five random fields were chosen under 400X magnification.

The number of cells in each treatment was then counted and mean of the cells calculated and % invasion was calculated using the following formula:

Percentage invasion = [(mean of cells in test)/ (mean of cells in the control)] x 100

8. Statistical Analysis

The software SigmaStat of the version 3.5 for Windows was used for statistical analysis. Continuous variables were expressed as X ± SEM (standard error mean). Statistical analysis was performed with One way ANOVA (analysis of variance) followed by Tukey test. Differences were considered statistically significant when p was less than 0.05. Graphs and bar diagrams were plotted using SigmaPlot 11 software in accordance with the statistics performed.

RESULTS

RESULTS

Silibinin modulates cell growth and proliferation induced by arsenic at low concentration

We first examined the effect of arsenic, silibinin and their combination on the cell proliferation via MTT and trypan blue exclusion assay. Arsenic treatment at a concentration of 0.5 μM resulted in a slight inhibition of cell proliferation of around 3% which could be considered negligible at 24 hrs while it enhanced cell proliferation by 8% at the same concentration for 48 hrs treatment time in the trypan blue exclusion assay **(Figure 6).** On the other hand, arsenic at 0.5 μM concentration resulted in 40% increase in cell growth for 24 hrs treatment in MTT assay while it did not show any marked change in cell proliferation for the same group treatment of 48 hrs in MTT assay. 5 μM As treatment for 24 hrs resulted in an inhibition of cell proliferation 19% in trypan blue exclusion assay **(Figure 7)** and 5% in MTT assay while its resulted in cell proliferation of 4% in trypan blue assay and 7% in MTT assay. Silibinin was found to significantly inhibit cell proliferation by 23% for 24 hrs treatment which further went up to 37% inhibition in total cell growth after 48 hrs treatment time for the trypan blue assay **(Figure 9).** In accordance with results obtained in cell counting assay, a significant decrease in the cell proliferative activity of the cells by silibinin (100 μM concentrations) treatment both for 24 and 48 hrs treatments in MTT assay was observed.

Silibinin was found to inhibit the cell proliferation by 19% and by 37% in DU145cells that was induced by 0.5 μM As treatment for 24 and 48hrs of treatment, respectively, in trypan blue assay. A similar observation was obtained for the same group treatments of 0.5 μM As and 100 μM SB during 24 and 48 hrs of treatment times in MTT assay. A significant inhibition of 30% and 70% in cell proliferation was observed in trypan blue cell counting assay when DU145 cells were treated with 100 μM SB and 5 μM As for 24 and 48 hrs respectively **(Figure 11).** In accordance, with results obtained in the trypan blue exclusion assay we observed a significant decrease in cell proliferative activity for the same group treatment of 100 μM SB and 5 μM As both for 24 and 48 hrs in the MTT assay.

The most significant observation of this study was that no significant cell death was obtained in any of the group treatments for 24 hrs in the trypan blue exclusion assay, except

for 100 μM Silibinin for 24 hrs treatment **(Figure 8)**. However, a significant percentage of dead cells were observed for combination group treatments for 48 hrs **(Figure 10).** Next, we investigated the effect to arsenic, silibinin alone and in combination on the colony forming ability of DU145 cells.

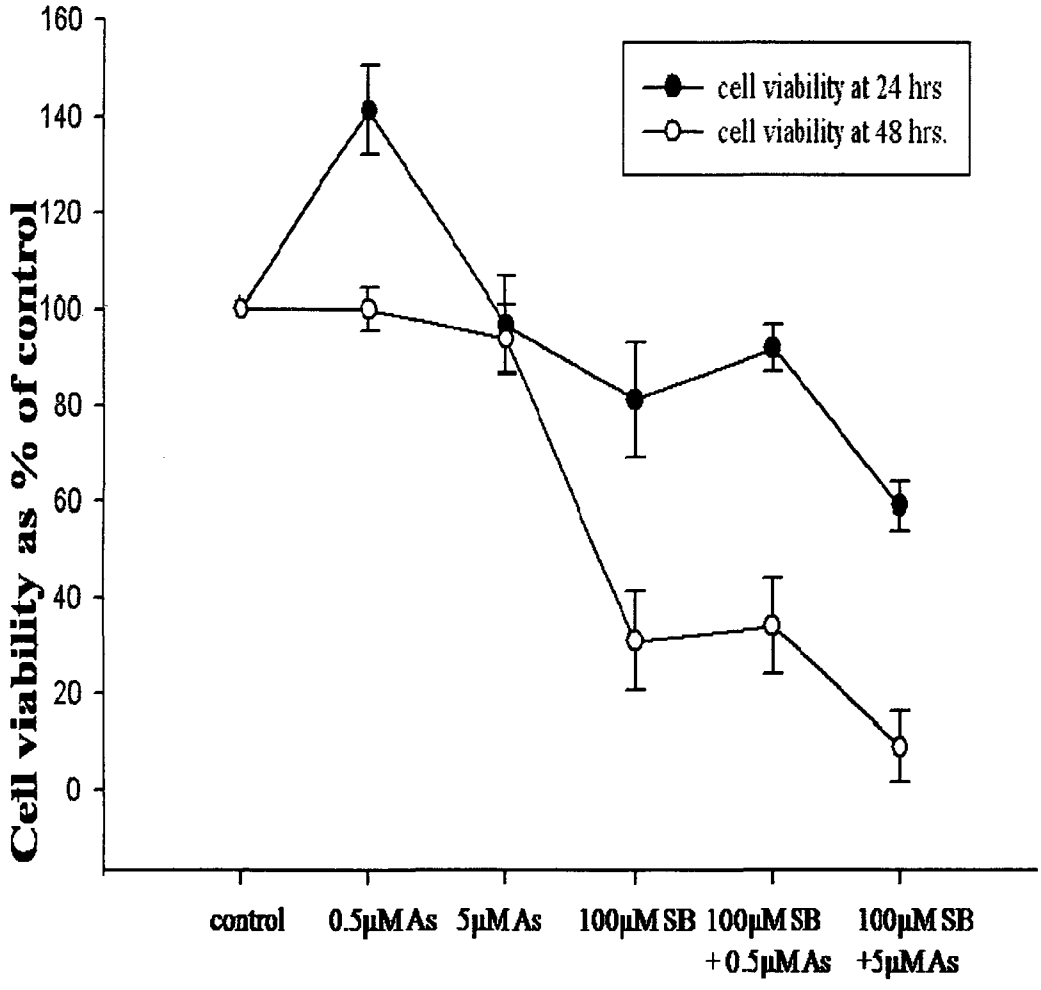


Figure6. Comparative analysis of the cell viability of DU-145 cells after 24 hrs and 48 hrs treatment with arsenic, silibinin alone and their combination *via* MTT assay. Columns represent, mean of three independent treatment samples and were reproducible in an additional independent experiment.

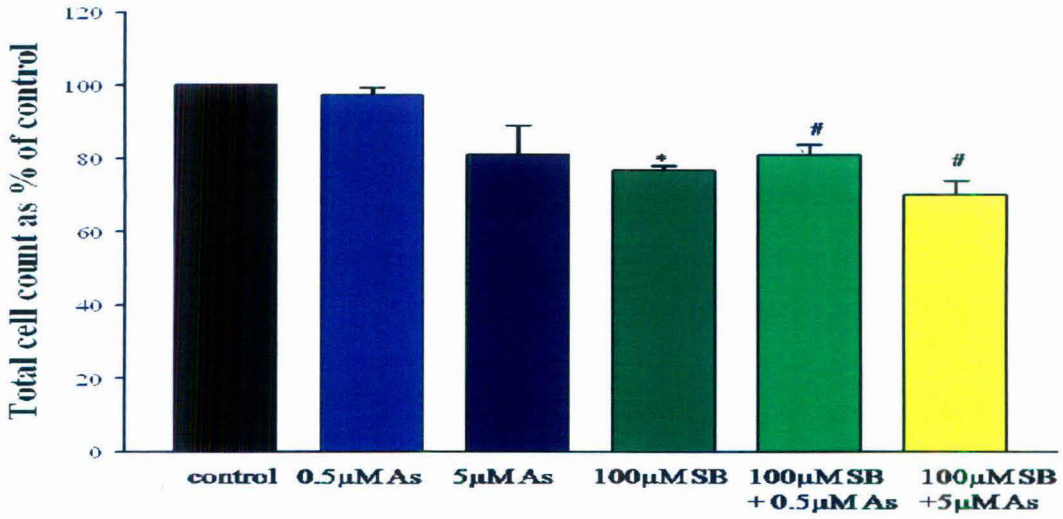


Figure 7. Analysis of the proliferative potential of DU145 cells after 24 hrs treatment with arsenic, silibinin alone and their combination *via* trypan blue exclusion assay . Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment. #, $P < 0.01$; *, $P < 0.001$.

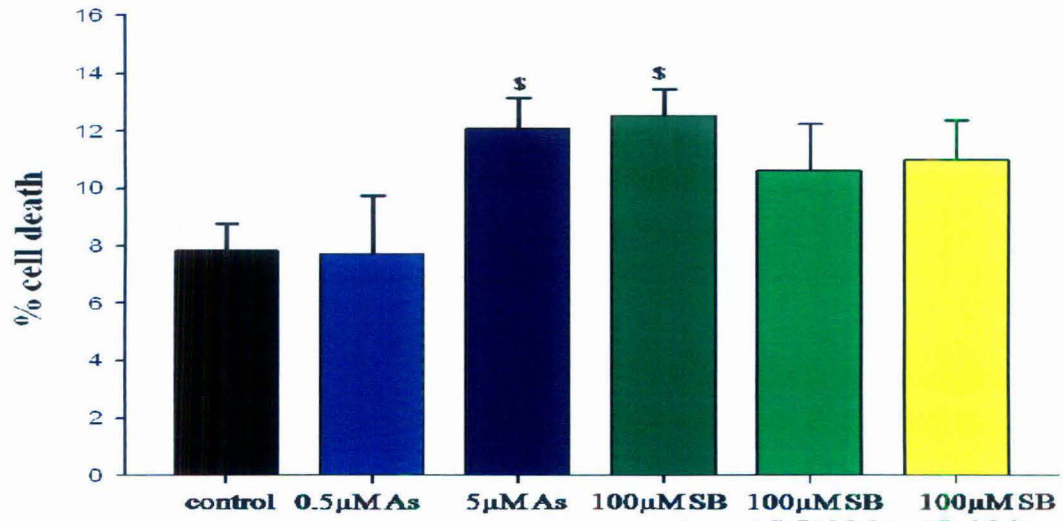


Figure 8. Analysis of % cell death of DU-145 cells after 24 hrs treatment with arsenic, silibinin alone and their combination *via* trypan blue exclusion assay. Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment. $, $P < 0.05$.

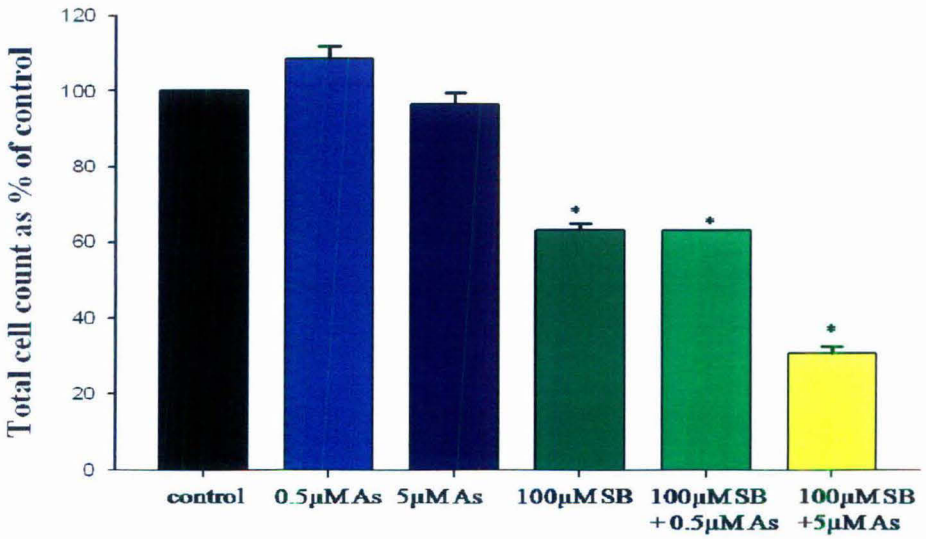


Figure 9. Analysis of the proliferative potential of DU145 cells after 48 hrs treatment with arsenic, silibinin alone and their combination *via* trypan blue exclusion assay . Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment. *, $P < 0.001$.

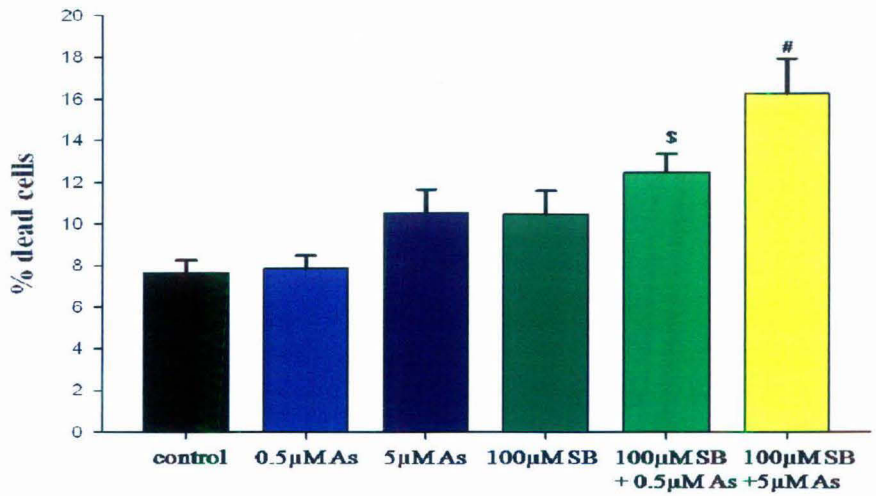


Figure 10. Analysis of % cell death of DU-145 cells after 48 hrs treatment with arsenic, silibinin alone and their combination *via* trypan blue exclusion assay. Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment. $, $P < 0.05$; #, $P < 0.01$.

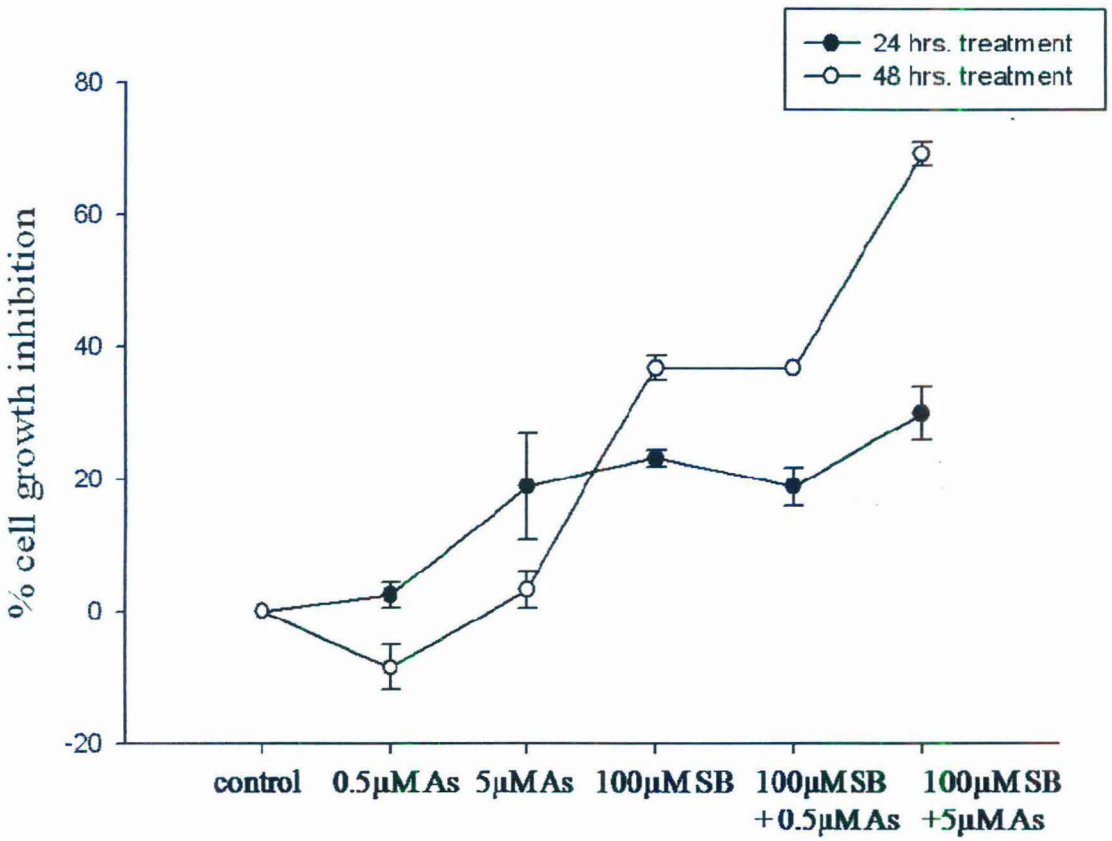


Figure 11. Comparative analysis of the proliferative potential of DU-145 cells as % inhibition after 24 hrs and 48 hrs treatment with arsenic, silibinin alone and their combination *via* trypan blue dye exclusion assay. Columns represent, mean of three independent treatment samples and were reproducible in an additional independent experiment.

Silibinin enhances the induction of apoptosis in DU145 cells treated with arsenic

To further investigate the possible reason for enhanced cell death for 48 hrs treatment of the cells with our agent arsenic (0.5 μM and 5 μM) and its combination with silibinin, we went for apoptosis assay using annexinV-FITC and PI stains (**Figure 12**). We obtained 3% and 4% increase in the % apoptotic cells for 0.5 μM and 5 μM arsenic treatment alone (**Figure 13**) which was not significant but correlated with the % dead cells obtained from trypan blue exclusion assay for 48 hrs treatment. Silibinin treatment alone showed a 16% increase in the apoptotic cells which showed that cell death induced due to silibinin treatment was due to apoptosis induction. Combination treatment of silibinin with 0.5 μM and 5 μM showed significant increase upto 13% and 26% in apoptotic cells respectively which was again in accordance with the increased cell death at 48 hrs as observed in trypan blue exclusion assay.

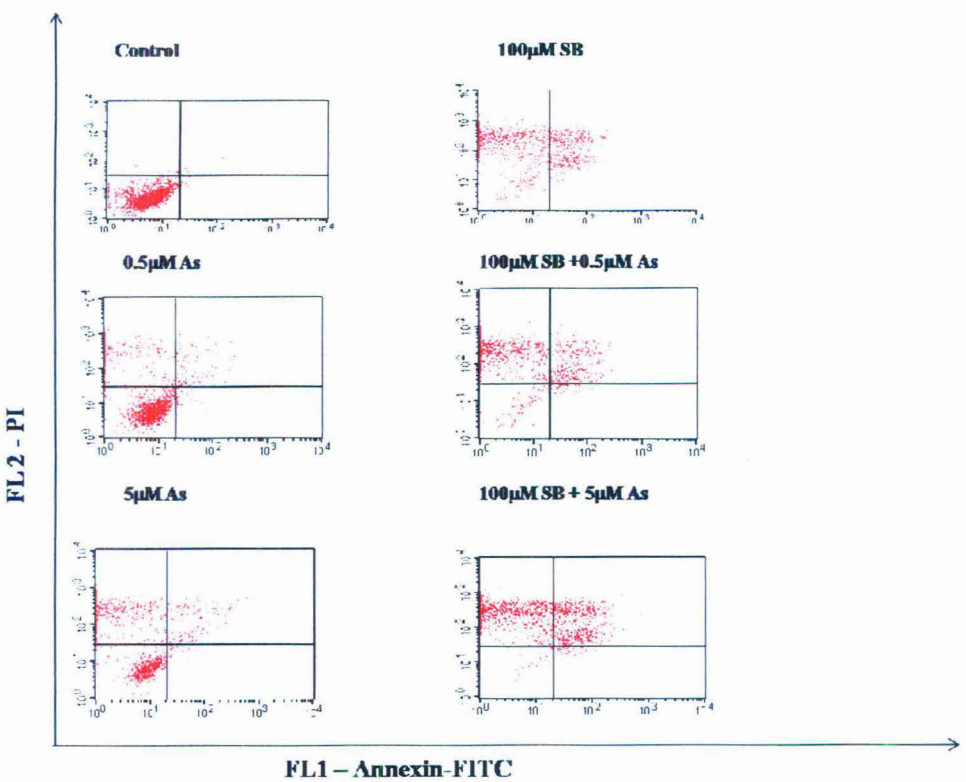


Figure 12. Representative figure of the analysis of the induction of apoptosis in DU145 cells on treatment with arsenic and silibinin alone and their combination after 48 hrs time point. Each graph represents the mean of three independent samples for each treatment.

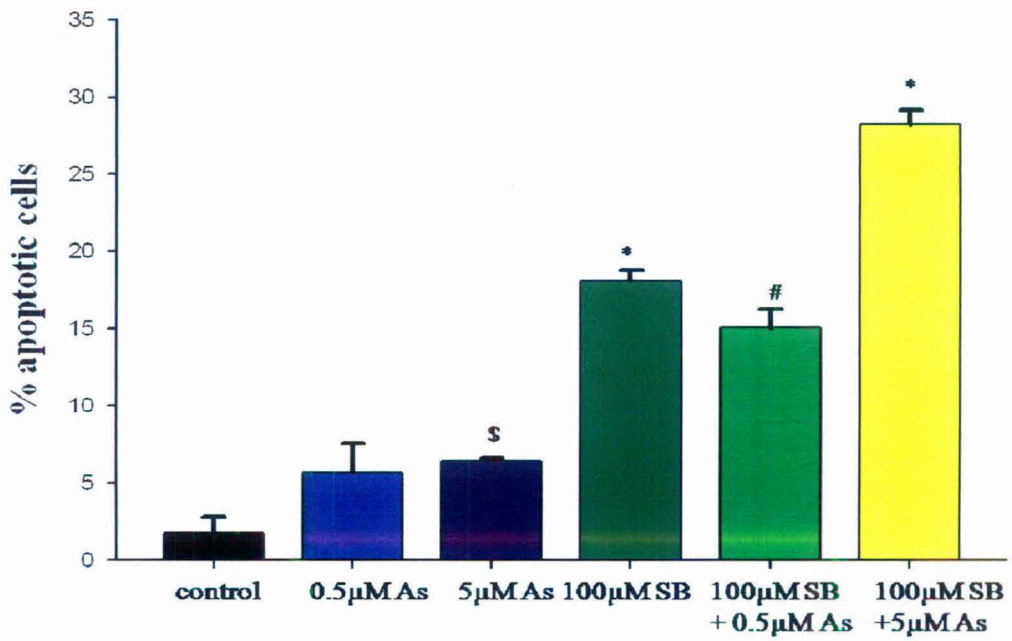


Figure 13. Analysis of the % apoptotic cells of DU-145 cells on treatment with arsenic, silibinin alone and their combination. Columns represent, mean of three independent treatment samples. $, $P < 0.05$;#, $P < 0.01$; *, $P < 0.001$.

Silibinin in combination with arsenic modulates and inhibits the colony-forming ability induced in DU145 cells

Our next aim was to analyze the modulatory effect of silibinin and arsenic alone and in combination on the colony forming ability of the advanced prostate carcinoma DU145 cells. We used 10 μM concentration of SB for this experiment. Arsenic treatment alone showed a significant decrease in the colony forming potential of the cells, both at 0.5 μM and 5 μM concentration which accounted for 27% and 74% inhibition respectively (**Figure 14.a and 14.b**). A significant repression in the colony forming potential was observed at 5 μM concentrations As treatment was possibly due to induction of cell death. Similarly, a significant inhibition in colony forming ability of the cells was observed by silibinin treatment alone at 10 μM concentration (71% inhibition) as well as silibinin in combination with 0.5 μM As (45% inhibition) and with 5 μM As (95% inhibition). On the whole, the results obtained from this experiment showed that silibinin (100 μM) alone and in combination with arsenic significantly represses the colony forming ability of DU145 cells.

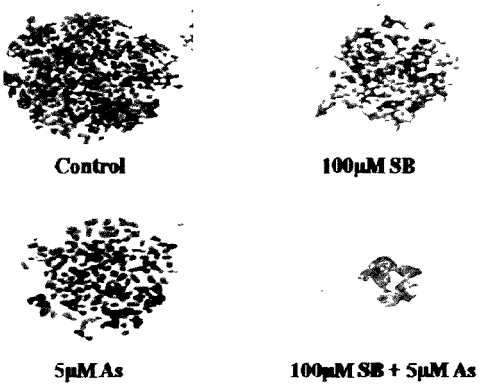


Figure 14.a. Representative figure of colony forming ability of DU145 cells on treatment with arsenic and silibinin alone and their combination. The experiment was done in triplicates for each of the samples of the group treatments as described in material and methods.

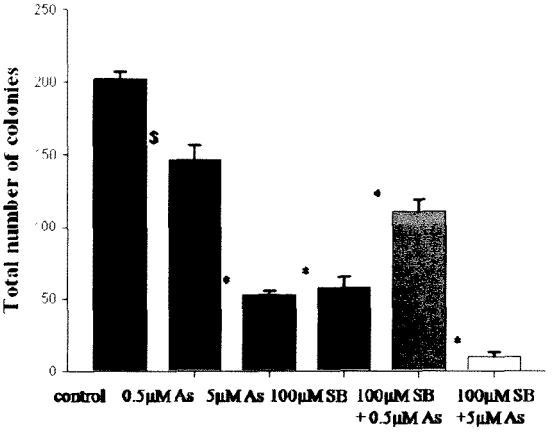


Figure 14.b. Analysis of the colony forming ability of DU-145 cells on treatment with arsenic, silibinin alone and their combination. Columns represent, mean of three independent treatment samples. $, $P < 0.05$; *, $P < 0.001$.

Arsenic induces G1 arrest and this effect is further enhanced in the presence of silibinin

To identify the underlying mechanism of the growth arrest mediated by arsenic, silbinin and their combination we next treated asynchronously growing DU145 cells with selected doses of the drugs both alone and in combination, and then stained with saponin/propidium iodide and cell cycle distribution was analyzed (**Figure 15**). At 24 hrs of treatment, higher dose of As (5 μM) or silibinin (100 μM) induced G1 arrest. However, in combination, at lower dose of As (0.5 μM) it remained at G1 phase of cell cycle but shifted to G2/M phase arrest at higher dose of As. Arsenic treatments (0.5 μM and 5 μM concentration) alone resulted in G2/M phase arrest while in combination with silibinin (100 μM concentration) induced strong G1 arrest or G2/M arrest ($P < 0.001$) in DU145 cells both for 24 and 48 hrs of treatment (**Figure 16**). Analysis showed that cells started accumulating in G1 phase at the expense of a decrease in S-phase population.

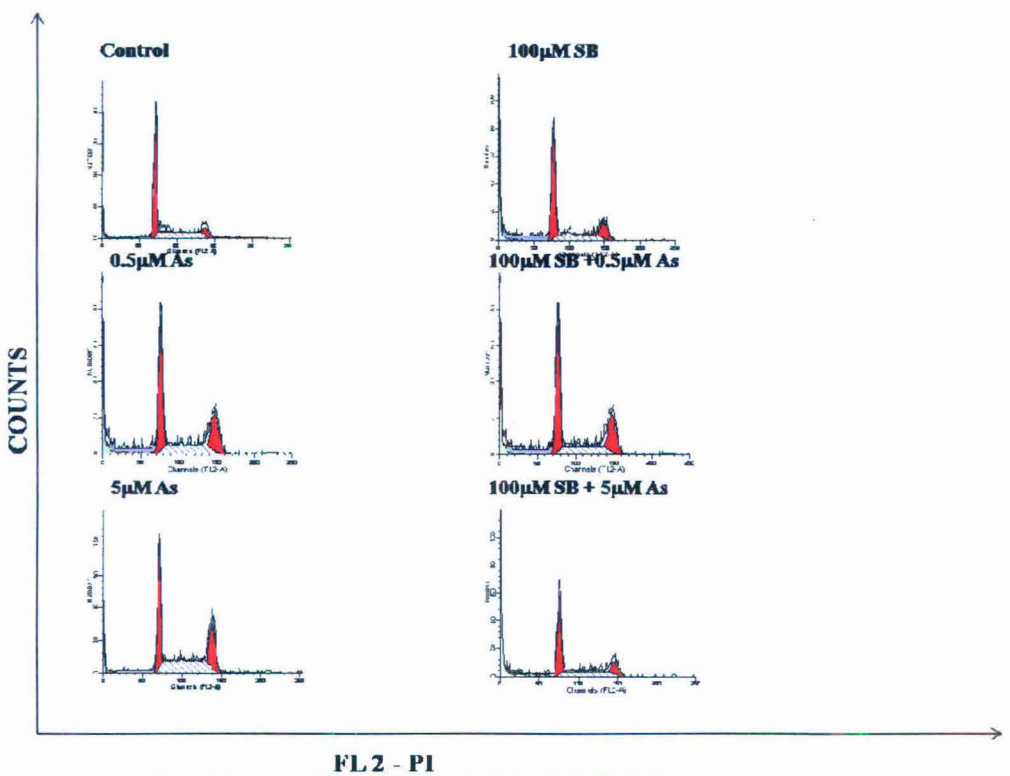


Figure 15. Representative figure of the analysis of cell cycle distribution in DU145 cells on treatment with arsenic and silibinin alone and their combination after 48 hrs time point. Each graph represents the mean of three independent samples for each treatment. Results were reproducible in an additional independent experiment.

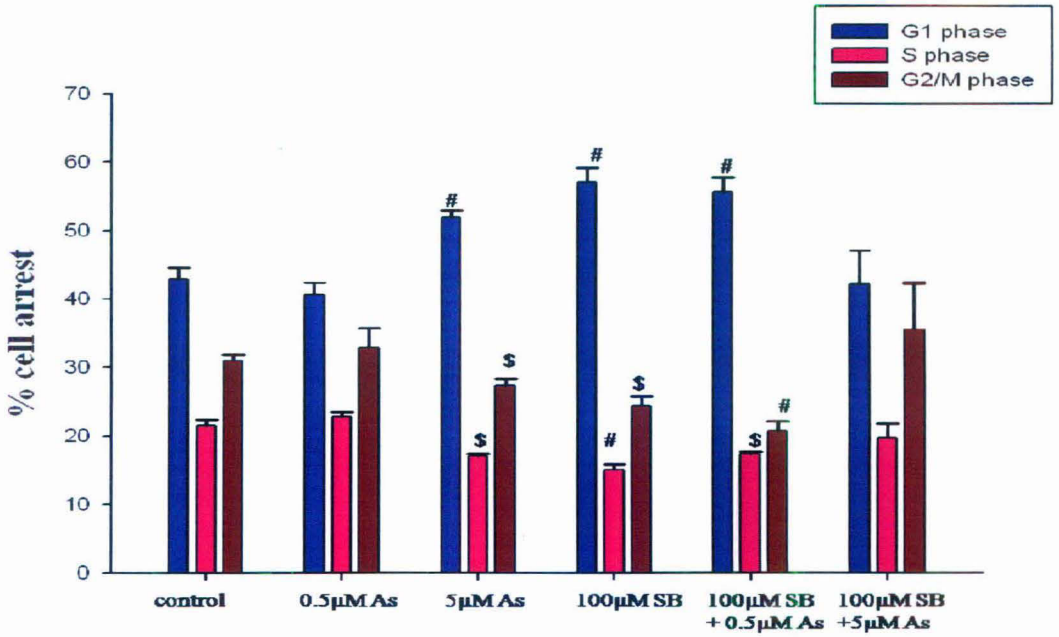


Figure 16. Analysis of the distriution of cell cycle of DU145 cells after 24 hrs treatment with arsenic, silibinin alone and their combination *via* FACS. Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment.. $, $P < 0.05$;#, $P < 0.01$.

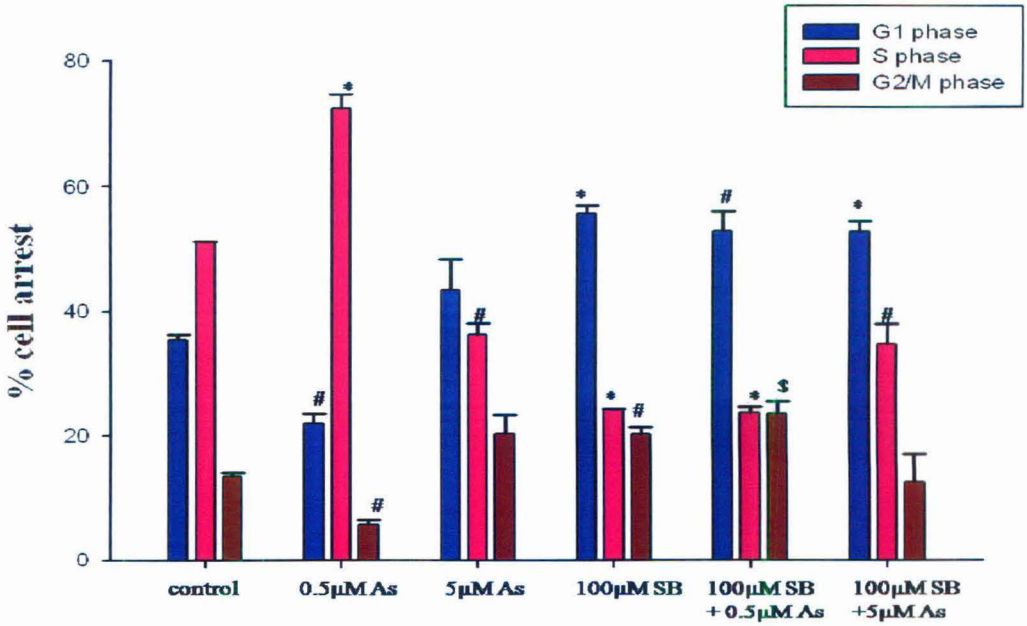


Figure 17. Analysis of the distriution of cell cycle of DU145 cells after 48 hrs treatment with arsenic, silibinin alone and their combination *via* FACS. Columns represent, mean of three independent treatment samples and were reproducible in an additional experiment. $, $P < 0.05$;#, $P < 0.01$; *, $P < 0.001$.

Silibinin treatment in combination with arsenic induces the expression of cyclin dependent kinase inhibitors p21 and p27

Cell cycle arrest at the G1 phase of DU145 cells due to silibinin and arsenic treatment led us to select cyclins and cyclin dependent kinases of G1 phase for analyzing the change in the level of expression of these proteins. An increase in the level of protein expression of cyclin dependent kinases, CDK2, CDK4 and CDK6 was observed in 0.5 μM As treatment both for 24 hrs and 48 hrs time periods **(Figure 18).** Corresponding to this, a slight increase or no alteration in the level of cyclin D3 and cyclin E was also observed for 0.5 μM As treatment both for 24 hrs and 48 hrs time periods. This indicated a possible involvement of arsenic at low dose of 0.5 μM in cell cycle progression. However, a slight increase in the expression CDKIs p21 and p27 was observed at this treatment dose of arsenic **(Figure 19).** This indicated that cell death at 0.5 μM As may possibly be due to cell cycle arrest at G1-S transition phase induced for 24 hr and48 hrs treatments.

Arsenic at 5 μM concentrations too increased the levels of CDK2, CDK4 and CDK6 protein expression both for 24 and 48 hrs treatments alongwith a slight increase in the levels of cyclin D3 for 48hrs **(Figure 19)** treatment and slight increase in cyclin E **(Figure 18)** levels for 24 hrs treatment. This clearly indicated that arsenic at 5 μM concenttration affects cell cycle arrest in the early G1 phase for 48 hrs of treatment while it arrest cell cycle at late G1 phase for 24 hrs of treatment in DU145 cells. A slight decrease in the expression of p21 and p27 was observed at 24 hrs treatment with 5 μM As while an increment in the expression of the two CDKIs was observed at 48 hrs treatment for this dose of arsenic.

Silibinin at 100 μM alone and in combination with 0.5 μM and 5 μM As strongly reduced the expression of CDK2, CDK4 and CDK6. This reduction in protein expression was higher for 48 hrs treatment as compared to that of 24 hrs of treatment. Moreover, the combination treatment of silibinin with 5 μM As showed a higher reduction in the expression of the CDKs as compared to treatment of DU145 by silibinin alone or in combination with 0.5 μM As both at 24 h and 48 hrs treatment time points. We observed a decrease in cyclin E expression for treatment by silibinin alone while there was no observed alteration in the expression of cyclin D3 treatment both at 24 hrs and 48 hrs which led to the conclusion that silibinin affects the late G1 phase of the cell cycle. These results are consistent with decrease

in the expression of CDK2 in our study and are in accordance with the previous studies on silibinin on DU145 cells documented in literature (Roy *et al.* 2007). However, we did not observe any significant change in the expression of both cyclin D3 and cyclin E in combination treatment of both silibinin with 0.5 μM and silibinin with 5 μM As both for 24 and 48 hrs treatment time points. Nervertheless, we observed a strong reduction in the expression of cyclin D3 in combination treatment of silibinin with 5 μM As in 24 hrs treatment.

A significant increase in the expression of CDKIs, p21 and p27 was obtained for combination treatment of silibinin with 5 μM As both at 24 hrs and 48 hrs treatment time points. The increase in expression of p27 was significant for combination of silibinin alone as well as for silibinin with 0.5 μM As both in 24 and 48 hrs of treatment time points. There was no observed enhancement in the expression of p21 protein for silibinin alone as well as for silibinin with 0.5 μM As in 24 hrs treatment while there was a marked increment in the expression of p27 protein for the same treatments in 24 hrs time point. This result is in agreement with the finding that the expression and role of p21 and p27 proteins is complementary as documented in literature (Roy *et al.* 2007).

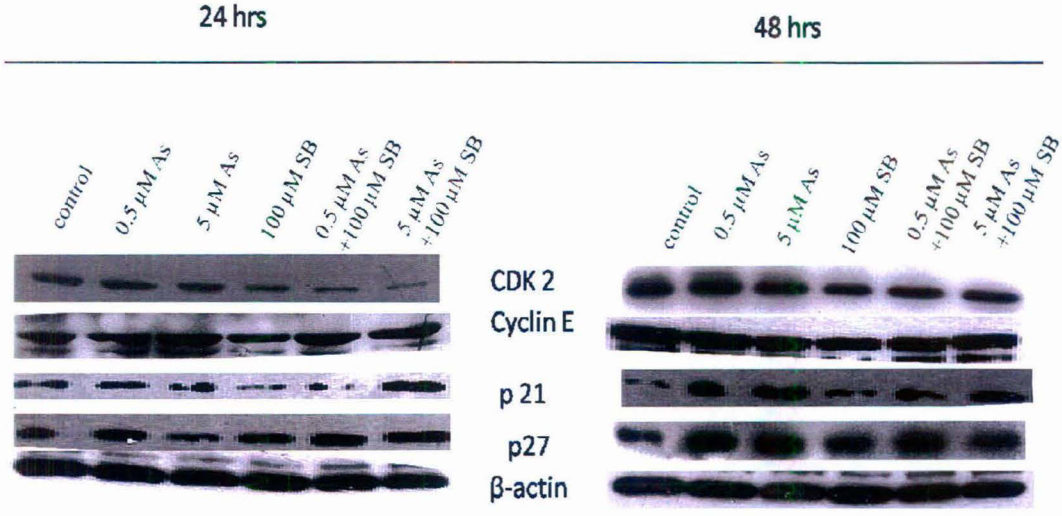


Figure 18. Immunoblot analysis cell cycle regualting proteins of G1 phase for DU145 cells on treatment with arsenic and silibinin alone and their combination after 24 and 48 hrs time points. The results were reproducible in an additional independent experiment.

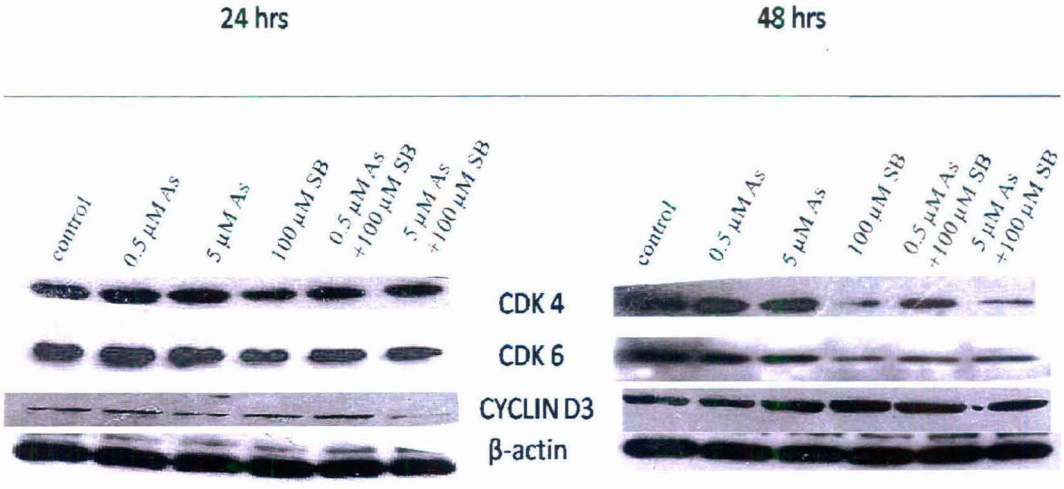


Figure 19. Immunoblot analysis cell cycle regualting proteins of G1 phase for DU145 cells on treatment with arsenic and silibinin alone and their combination after 24 and 48 hrs time points. The results were reproducible in an additional independent experiment.

Silibinin inhibits the migration and invasion induced by arsenic treatment in DU145 cells

To test whether arsenic alone or /and in combination with silibinin affected motility of our model cell line, DU145 cells, we carried out standard *in vitro* chamber assay. Treatment with 0.5μM As led to a slight decrease in the motility of the cells whereas treatment with 5 μM As increased the motility of DU145 cells by 4% (**Figure 20.a and 20.b**). A significant repression in the migration ability of the cells was observed on treatment with silibinin (20% repression) alone as well as silibinin in combination with 0.5 μM As (30% repression) and 5μM As (39% repression).

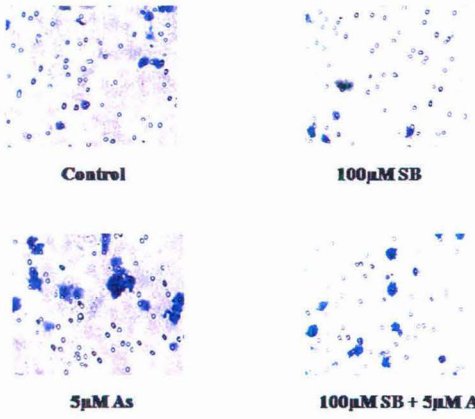


Figure 20.a. Representative figure of the analysis of the migratory potential of DU145 cells on treatment with arsenic and silibinin alone and their combination. The experiment was done in duplicates for each of the samples of the group treatments as descried in material and methods.

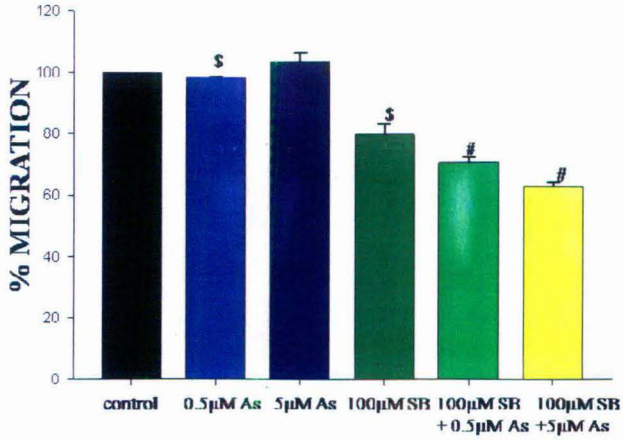


Figure 20.b. Analysis of the migratory potential of DU145 cells on treatment with arsenic, silibinin alone and their combination. Columns represent, mean of two independent treatment samples . $, $P < 0.05$;#, $P < 0.01$.

To extend our studies, we assayed the capacity of drug-treated cells to invade through matrigel coated transwell inserts. Consistent with the results of migration assays, DU145 cells were found to have enhanced invasive potential by 3% when treated with 0.5 μM As and 2% when treated with 5μM As alone (**Figure 21.a and 21.b**). Treatment with silibinin significantly repressed the invasive potential of cells by 12% while its combination with 0.5 μM As and 5 μM As resulted in 22% and 23% repression respectively. Taken together, these data indicated that silibinin alone and in combination with arsenic repressed motility and matrigel invasive potential of DU145 cells.

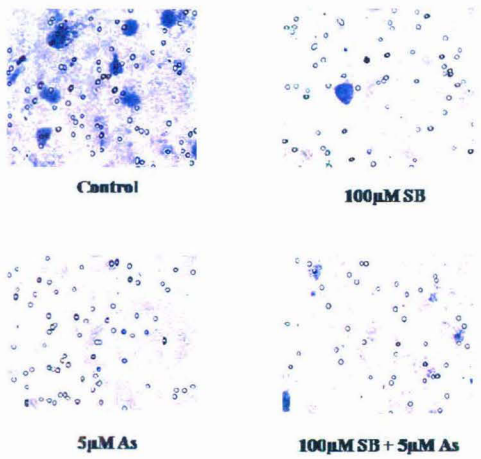


Figure 21.a. Representative figure of the analysis of the matrigel invasive potentialof DU145 cells on treatment with arsenic and silibinin alone and their combination. The experiment was done in duplicates for each of the samples of the group treatments as descried in material and methods.

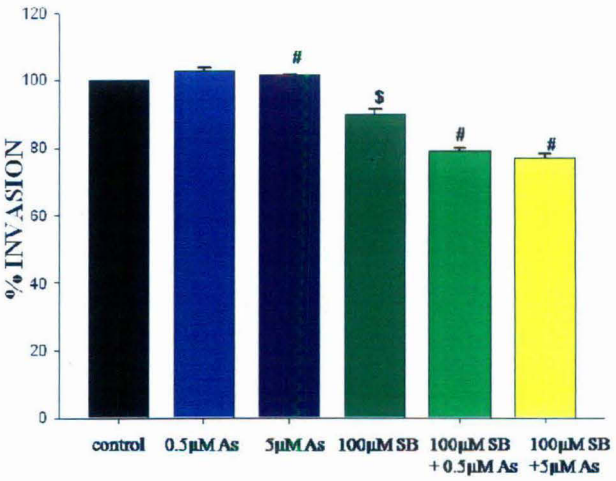


Figure21.b. Analysis of the invasive potential of DU145 cells on treatment with arsenic,silibinin alone and their combination.Columns represent, mean of two independent treatment samples. $, $P < 0.05$;#, $P < 0.01$.

DISCUSSION

DISCUSSION

Arsenic has been documented to be a carcinogen and one of the most toxic elements. Its intake in low doses in drinking water leads to chronic arsenic toxicity that enhances the risk of developing cancer. On the other hand its potential to be an anticancer agent has been studied in cancers such as APL (acute promyelocytic leukemia) but not in prostate cancer. Arsenic is a prime example of the prototype that "poisons in small doses are the best medicines and the best medicines in too large doses are poisonous" (Zhu *et al.* 2002).

As_2O_3 and $NaAsO_4$ (sodium metaarsenite) are identical substances in solution. Since As_2O_3 is poorly soluble in water it should be dissolved in NaOH and then adjusted to physiological pH yielding sodium metaarsenite (Phatak *et al.* 2008). Most of the investigators who have studied arsenic trioxide have directly used sodium metaarsenite for their experiments (Yih *et al.* 2006). They have also compared the *in vitro* cytotoxicity of sodium metaarsenite and that of solubilized As_2O_3 in tumor cell lines and hardly found any difference in the potency of both the drugs suggesting that they might both act in a very similar manner. Sodium metaarsenite was therefore chosen for this study.

Silibinin is well documented for its chemopreventive potential against various types of cancer models including prostate, skin cancer etc. It is a polyphenolic antioxidant that exhibits little or no systemic toxicity. Silibinin has been observed to show all its significant biological effects at 100 µmol/L concentration and clinical trials have shown that of 100 µmol/L of free silibinin is easily achievable in prostate cancer patients (Flaig *et al.* 2006). Silibinin has shown promising efficacy against prostate cancer where it has entered the phase II clinical trial. Further, silibinin has also been studied in combination with other chemotherapeutic drugs to study the efficacy of combination therapy on growth inhibition, cell cycle regulation and apoptotic induction in prostate, breast and lung cancer cells. Together, the results indicated a synergistic effect of silibinin on growth inhibition, the reversal of chemoresistance, and apoptosis induction when given in combination with these drugs. This formed the objective of our present study of analyzing the modulatory effects of silibinin on arsenic mediated biological and molecular events in prostate cancer.

Arsenic in low doses of 0.5 μM was found to enhance the proliferative potential of the advanced prostate carcinoma DU145 cells. This was in accordance with the cell cycle distribution result that showed arrest at synthetic phase (S phase) of the cell cycle. Similarly, growth was slightly but not significantly altered by 5μM of arsenic treatment. This was possibly due to the arrest in the G1 phase at 24 hrs treatment and G1 and/or G2/M arrest for 48 hrs treatment. Silibinin showed a significant inhibition in the cell growth potential of the cells both at 24 and 48 hrs treatment which might be due to the arrest of the cells at G1 phase of the cell cycle. This finding was in accordance with the previous studies in literature (Roy *et al.* 2007). However, our combination treatment (0.5 μM As with SB and 5 μM As with SB) showed a significant inhibition of cell proliferation both at 24 and 48 hrs of treatment. This was possibly due to the arrest of the cells at G1 phase for 0.5μM arsenic in combination with silibinin which further shifted to G2/M arrest for 5μM arsenic with silibinin for 24 hrs treatment. Combination of silibinin with 0.5 μM arsenic showed strong G1 and G2/M arrest at 48 hrs while 5μM arsenic combination with silibinin strongly inhibited G1 phase of the cell cycle for 48 hrs treatment.

We also observed an increase in cell death from 0.5 μM and 5 μM arsenic treatment to silibinin treatment alone and their combination for 48 hrs treatment. This was in accordance with the results obtained from apoptosis assay where % apoptotic cells increased in a similar manner as % dead cells for trypan blue assay for 48 hrs treatment. We obtained 16%, 13% and 26% ($p < 0.001$) more apoptotic cells as compared to control for silibinin alone and siliibnin in combination with 0.5μM and 5μM arsenic treatment respectively. This explains that the enhanced cell death as result of combination treatment might have been due to the induction of apoptosis.

Similar to the above findings, we obtained a strong repression in the colony forming ability of the cells after treatment with the drugs. Colony forming ability of the cells was inhibited upto 74% with 5 μM arsenic treatment which was further elevated to 95% inhibition in its combination with silibinin. The enhanced inhibition in colony forming ability of the cells due arsenic treatment alone was according to the previous studies documented in literature (Lu *et al.* 2004).

Deregulation in cell cycle progression is a hallmark of cancer cells. Cell cycle progression is regulated by the activity of CDKs in non- covalent association with their

regulatory subunits cyclins and CDKIs (Sherr 2000). We therefore, carried out immunoblot analysis of cell cycle regulating proteins. Consistent with the above findings, we obtained enhanced expression of CDK2 on treatment with arsenic (0.5 and 5 μM) alone while a decrease in its expression was observed for silibinin alone and in combination both at 24 and 48 hrs treatment. We observed a decrease in the expression of cyclin E for silibinin treatment alone (24 and 48 hrs treatment) while there was hardly any change in its expression for the arsenic alone and combination treatment (both for 24 and 48 hrs treatment). Our immunoblot analysis for the expression of CDK4, CDK6 and cyclin D3 also correlated with the results obtained for CDK2 and cyclinE treatment. We observed hardly any alteration in the expression of CDK4 (both 24 and 48 hrs treatment), CDK6 (48 hrs treatment) and cyclin D3 expression for arsenic treatment alone (both 24 and 48 hrs). However, a slight increase in the expression of CDK6 for arsenic treatment alone (0.5 and 5 μM) was observed for 24 hrs treatment. A significant decrease in the expression of silibinin and the combination treatment was obtained for CDK4 and CDK6 proteins both for 24 and 48 hrs treatment. On the other hand a marked decrease in cyclinD3 was observed for combination treatment (5 μM As with silibinin) for 24 hrs treatment while there hardly any difference in its expression for other treatments both at 24 and 48 hrs. These results indicate that the alterations in the expression of these proteins are possibly due to the effect of our agents (arsenic and SB alone and their combination) on cell cycle regulators of the G1 phase of the cell cycle.

p21, the universal inhibitor of cell cycle as well as p27 showed an enhanced expression for the arsenic treatment (0.5 μM and 5 μM) for 48 hrs treatment which suggested that enhanced cell death at both these treatments may possibly be due to enhanced induction of cell cycle arrest. However, we also obtained a slight increase in CDK2 levels for arsenic treatment alone for 48 hrs time point suggesting that although CDKIs might be increasing in their concentration but they might have not been able to bind to the ATP binding sites of the kinases and consequently could not inhibit cell proliferation. In correlation with the results obtained for the expression of CDKs for slibinin treatment alone and combination treatments, we obtained enhanced levels of p27 both for 24 and 48 hrs treatment suggesting that cell cycle arrest induced by silibinin might have been due to binding of CDKI, p27 with CDKs. We also noticed a decrease in the expression of p21 while an enhanced expression for p27 for silibinin alone and combination of

silibinin with 0.5 μM arsenic for 24 hrs treatment. This might possibly be due to complementary role of p21and p27 in cell cycle arrest (Roy *et al.* 2007). In other combination treatment (5μM As with SB) we obtained an enhanced expression of p21 both for 24 and 48 hrs suggesting that p21 negatively regulates the activity of CDKs which further results in induction of cell death *via* cell cycle arrest.

Our results of migration and matrigel invasion assays indicated a significant repression in the motility and matrigel invasive potential of DU145, a high-grade metastatic prostate cancer cell line. However, further studies are required to unfold the molecular mechanisms involved in repressing the migratory and matrigel invasive potential of the cells due to the treatment with the above agents, arsenic (0.5 μM and 5 μM arsenic) and silibinin alone and their combination.

The central finding of this study was that silibinin strongly modulates the effect of arsenic in the advanced human prostate carcinoma DU145 *via* induction of cell cycle arrest and cell death. This is a significant finding since arsenic has been known to cause systemic toxicity and carcinogenesis. On the other hand, its prowess to be an anticancer agent has also been proved in various cancers (such as APL) both alone and in combination with other chemotherapeutic drugs. However, clinical trials have indicated an increase in systemic toxicity and other side effects when arsenic is given alone or in combination with other drugs. To overcome this problem of systemic toxicity and enhanced chemoresistance extensive research has been directed towards combination chemotherapy for the purpose of finding compounds with a known mechanism of action that could increase the therapeutic index of clinical anticancer drugs. A number of studies have shown that silibinin is one such agent, which is nontoxic, consumed widely as a dietary supplement, and possesses strong anticancer activity. Consequently, we chose this combination of arsenic and silibinin for the present study. Our findings suggests that a pre-clinical study can be initiated for this combination of drugs once, and positive outcomes of such *in vivo* study could form the basis for the clinical studies.

SUMMARY

AND

CONCLUSION

Summary and conclusion

Arsenic is an atypical carcinogen as it is classified neither in the initiator nor in the promoter categories of carcinogenic agents. On the other hand, studies have displayed its role as a valuable therapeutic in cancer treatment. Therefore, it is regarded to be a paradox. However, As alone as well as in combination with other chemotherapeutic drugs has shown its anticancer prowess. In this regard, we attempted to unfold its paradoxical nature by analyzing its effect on advanced prostate cancer DU145 alone and in combination with the chemopreventive agent, silibinin.

Arsenic at low concentration of 0.5 μM enhanced cell proliferation while at 5μM had no significant change in the growth potential of the cells. On the other hand silibinin alone and in combination with arsenic significantly reduced the cell proliferative potential of the advanced prostate carcinoma DU145 cells as indicated by the trypan blue dye exclusion assay. Further, we found marked inhibition in the colony forming ability of the cells both by arsenic and silibinin alone and their combination treatments.

In accordance with the cell growth inhibition, our results of apoptosis assay indicated that increased cell death for 48 hrs treatments with silibinin alone and with the combination treatment (0.5μM As with SB and 5μM with SB) was due to increment in the induction of apoptosis in the cells.

Results obtained from cell cycle distribution indicated that the enhanced growth of the cells due to arsenic treatment at 0.5μM as there were more cells in the synthetic phase after 48 hrs treatment, which further shifted to G2/M phase at 5 μM arsenic treatment. Silibinin on the other hand arrested the cells at G1 phase both at 24 and 48 hrs. However, we obtained significant G1 arrest in the combination treatment of silibiinin with arsenic at 0.5 μM for 24 hrs treatment while G2/M and G1 arrest at 48 hrs treatment. A significant G1 arrest was obtained in combination treatment of silibinin with 5 μM arsenic at 48 hrs while G2/M for 24 hrs treatment. On the whole the results indicated that the growth inhibition in combination was mainly due to induction of cell cycle arrest.

On further investigating the molecular mechanism underlying the effect of the combination treatment, we were able to show that cell cycle arrest was due to enhanced expression of the cell cycle regulating proteins of the G1 phase of the cell cycle, through

immunoblot analysis. Accordingly, we obtained a decrease in the expression of cyclin dependent kinases, CDK2, CDK4 and CDK6 alongwith an enhanced expression of CDKIs, p21 and p27 proteins for the combination treatments.

Analysis of the migration and matrigel invasion assay showed a significant repression in the motility and matrigel invasive potential of DU145 cells due to the combination treatment.

Thus, the present study provided us with a new insight into the area of combined chemotherapy since the results obtained were highly significant with respect to the combined chemotherapy approach. The major criteria for combination therapy are that the response has to be synergistic and that the drugs should not share common mechanisms of resistance and their major side-effects should not overlap. Accordingly, we emphasize that silibinin in combination with arsenic can be an ideal candidate to be used in clinical combination once the efficacy of this combination is proved in the *in vivo* studies.

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APPENDIX

APPENDIX

Acrylamide (30%) Stock Solution

Weigh 29g of Acrylamide (Sigma Aldrich) and 1g of NN Methylene Bis Acrylamide (Sigma Aldrich). Dissolve it in 100ml D.D H_2O. Store it in dark place in 4°C.

Blocking Buffer (5%)

Dissolve 5g Non-fat dry milk in 100ml 1X Wash buffer.

Bradford (5X) Stock Reagent

Weigh 50mg of Coomassie Brilliant Blue G-250 (CBGG-250) in 25ml 95% ethyl alcohol.
Add 50ml 85% Orthophosphate. Mix properly and make up the final volume to 100ml with D.D water.

BSA (1mg/ml) Stock Solution

Dissolve 10mg of BSA powder in 5ml of distilled water. Mix properly and make up to the final volume. Keep it at 4°C.

0.5M EDTA (Ethylene Diamine Tetra-Acetate)

MW 292.25g, 1Litre (pH=8)

Weigh out 146.13g of EDTA. Add 800 ml double distilled water. Stir vigorously and then add approximately 20g (not more) of sodium hydroxide pellets. Stir for 10 minutes. Adjust the pH to 8.0 with more pellets or using a 10N sodium hydroxide solution. The EDTA will not go into solution until almost pH 8.Once the EDTA solution has become clear, check the pH. Adjust the volume to 1 litre.

10X PBS (Phosphate- Buffered Saline):

Dissolve in 800ml D.D H_2O:

80g NaCl

2g KCl

14.4g Na_2HPO_4

2.4g (17.64 from solution) KH_2PO_4

Adjust the pH to 7.4 with HCl or NaOH. Make up the volume to 1L and autoclave.

10X Running Buffer

29g Tris base

144g Glycine

10g SDS

Add distilled water to make up 1 litre of the buffer. Stir it till get a clear solution.

RNase A (10mg/ml)

Dissolve 10 mg RNase A, 10mM Tris HCl(pH 7.5) and 15mM NaCl in 1ml PBS (Ca, Mg-free).

2X Sample Buffer (For 10ml Solution)

2.5 ml 0.5 M Tris Buffer

2.0 ml 20% SDS

2.0 ml Glycerol

1.0 ml Beta- Mercaptoethanol

4.0 mg Bromophenol Blue

Make up the volume to 10ml with distilled water, aliquot in eppendorf tubes and store at -20°C.

20% SDS (Sodium Dodecyl Sulphate)

(MW: 288.38)

Weigh 20g and dissolve in 80 ml distilled water. It will be necessary to heat the solution to 68°C to assist in dissolution. Adjust the volume to 100ml. Filter sterilize, do not autoclave.

1X Stripping Buffer

For 500ml:

1. Tris HCl (pH 6.7)

   Take 12.5 ml of 2.5M Tris HCl (pH 6.7)

2. Beta- Mercaptoethanol

   Take 3.5 ml of BME liquid with density 1.11

3. Sodium Dodecyl Sulphate
   50µl of 20% SDS

10X Transfer Buffer

Weigh 30g Tris base and 144g Glycine. Dissolve in 1 litre distilled water.

1X Transfer Buffer

Dissolve 100 ml 10X Transfer buffer, 200ml Methanol in 700 ml D.D H_2O.

10X Wash Buffer

Weigh 12.11g Tris Base (100mM) of pH 7.5, 58.44g NaCl. Dissolve it in 700ml of D.D H_2O and adjust the pH with HCl and make up the final volume 1000ml.

1X Wash Buffer

Dissolve 100ml of 10X Wash Buffer and make up the volume to 1 litre with distilled water. Add 1ml of Tween-20 (0.1%). Dissolve it properly by shaking.

LIST OF TABLES

LIST OF FIGURES