

## RESEARCH COMMUNICATION

# Therapeutic Profile of T11TS vs. T11TS+MiADMSA: A Hunt for a More Effective Therapeutic Regimen for Arsenic Exposure

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### Abstract

Arsenic exposure is a serious health hazard worldwide. We have previously established that it may result in immune suppression by upregulating Th2 cytokines while downregulating Th1 cytokines and causing lymphocytic death. Treatment modalities for arsenic poisoning have mainly been restricted to the use of chelating agents in the past. Only recently have combination therapies using a chelating agent in conjunction with other compounds such as anti-oxidants, micronutrients and various plant products, been introduced. In the present study, we used T11TS, a novel immune potentiating glycopeptide alone and in combination with the sulfhydryl-containing chelator, mono-iso-amyl-dimercaptosuccinic acid (MiADMSA) as a therapeutic regimen to combat arsenic toxicity in a mouse model. Results indicated that Th1 cytokines such as TNF- $\alpha$ , IFN $\gamma$ , IL12 and the Th2 cytokines such as IL4, IL6, IL10 which were respectively downregulated and upregulated following arsenic induction were more efficiently restored to their near normal levels by T11TS alone in comparison with the combined regimen. Similar results were obtained with the apoptotic proteins studied, FasL, BAX, BCL2 and the caspases 3, 8 and 9, where again T11TS proved more potent than in combination with MiADMSA in preventing lymphocyte death. The results thus indicate that T11TS alone is more efficient in immune re-establishment after arsenic exposures compared to combination therapy with T11TS+MiADMSA.

**Keywords:** Arsenic - carcinogenesis - T11TS - MiADMSA - cytokines - apoptosis

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### Introduction

Arsenic exposure continues to be a worldwide environmental concern and a serious widespread health hazard. Our previous work published in this journal (Acharya et al., 2010) has established the arsenic-induced downregulation of the Th1 cytokines and an upregulation of the Th2 cytokines result in immune suppression. The apoptotic cascades of the lymphocytes are also thrown amuck in the face of arsenic induction. Arsenic-induced carcinogenesis was strongly indicated in this study (Acharya et al., 2010). Exposure to low concentrations of arsenic suppresses the overall innate immune function in zebrafish by effecting declines in interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  mRNA levels (Nayak et al., 2007).

Medical treatment of acute and chronic arsenic toxicity is furnished by sulfhydryl-containing chelating agents like British Anti Lewisite (BAL), sodium 2, 3-dimercaptopropane 1-sulfonate (DMPS), meso 2, 3-dimercaptosuccinic acid (DMSA) etc. which are organic compounds capable of linking together metal ions to form complex ring like structures. Meso-2-3-dimercaptosuccinic

acid (DMSA) has been tried successfully in animals as well as in a few cases of human arsenic and lead poisoning (Kalia and Flora, 2005). Monoesters of DMSA are more effective in the treatment of experimentally induced metal intoxication. Mono-iso-amyl DMSA (MiADMSA) may be a future drug of choice owing to its lipophilic characters and absence of any metal redistribution. Moderate toxicity after repeated administration of MiADMSA may be reversible after the withdrawal of the chelating agents (Kalia and Flora, 2005). The treatment with these chelating agents however is compromised with certain serious drawbacks/ side effects although chelation therapy with sulfhydryl-containing chelating agents is considered to be the best known treatment against arsenic poisoning.

A new and novel trend in chelation therapy has emerged recently which is to use combination therapy instead of mono-therapy with chelating agents. Some workers have shown that supplementation of antioxidants along with a chelating agent prove to be a better treatment regimen (Flora et al., 2007). For example, a study has shown that combined administration of vitamin C plus DMSA and vitamin E plus MiADMSA led to a more

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pronounced depletion of brain arsenic in rat model. Such coadministration was found to be useful in the restoration of altered biochemical variables (particularly the effects on heme biosynthesis and oxidative injury), although its role in depleting the arsenic burden is limited (Kannan and Flora, 2004). Another study however, showed that Se administration during chelation with DMSA had some beneficial effects on oxidative stress but no major additional beneficial effect on arsenic depletion (Modi et al., 2007). These and several other combination therapies have been suggested by workers from time to time to counteract the effects of arsenic toxicity.

Though various kinds of combination therapy have been tried out in the past using chelators, micronutrients, antioxidants and various plant products, a new combination therapy using the arsenic chelator, monoisoamyl dimercaptosuccinic acid (MiADMSA) and immunopotentiator T11TS / SLFA-3 is being proposed in this study for the effective therapeutic remedy in arsenic induced carcinogenesis. T11 target structure (T11TS) (Chaudhuri and Ghosh, 2006) or sheep form of Leucocyte Function Antigen 3 (SLFA-3) (Hunig et al., 1987) derived from sheep red blood cell (SRBC) membrane, has been isolated, purified and characterized in our lab. It has been established in our earlier work that T11TS / SLFA-3 is a potent immune potentiator and has anti neoplastic activity (Sarkar et al., 2004). It also acts as a cytokine modulator (Ghosh et al., 2010), a cell cycle modulator (Acharya et al., 2010), a specific apoptotic inducer (Bhattacharjee et al., 2008), and an anti-angiogenic agent. The present study is novel because it aims to combine the effects of immune potentiation with T11TS with chelation therapy in the eradication of arsenic induced carcinogenesis.

## Materials and Methods

### Animal Grouping

Healthy age matched adult Swiss albino mice were taken for the study. A single control group sufficed for the entire study. The animals were grouped in batches of 6 for each experimental group, which are as follows: I. Normal control (N), II. 4 Month Arsenic-induced animal. III. 4 Month arsenic-induced animal treated with 1<sup>st</sup> dose of T11TS [T1], IV. Treated with 1<sup>st</sup> and 2<sup>nd</sup> dose of T11TS [T2], V. Treated with 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> dose of T11TS [T3]. VI. After an interval of 6 days after 3<sup>rd</sup> dose of T11TS (after 18 days of T11TS treatment), 1<sup>st</sup> dose of miADMSA was administered orally, given rest for 7 days and then 2<sup>nd</sup> course of miADMSA was given [miADMSA+T11TS].

### Arsenic dosimetry

Sodium metaarsenite [NaAsO<sub>2</sub>] (Mol wt. - 129.91) was taken as the source of Arsenic. 50 ppm of Sodium Meta arsenite was administered in drinking water (1 liter water contained 86.72 mg of NaAsO<sub>2</sub>) of the animals after the animals attain adulthood from 2 months of age onwards.

### miADMSA dosing

miADMSA was kindly supplied by Dr SJS Flora, DRDE. 50 mg/kg/4mL was administered orally for 5 days. Rest for 7 days followed by 2<sup>nd</sup> course of miADMSA.

### Isolation of T11TS and administration in animals

T11TS was isolated from sheep erythrocyte (sheep red blood cell) membranes. Briefly, sheep red blood cells were trypsinized, treated with TCA and neutralized. The glycopeptides were separated by ion exchange chromatography on a DEAE-cellulose column and eluted with a five-gradient system. Elute fraction III was selected as the fraction of choice. The first dose of 1 ml of T11TS (0.4 mg/kg body weight) was administered to mice i.p. from the third elute fraction III, which was followed by a second booster dose on the sixth day and a third booster dose on the 12th day, making a dose schedule of 1, 2, and 3 ml to the T1, T2, and T3 animals, respectively (Mukherjee et al., 2002).

### Antibodies

Mice monoclonal antibody specific for IL-4, IL-6, IL-10, IL-12, IFN- $\gamma$ , CD178 (Fas L), Bcl-2 were purchased from BD Biosciences, USA. Mice monoclonal antibody for Bax and TNF- $\alpha$  was purchased from Santacruz Biotechnology, Santacruz, California. Mice monoclonal antibody specific for Caspase-8 raised against epitope mapping at the N terminus caspase 8 p20 subunit of human origin used for detection of the precursor of caspase -8 were purchased from Santacruz Biotechnology, Santacruz, California. Mice monoclonal antibody specific for Caspase-3 raised against epitope corresponding to amino acids 1-277 representing the full-length precursor form of caspase 3 of human origin and rat monoclonal antibody specific for Caspase-9 raised against epitope corresponding to amino acids 315-397 mapping within the C terminus of caspase-9 of human origin used for detection of the precursor of caspase-9 were purchased from Santacruz Biotechnology, Santacruz, California. The primary and the secondary antibodies were diluted in PBS/azide (1% FBS 1% sodium azide in PBS) plus 10 % autologous mice serum (Sarkar et al., 2004).

### Labeling of cells and flow cytometric analysis

Single color analysis was used for all the antibodies. For the surface antigen CD178, 1x10<sup>6</sup> cells/ml were directly incubated with the primary antibodies for 45 mins at 4°C. The cells were then washed and resuspended in PBS after which they were stained with the secondary antibody FITC, in the next step (30 mins. at 4°C in dark). After washing, cells were fixed with 1ml ice-cold 1% paraformaldehyde in PBS (pH 7.2) overnight at 4°C in dark. For intracellular antigens Bcl-2, Bax, p53, caspase-8, caspases-9 and Caspase-3, 4 batches of 1x10<sup>6</sup> cells/ml were fixed in 0.5 ml of 0.3% ice cold paraformaldehyde in PBS (30 mins at room temperature). Cells were then washed with PBS and permeabilized by being gently resuspended in 1% Triton-X 100 in PBS and incubated at 37°C for 30 mins. The samples were then labeled with the corresponding primary and secondary antibodies and fixed as described above. After overnight incubation, samples were taken out and resuspended in PBS and kept in ice on dark and analyzed within 1 hour. Immunofluorescence was performed in a FACS caliber using Expo 32 software (Beckman Coulter, USA). For each sample, 40,000 events were scored. For surface and intracellular antigen labeling,

the Ig control sample values were subtracted from all other sample values to remove FITC background fluorescence.

### Statistical analysis

The results of flow cytometry were analyzed using t-test for paired observations. The computed t score was then compared with the critical t scores with the same df. The difference between the paired observations was considered significant if the computed t equaled or exceeded the critical t for the chosen level of significance ( $P < \alpha$ ). On the contrary, the difference was considered not significant if the computed t was lower than the critical t for the chosen significance level ( $P > \alpha$ ). All results were evaluated statistically by applying the SPSSPC package (version 9.0, SPSS, Chicago, IL, USA).

## Results

### Cytokine production profile with the therapeutic regimen

Therapeutic scheduling was started in the 4<sup>th</sup> month of arsenic induction. 4<sup>th</sup> month Arsenic-induced animals were taken since carcinogenic changes were evident in various organs like liver, G.I tract and kidney (Acharya et al., 2010). Data for interleukins are shown in Figure 1.

### Th-2 cytokines

Interleukin 4 expression was highest in the 4<sup>th</sup> month of arsenic dosing as compared to the normal group, which was significantly ( $P < 0.0001$ ) reduced with the 1<sup>st</sup> dose of T11TS and further decreased with 2<sup>nd</sup> and 3<sup>rd</sup> dosing. But miADMSA after the 3<sup>rd</sup> T11TS dosing increased the secretion of this immunosuppressive cytokine to a level higher than the 1<sup>st</sup> T11TS dose, though it was significantly ( $P < 0.001$ ) higher than in the arsenic-induced animal.

Interleukin 10, the immunosuppressive cytokine, IL-10 is highly augmented after 4 months of arsenic treatment as compared to normal. The three doses of T11TS cause significant ( $P < 0.0001$ ) reduction of IL-10 expression in a linear fashion. However, miADMSA augmented the reduced IL-10 secretion due to T11TS to a much higher level.

### Interleukin 6

A near 5 fold increase of IL-6 expression occurs after 4 months of arsenic dosing as compared to the finding in normal animals. 3 successive doses with T11TS bring the value down to near normal levels. IL-6 expression however again increases to about twice that of normal values even after miADMSA treatment has been combined with T11TS.

### Th-1 cytokines

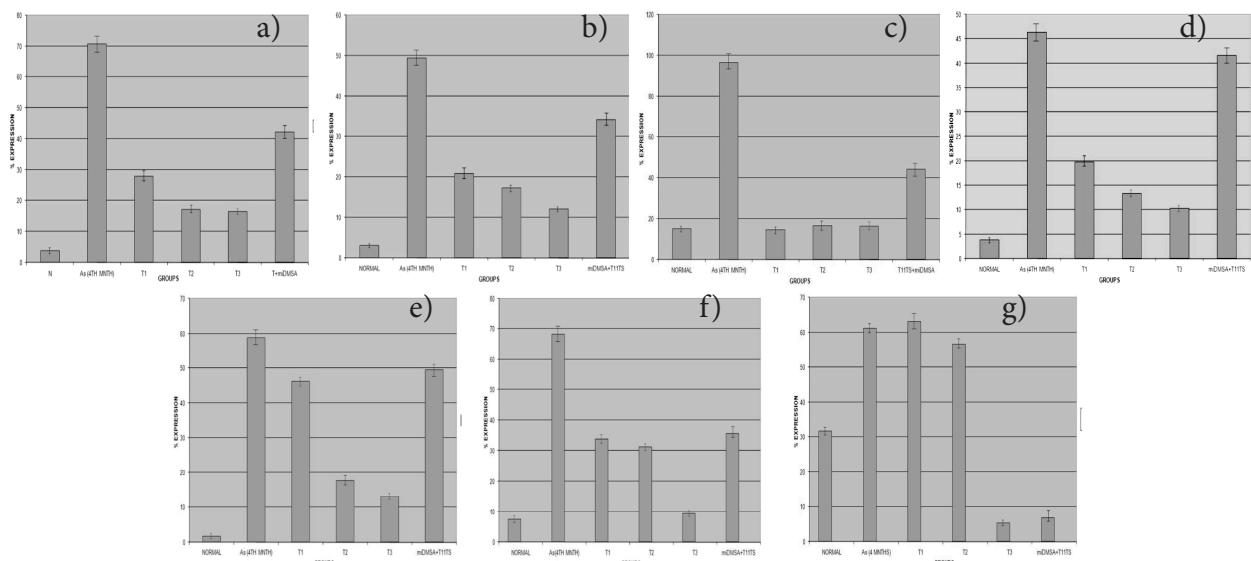
Interleukin 12, 4 months of arsenic treatment causes the expression levels of IL-12 to rise to over 9-fold its normal expression levels: the 1<sup>st</sup> dose of T11TS brings this value down to nearly 4-folds the normal value & by the time the 3<sup>rd</sup> dose has been administered, IL-12 values further fall to only less about 2-fold the normal expression values. This situation is markedly altered by the administration of miADMSA along with T11TS, which again shoots up IL-12 expression to around 8-folds that of normal levels, i.e., to nearly similar levels as arsenic-induced condition.

IFN- $\gamma$ , a near 6-fold increase of IFN- $\gamma$  is seen to occur with arsenic treatment for 4 months compared to normal expression levels. The 3<sup>rd</sup> dose of T11TS brings down these levels to significantly ( $P < 0.0001$ ) low values as compared to arsenic-induced state. miADMSA combination raises this T11TS value to the values comparable to the 1<sup>st</sup> dose of T11TS.

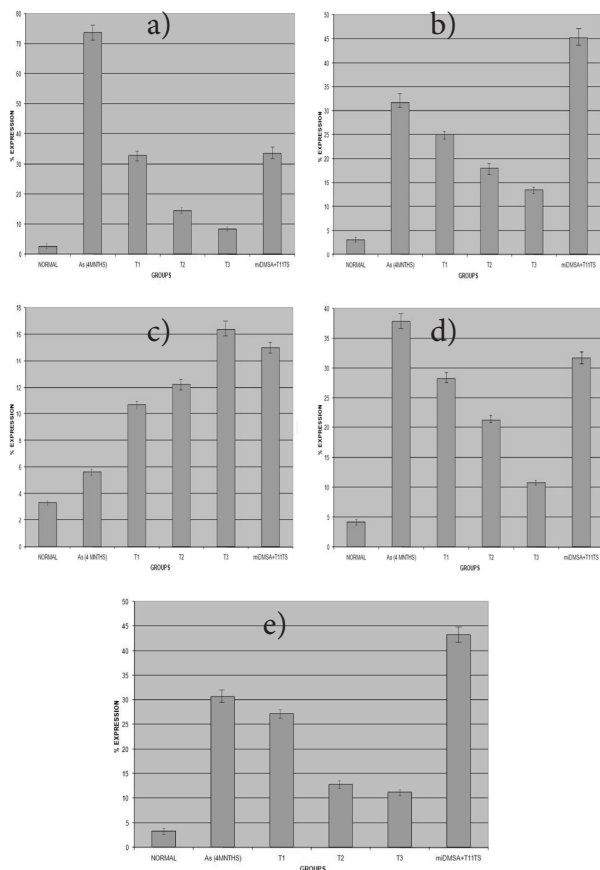
TNF- $\alpha$ , the highly significant ( $P < 0.0001$ ) increase of TNF- $\alpha$  is after 4 months of arsenic dosing as compared to normal is brought down significantly to near normal levels after 3 doses of T11TS. miADMSA combined with T11TS causes expression levels of TNF- $\alpha$  to rise to similar levels as the values obtained after 1<sup>st</sup> dosing with T11TS.

### Analysis of the apoptotic regulators of the peripheral lymphocytes under the therapeutic regimen

Estimation of death proteins of lymphocytes involved in extrinsic and intrinsic apoptotic pathways was conducted. Data are summarized in (Figure 2).



**Figure 1. Comparative Expression of Interleukins in Normal, 4 Month As-Induced, T1, T2, T3 AND T11TS+miADMSA. a) IL-4, b) IL-10, c) IL-6, d) IL-12, e) IFN- $\gamma$ , f) TNF- $\alpha$ , g) Fas-L.**



**Figure 2. Comparative Expression of Caspase 8 in Normal, 4 Month As-Induced, T1, T2, T3 and T11TS+miADMSA.** a) Caspase 8, b) Caspase 3, c) BCL-2, d) BAX, e) Caspase 9.

**Extrinsic.** Fas-L, the 1<sup>st</sup> dose of T11TS causes Fas-L expression levels to rise above that obtained in the 4<sup>th</sup> month of arsenic dosing. However, this level falls to significantly low levels on 3<sup>rd</sup> dosing with T11TS compared to 4<sup>th</sup> month of Arsenic dosing. The decrease in Fas-L expression is also significant for combination therapy with T11TS and miADMSA; however, it is slightly at a higher level than treatment with T11TS alone.

A 73% increase of Caspase 8 compared to normal expression levels noted on the 4<sup>th</sup> month of arsenic treatment falls to about 32% expression with 1<sup>st</sup> dosing of T11TS, which decreases to further lower levels with 2<sup>nd</sup> and 3<sup>rd</sup> doses of T11TS treatment. In the 3<sup>rd</sup> dose it is only 8% while the normal level was 2%. When the combined dose was administered the death protein level showed an increase to about 33%.

Caspase-3, expression of the executioner caspase, i.e. caspase 3 rises sharply with arsenic dosing indicating death of lymphocytes. This increase gradually subsides with the three consecutive doses of T11TS though the level remains higher than the normal level. But with the combination therapy with miADMSA and T11TS this level very significantly surpasses even the level of 4<sup>th</sup> month Arsenic.

**Intrinsic.** Bcl-2, the anti apoptotic protein Bcl2 increases to some extent with arsenic dosing as compared to the normal but the level sharply rises with T11TS dosing with a 14 fold increase in the 3<sup>rd</sup> dose and with

the combination therapy there is a slight decrease in the Bcl-2 level than that of the 3<sup>rd</sup> dose T11TS .

Bax, death of lymphocytes is eminent in the Arsenic group where 38% Bax expression is seen as compared to the normal level 4.5%. This level of pro- apoptotic protein gradually decreases with T11TS administration until a level of about 11% is noted with the 3<sup>rd</sup> dosing of T11TS. Combination therapy with miADMSA+ T11TS causes Bax levels to rise above the T11TS induced levels to about 32%.

Caspase 9, with 4 months of Arsenic dosing, Caspase 9 expression levels rise to 31% compared to the normal expression levels of around 3%. T11TS dosing results in a gradual fall from 27% with 1<sup>st</sup> dosing to around 12% with the 3<sup>rd</sup> dose. Combination therapy with miADMSA along with T11TS dosing overshoots the arsenic-induced levels to around 43%

## Discussion

A counter-regulatory balance is maintained by the Th-1 & Th-2 cytokines. The Th-2 cytokines like IL-4 & 10 are immunosuppressive in nature (Penn, 1994; Liotta and Kohn, 2001; Dranoff, 2004). The highly immunosuppressive milieu obtained by the large amount of IL-10 secretion (50%) in Arsenic-induced neoplastic conditions is controlled to a large extent (12%) after the 3<sup>rd</sup> dose of T11TS. But this level is raised to about 33% in the combination therapy. Nearly similar results are obtained with IL-4, the 70% increase with arsenic treatment showed a 17% level with T11TS treatment & a 42% level with combination therapy (T11TS+MiADMSA). Though T11TS treatment combats the immunosuppressive nature of these two cytokines by significant suppression of their expression levels, MiADMSA even under this condition, when administered after T11TS therapy raises the levels of these cytokines to a high extent, though it is still lower than the arsenic group.

The other Th2 cytokine IL6, which is a pleiotropic cytokine showed a 95% secretion in arsenic dosing as compared to a normal dosing of 15%. IL6 influences antigen-specific immune responses and inflammatory reactions (Penn, 1994). It is one of the major physiological mediators of reaction, which is induced in mice exposed to arsenic which develop neoplasia. IL6 has been shown to be an autocrine modulator of growth for in vitro cervical tumor cell growth. IL6 at concentrations of only 0.002 ng/mL is one of the major autocrine growth modulator for many human myelomas. IL6 may function also as an autocrine growth modulator for other tumor types, some of which have been found to secrete IL6 constitutively (Paul et al., 1989; Hilbert et al., 1995). All 3 doses of T11TS ties down this cytokine back to the normal level but MiADMSA along with T11TS raises the value to above 40%. So inflammatory conditions which are also indicative of the carcinogenic condition is reduced with T11TS, but are heightened with the combination of MiADMSA, indicating that T11TS alone is more effective than the combined regimen is protective in the immunological perspective of arsenic induced cancers.

In peripheral lymphocytes of the Th1 T-helper cell

type IL12 induces the synthesis of IFN-gamma and IL2, and TNF  $\alpha$ . TNF-alpha also appears to be involved in mediating the effects of IL12 on natural killer cells since the effects of IL12 are inhibited by an antibody directed against TNF-alpha (Sabel et al., 2007). IL12 and TNF-alpha are costimulators for IFN-gamma production with IL12 maximizing the IFN-gamma response; the production of IL12, TNF, and IFN-gamma is inhibited by IL10. In Th2 T-helper cells IL12 reduces the synthesis of IL4, IL5, and IL10 (Dranoff et al., 2002). The T11TS-induced fall in IL-12 expression levels points towards a progress towards cessation of the inflammatory condition brought about 4 months of arsenic dosing. Concurrent falls in the expression levels of IFN-gamma as well as TNF-alpha also points towards a reversion of the inflammatory condition. MiADMSA administered after the three doses of T11TS have been applied brings the levels of all three Th-1 cytokines to levels higher than those obtained with T11TS dosing, hinting at the re-establishment of the inflammatory state prompting carcinogenic invasion. This further elaborates that T11TS alone is more potent than T11TS+ MiADMSA in overcoming the inflammatory condition brought about by arsenic.

In the arsenic-induced state, lymphocytic death has been strongly indicated (Acharya et al., 2010). High levels of FasL and caspase 8 obtained in the 4<sup>th</sup> month of Arsenic induction are consistent with this finding. The cysteine protease, caspase 8 overshoots normal expression levels by over 70%. The soluble form of the Fas ligand has been known to actively trigger apoptosis by binding to Fas (Debatin et al., 2004; Schnitz et al., 2006). Although the 1<sup>st</sup> two doses of T11TS still keep up the high levels of Fas L expression, the 3<sup>rd</sup> dose brings it down to sub-normal levels. Combination therapy with T11TS and MiADMSA also maintain the decline of Fas-L expression below normal levels. This finding coupled with the successive lowering of Caspase 8 levels with the 3 consecutive doses of T11TS leads us to believe that T11TS is strongly abrogating lymphocytic death along the extrinsic proteolytic cascade of apoptosis. MiADMSA coupled with T11TS therapy raises the levels obtained with the 3<sup>rd</sup> dosing of T11TS but still maintains levels obtained with the 1<sup>st</sup> dose of T11TS.

The induction of anti-Fas mediated apoptosis appears to be correlated with BCL2 mRNA down-regulation. The overexpression of BCL2 prevents death by apoptosis of B-cells and T-cells and counteracts apoptotic mechanisms elicited by cross-linking of the APO-1 cell surface protein (Saini and Walker, 1998; Borner et al., 2003). The high expression levels of Bcl2 obtained after 3 successive doses of T11TS alongwith the downregulation of Fas-L corroborates the fact that T11TS is pro-lymphocytic survival. MiADMSA on combination with T11TS therapy also keeps up the high expression levels of Bcl-2, though to a lesser extent than with T11TS alone.

Overexpression of BAX per se can accelerate apoptosis and counters the death repressor activity of BCL2 and BCLxL (Finucane et al 1999; Antonsson et al., 2000). The pro-apoptotic Bax whose expression levels had risen to 37% after 4 months of arsenic induction showed gradually declining levels with 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> dosing of T11TS and

after the 3<sup>rd</sup> dose, values were about 11% compared to the 4% normal expression levels. MiADMSA in combination with T11TS again pushed up Bax expression levels to 32%, indicating apoptotic milieu for lymphocytes.

Caspase 9 and the executioner caspase 3 both record high expression levels with arsenic induction, which were brought down significantly by T11TS dosing, hinting effectively at the role of T11TS in preventing lymphocytic apoptosis. However, in both the cases of caspase 9 and also for the executioner caspase 3, expression levels significantly overshoot even the arsenic induced expression levels of these caspases on combination therapy with MiADMSA. These findings strongly suggest that the combined action of MiADMSA masks the anti-apoptotic action of T11TS and renders a cytotoxic environment for the lymphocytes, pushing them towards apoptotic death.

The experimental approach of the present study indicated that T11TS acts as an immunopotentiator and counteracts lymphocytic death after arsenic induced neoplastic state, but MiADMSA administered after T11TS DOSING reverses the situation ensuing a counter immunosuppressive milieu with Th2 cytokine upregulation and enhancing lymphocytic death. Hence, although MiADMSA is an effective chelator of Arsenic with antioxidant properties, it may not be an ideal candidate for combination therapy with T11TS for Arsenic intoxication. In this regard, the glycopeptide T11TS when administered alone in three consecutive doses, may be more beneficial in reversing the immune suppression induced by arsenic towards a normal state.

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