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Ha, CS
Michalek, JE
Elledge, R
[et al.](#)

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p53-Based Strategy to Reduce Hematological Toxicity of Chemotherapy: A Proof of Principle Study

Chul S. Ha^a, Joel E. Michalek^b, Richard Elledge^c, Kevin R. Kelly^{c,1}, Suthakar Ganapathy^{a,2}, Hang Su^a, Carol A. Jenkins^a, Athanassios Argiris^{c,3}, Ronan Swords^{c,4}, Tony Y. Eng^a, Anand Karnad^c, Richard L. Crownover^a, Gregory P. Swanson^a, Martin Goros^b, Brad H. Pollock^{b,5}, and Zhi-Min Yuan^{a,6}

^aDepartment of Radiation Oncology, 7703 Floyd Curl Drive, University of Texas Health Science Center at San Antonio, TX 78229

^bDepartment of Epidemiology and Biostatistics, 7703 Floyd Curl Drive, University of Texas Health Science Center at San Antonio, TX 78229

^cDivision of Hematology/Oncology, Department of Medicine, 7703 Floyd Curl Drive, University of Texas Health Science Center at San Antonio, TX 78229

Abstract

P53 activation is a primary mechanism underlying pathological responses to DNA-damaging agents such as chemotherapy and radiotherapy. Our recent animal studies showed that low dose arsenic (LDA)-induced transient p53 inhibition selectively protected normal tissues from chemotherapy-induced toxicity.

Study objectives were to: 1) define the lowest safe dose of arsenic trioxide that transiently blocks p53 activation in patients and 2) assess the potential of LDA to decrease hematological toxicity from chemotherapy.

Corresponding Author: Chul S. Ha, M.D., Department of Radiation Oncology, MSC 7889, 7703 Floyd Curl Drive, San Antonio, TX 78229, HAC@UTHSCSA.EDU, Phone: (210) 450-5566, Fax: (210) 450-0413.

¹Current address: Jane Anne Nohl Division of Hematology, Keck School of Medicine of University of Southern California, 1975 Zonal Ave., Los Angeles, CA 90030

²Current address: Center for Drug Discovery, Northeastern University, 360 Huntington Ave., Boston, MA 02115

³Current address: Hygeia Hospital, 5 Erythrou Stavrou, 6th Floor, Office 6.12, Marousi 15123, Athens, Greece

⁴Current address: Leukemia Service, Sylvester Comprehensive Cancer Center, Division of Hematology/Oncology, University of Miami Miller School of Medicine, 1600 NW 10th Ave., Miami, FL 33136

⁵Current address: Department of Public Health Sciences, School of Medicine, University of California-Davis, One Shields Ave., Davis, CA 95616

⁶Current address: Department of Genetics and Complex Diseases, Harvard School of Public Health, 677 Huntington Ave., Boston, MA 02115

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Patients scheduled to receive minimum 4 cycles of myelosuppressive chemotherapy were eligible. For objective 1, dose escalation of LDA started at 0.005mg/kg/day for 3 days. This dose satisfied objective 1 and was administered before chemotherapy cycles 2, 4 and 6 for objective 2. P53 level in peripheral lymphocytes was measured on day 1 of each cycle by ELISA assay. Chemotherapy cycles 1, 3, and 5 served as the baseline for the subsequent cycles of 2, 4 and 6 respectively. If p53 level for the subsequent cycle was lower (or higher) than the baseline cycle, p53 was defined as “suppressed” (or “activated”) for the pair of cycles. Repeated measures linear models of CBC in terms of day, cycle, p53 activity and interaction terms were used. Twenty-six patients treated with 3 week cycle regimens form the base of analyses. The mean white blood cell, hemoglobin and absolute neutrophil counts were significantly higher in the “suppressed” relative to the “activated” group.

These data support the proof of principle that suppression of p53 could lead to protection of bone marrow in patients receiving chemotherapy.

Keywords

p53; arsenic; myelosuppression; hematological toxicity; chemoprotection

1. Introduction

One of the major pathways by which DNA damaging radiation therapy or chemotherapy causes toxicity in normal tissues is the activation of p53, which induces cascade of events that eventually leads to cell senescence or cell death (Baskar et al., 2012; Junttila and Evan, 2009). We have recently demonstrated that very LDA, by temporarily and reversibly suppressing p53 activation at the time of treatment with radiation or chemotherapy, reduces the normal tissue toxicity without compromising tumor response to treatment (Ganapathy et al., 2014a; Ganapathy et al., 2014b). Our *in vitro* and *in vivo* studies have shown that pretreatment of untransformed cells with LDA induces concerted p53 suppression and NF- κ B activation, which elicit a marked induction of glycolysis. This metabolic shift provides cells with effective protection against cytotoxic radiation or chemotherapy, coupling the metabolic pathway to cellular resistance. The selective protection of normal tissues is possible because this strategy requires normal functioning p53 (Ganapathy et al., 2014a; Ganapathy et al., 2014b). Essentially all of the cancer cells have either mutated or dysfunctional p53 and therefore are not protected (Junttila and Evan, 2009). Importantly, it has been demonstrated that DNA damaging agent-induced p53 activity is inconsequential to the tumor suppressor function of p53, negating the concern that suppression of p53, even though temporary, could contribute to tumor development or progression (Christophorou et al., 2005; Christophorou et al., 2006). The other concern about using LDA is possible tumorigenesis as arsenic is a known carcinogen. However, epidemiological data suggest that certain cumulative threshold doses need to be reached for carcinogenic effect over a long period of time, whereas we use LDA only for a very short period of time and have not observed any secondary malignancy with LDA in our mouse model (Ganapathy et al., 2014a; Snow et al., 2005). It has been well documented *in vitro* that arsenic has different biological effects and expresses different sets of genes depending on the dose, supporting our strategy (Andrew et al., 2003).

We have taken this strategy from our preclinical studies to the current clinical trial. The primary objectives of this trial were: 1) to define the lowest safe dose of arsenic trioxide that blocks p53 activity and 2) to assess the activity of arsenic trioxide in decreasing hematological toxicity in patients receiving myelosuppressive chemotherapy as measured by CBC. For the primary objective 1, p53 activity in patients' peripheral lymphocytes served as a surrogate marker for patients' normal tissue p53 activity. The basal p53 activity in lymphocytes of healthy individuals is usually very low (Salazar et al., 2004). p53 activity can be induced, however, by treating freshly isolated lymphocytes from healthy individuals with 2 Gy of radiation. We were to define the lowest safe dose of arsenic trioxide administered to patients that suppresses this p53 activity induced by radiation *ex-vivo*. Patients who were to be treated with at least 4 cycles of myelosuppressive chemotherapy were pretreated with this dose of arsenic trioxide before chemotherapy cycles 2, 4 and 6 (if applicable) but not cycles 1, 3 and 5 (if applicable). Blood counts (WBC, ANC, HgB, and platelet) from cycles 2, 4, and 6 were to be compared to those from cycles 1, 3 and 5 for the primary objective 2. The secondary objectives were to correlate p53 activity with blood arsenic concentration and to monitor the toxicity profile of arsenic trioxide at the low doses used in this trial.

2. Patients and Methods

2.1. Patient Eligibility

The protocol was approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio and all patients provided signed consent forms. Patients 18 years of age with a cancer diagnosis (excluding leukemia) who were to undergo myelosuppressive chemotherapy were eligible. The required interval between each cycle of chemotherapy was a minimum of 2 weeks. The minimum required number of planned chemotherapy cycles was 4. Radiation therapy during chemotherapy was allowed as long as less than 10% of the total bone marrow was radiated. Previous treatment, if any, should have been completed at least 2 weeks prior to registration to the study. Other eligibility criteria included an ECOG performance status 2, an expected life expectancy greater than 6 months and good organ function. Patients were excluded for pregnancy, HIV infection, or if they had circulating tumor cells in the peripheral blood. Once the patients agreed to participate in the protocol by signing the consent form, their peripheral blood was collected and lymphocytes were isolated. The patients were required not to have baseline p53 activation in peripheral lymphocytes in culture but p53 activation had to be inducible in culture upon 2 Gy of radiation to proceed with the rest of the protocol.

2.2. Study Design and Treatment

This study had two stages as summarized in Figure 1. The goal of the first stage was to determine the lowest safe dose of arsenic trioxide that suppresses p53 activation as measured by western blot of patients' lymphocytes that were radiated *ex-vivo*, as described below.

The first cohort of 5 patients was treated with arsenic trioxide at the starting dose of 0.005 mg/kg intravenously for 3 consecutive days. This starting dose was determined based on a preclinical animal model (Ganapathy et al., 2014a; Ganapathy et al., 2014b). Dose escalation

was to proceed in cohorts of 5 patients each to 0.01 mg/kg, 0.02 mg/kg, 0.04 mg/kg and so on. The patients whose lymphocyte p53 activation was suppressed by the *in vitro* assay were to receive the same dose of arsenic on days -3, -2 and -1 before chemotherapy cycles 2, 4 and 6 (if applicable) as described in the second stage of the study. The patients whose lymphocyte p53 activation was not suppressed by the *in vitro* assay were not eligible to receive the dose of arsenic before chemotherapy and did not count toward the final accrual goal of 32 evaluable patients for this study. They were, however, counted as a part of the dose-defining study. If more than 2 patients in the cohort had elevated lymphocyte p53 activity prior to *ex-vivo* radiation, dose escalation was not to proceed since the corresponding dose of arsenic trioxide was considered too toxic to be used as a chemotherapy protector. Once the lowest dose of arsenic trioxide that could safely suppress the radiation induced p53 activation *in vitro* was defined, the second stage of the study was to accrue patients at this dose until a total of 32 evaluable patients were accrued.

When the lowest safe dose of arsenic trioxide was being determined during the first stage of the study, arsenic trioxide was administered on days -3, -2, and -1. The first day after the last dose of arsenic was counted as day 1. An electrocardiogram was performed on days -3, -2 and -1. Peripheral blood was collected for measurement of arsenic concentration and p53 expression on days 2, 4, 6, 8, 10 and 12. The patients did not receive any chemotherapy during this period while their p53 status was followed in the first stage of the study. This was to allow monitoring of p53 status without potential interference from chemotherapy. Chemotherapy was started after this period for these patients.

Once the lowest safe dose of arsenic trioxide was defined, the second stage of the protocol started as follows. Patients were treated with this dose on days -3, -2, and -1 prior to chemotherapy cycles 2, 4 and 6 (if applicable). The first day of chemotherapy was defined as day 1. Chemotherapy cycles 1, 3 and 5 (if applicable) were administered without pretreatment with arsenic trioxide. CBC with differential was measured on days 1, 8, 15 (if applicable) and 22 (if applicable) of chemotherapy. The peripheral blood for day 1 was obtained before chemotherapy was started. This was to ensure the blood counts from this day could serve as the baseline for the rest of the cycle. Ten ml of peripheral blood was collected on days 1, 2, 5, 8 and 15 (if applicable) of chemotherapy for the measurement of p53 expression. Patients were to be removed from study for grade 3 or 4 toxicities considered attributable to arsenic trioxide administration. For the first stage of the study, a western blot was used to assess the expression of p53. The first five patients who were enrolled for the dose escalation study moved on to the second stage of the study and continued to have their p53 measurement performed by western blot. However, upon completion of the dose defining stage of the study, it was decided to use ELISA assay to better quantify the expression of p53 the rest of the study. Arsenic concentration was measured using atomic absorption spectroscopy as described below.

There was no restriction in the standard of care for the patients due to participation on this study. Specifically there was no restriction in the use of growth factors, red blood cell or platelet transfusions.

2.3. *In-vitro* and *ex-vivo* experiments

Lymphocyte Isolation—Lymphocytes were isolated from 8-10 mL of freshly drawn blood from patients by Ficoll-Paque PLUS (Amersham Pharmacia, Piscataway, NJ) gradient centrifugation according to the product instruction as previously described (Colognato et al., 2008).

Detection of p53 activity—An X-ray (2 Gy) treatment or a sham treatment was given to either dish of the isolated lymphocytes using the Faxitron X-ray System (Faxitron XRay Corporation, Buffalo Grove, IL). The activity of p53 was examined by western blotting and ELISA 3 hours after radiation. Western blotting was performed as previously described (Ganapathy et al., 2014b). For ELISA, the activity of p53 was detected using a commercial PathScan® Phospho-p53 Sandwich ELISA kit (Cell Signaling Technology, Beverly, MA) and following the procedure as described in the user's manual. The p53 level before 2 Gy of radiation was used as a control for the measurement of p53 activity level at each time point. For positive control for p53 activation after 2 Gy of radiation, commercially obtained GM03798 cells (Coriell Institute, Cat# NA03798DNA) were used.

Measurement of arsenic level—Arsenic was measured by using atomic absorption spectrophotometer according to the manufacturer's instruction (model 210 VGP, Buck scientific Co, CT, USA).

2.4. Statistical Methods

2.4.1 Primary objective 1 to define the lowest safe dose of arsenic trioxide that blocks p53 activity—We were to use a 5+5 Fibonacci design and determine the dose at which the peripheral lymphocytes from all subjects in the cohort of 5 from the first stage of the study exhibit p53 inactivation after treatment in culture with 2 Gy of radiation. If more than 2 patients in the cohort had elevated p53 activity in lymphocytes without treatment with 2 Gy of radiation in culture, the dose escalation was to be stopped and the regimen was to be deemed too toxic.

2.4.2 Primary objective 2 to assess the activity of arsenic trioxide in decreasing hematological toxicity in patients receiving chemotherapy as measured by complete blood counts—We assessed complete blood count parameters (WBC, ANC, platelet and HgB) as primary outcome (dependent) variables; each was modeled separately. We used repeated measures linear models to address the “ABABAB” design where the “A” cycles serve as the baseline (non-arsenic) for the subsequent “B” cycle treatment (arsenic). We assessed the significance of the association between categorical outcomes with Fisher's Exact test. We summarized blood counts in log base 10 with box and whisker plots by cycle, day of the cycle, and p53 expression (activated, suppressed); boxes are determined by quartiles and whiskers extend to 1.5 times the interquartile range; values beyond the whiskers are indicated with dots. The significance of the relation between blood counts was assessed with repeated measures linear models of blood count in terms of p53 expression, day of the cycle, and cycle and pairwise interactions with an autoregressive order 1 autocorrelation matrix. We report least square means and standard errors for the main effect of arsenic.

2.4.3 The secondary objectives to correlate p53 activity with blood arsenic concentration and to monitor the toxicity profile of arsenic trioxide at the low doses used in this trial—The relationship between arsenic concentration and p53 activity was to be assessed with appropriate scaling (e.g. transformations).

All statistical testing was two sided with a significance level of 5% and SAS Version 9.4 for Windows (SAS Institute, Cary, North Carolina) was used for analysis and R was used for graphics.

3. Results

A total of 50 patients were accrued between April 2011 and November 2012. Ten of them were not eligible for the study due to elevated p53 expression at baseline. Five patients were not evaluable for the following reasons: a) withdrawal after cycle 1 (n=1); b) inability to start cycle 2 due to hospitalization (n=1); c) inability to start chemotherapy due to socioeconomic reasons (n=1); d) lack of certainty about starting chemotherapy (n=1); e) withdrawal after cycle 1 day 2 (n=1). The remaining 35 patients had at least two cycles of chemotherapy. The first 5 patients who were on the dose escalation phase of the study and moved on to receive chemotherapy had their p53 expression levels analyzed by western blot as described above. These patients' CBC data were not used to correlate with p53 suppression or activation due to difficulty in quantifying p53 expression levels. Of the remaining 30 patients, 26 patients completed 3 week cycle regimens of chemotherapy, 3 patients 4 week cycle regimens and 1 patient 2 week cycle regimen. Given a very small number of patients enrolled with 2 or 4 week regimens and the different patterns of nadirs for blood counts depending on the length of each cycle, we decided to limit our analyses to the 26 patients treated on 3 week regimens. Figure 2 is the CONSORT diagram for the study.

The chemotherapy regimens, all with a 3 week cycle, used for these 26 patients were; rituximab, doxorubicin, cyclophosphamide, vincristine, prednisone (Rummel et al., 2013) (n=1), docetaxel, cyclophosphamide (Jones et al., 2009) (n=7), doxorubicin, cyclophosphamide (Jones et al., 2009) (n=7), carboplatin, bevacizumab, premetrexed (Patel et al., 2009) (n=2), bortezomib, lenalidomide, dexamethasone (Richardson et al., 2010) (n=1), docetaxel, prednisone (Tannock et al., 2004) (n=5), doxorubicin, vincristine, etoposide, ifosfamide (Wexler et al., 1996) (n=1), carboplatin, etoposide (Klastersky et al., 1990) (n=1), cisplatin, etoposide (Klastersky et al., 1990) (n=1).

One patient was also treated with radiation therapy in the forms of radiosurgery while on this study for metastatic lung cancer in the brain.

3.1. Safety

There were no adverse events attributable to arsenic trioxide and therefore no patient was removed from the study due to toxicity. There were no clinically significant changes in electrocardiograms, including changes of the QT interval, in any subject.

3.2. Dose Escalation

3.2.1 Primary objective 1 to define the lowest safe dose of arsenic trioxide that blocks p53 activity—The first 5 patients accrued for the first stage of the study had suppression of p53 activation on days 2 and 4 at the starting dose of 0.005 mg/kg for 3 consecutive days. This suppression was temporary and reversed after day 4 or 5 for all 5 patients. The western blot data for the first patient accrued for the dose defining phase of the trial are presented in Figure 3. At the baseline, this patient's p53 is activated upon 2 Gy of radiation to his peripheral lymphocytes ex-vivo. However, p53 activation is suppressed on days 2, 4, and 5 in spite of 2 Gy of radiation ex-vivo. All of these 5 patients stayed on the study and received this dose of arsenic trioxide on days -3, -2 and -1 prior to chemotherapy cycles 2, 4 and 6. As none of these patients experienced any arsenic trioxide related toxicity, this dose of arsenic trioxide was determined to be the lowest safe dose of arsenic that suppressed the activation of p53 and used to treat the patients the rest of the study.

3.3 Efficacy

3.3.1 Primary objective 2 to assess the activity of arsenic trioxide in decreasing hematological toxicity in patients receiving chemotherapy as measured by complete blood counts—When the p53 activities (expression levels) measured by ELISA assay were plotted as a function of cycles, we did not observe a clear period of p53 inactivation during the first few days of cycles 2, 4 and 6 as would have been expected from the *in-vivo* model and from the first stage of the study. Because the competing and compounding effects of multi-drug chemotherapy on p53 were felt to affect the p53 activity even in the presence of LDA pretreatment, we decided to focus on the p53 activity obtained on day 1 of chemotherapy immediately before administration of chemotherapy. As described in the statistics section, cycles 1, 3 and 5 served as the baseline for the subsequent cycles of 2, 4 and 6 respectively. If the p53 activity level for the subsequent cycle was lower than the baseline cycle (e.g. day 1 of cycle 2 for day 1 of cycle 1 and day 1 of cycle 4 for day 1 of cycle 3), the patient's p53 was defined as “suppressed” for the pair of cycles. If the p53 activity level for the subsequent cycle was higher than the baseline cycle, p53 was defined as “activated” for the pair of cycles. Therefore a patient's p53 could be “suppressed” between the cycles 1 and 2 but “activated” between cycles 3 and 4.

Table 1 summarizes the baseline patient characteristics (n=26). The average age was 52.7 ± 11.3 , a majority was female (65.4%), breast cancer (53.9%) was the predominant diagnosis, and 46% had previously received chemotherapy and/or radiation therapy.

The repeated measures analyses of variation in ANC, WBC, HgB and platelets were carried out for the 26 patients. Among them, six patients were treated with a total of 19 doses of filgrastim or pegfilgrastim. While one patient received a packed red blood cell transfusion once, no one received platelet transfusion. WBC and ANC measured after filgrastim or pegfilgrastim administration and HgB after packed red blood cell transfusion were not used for the rest of the cycle for our data analyses. Of note, sample sizes are summarized by cycle in Table 2. The repeated measures modeling across all six cycles was prevented due to small or zero data counts in cycles 5 and 6. Therefore all modeling was carried out with restriction

to cycles 1, 2, 3, and 4 (Figure 4). As mentioned above, one needs to keep in mind for figure 4 that the designation of “activated” and “suppressed” status is specific for the pairs of the cycles (i.e. between cycles 1 and 2 and between cycles 3 and 4) for the patients and may not necessarily stay the same throughout the entire 4 cycles. The mean WBC, HgB and ANC were significantly higher in the suppressed group relative to the activated group (Table 3).

3.3.2 The secondary objectives to correlate p53 activity with blood arsenic concentration—The arsenic could not be detected from the patients' blood in any of the time points collected since its concentration was under the detection limit of the atomic absorption spectroscopy. Therefore we could not perform the studies to correlate arsenic concentration in blood with p53 activity.

3.3.3 The secondary objectives to monitor the toxicity profile of arsenic trioxide at the low doses used in this trial—There were no adverse events attributable to arsenic trioxide. There was no significant change in electrocardiogram including change of the QT interval.

4. Discussion

To date, very few limited options exist to protect normal tissues during chemotherapy or radiation. To this end, Amisfostine, basically a reactive oxygen species scavenger, was previously investigated in clinical studies. Its widespread use has been difficult, however, due to somewhat unfavorable risk/benefit ratio (Eisbruch, 2011; Zhou and Bartek, 2004). The cytoprotective effect from mesna comes from reaction with urotoxic ifosphamide metabolites and is limited to ifosphamide-induced cystitis (Hensley et al., 2009). The application of palifermin and dexrazoxane has been also extremely limited to very specific situations (Hensley et al., 2009). Although the therapeutic use of p53 inhibitors such as pifithrin- α has been proposed (Gudkov and Komarova, 2005, 2007), no clinical trial testing the use of p53 inhibitors as a cytoprotector from chemotherapy or radiation therapy has been conducted. To the best of our knowledge our current trial is the first to investigate a p53 inhibitor for such purpose. Our approach utilizing LDA takes advantage of p53 inhibition, but it is different from pifithrin- α in regard to the mechanism of action because it induces activation of NF- κ B and metabolic shift to glycolysis, thus enhancing survival potential in normal cells. P53 is at a resting state under non-stress condition in the general population (Salazar et al., 2004), but it is activated by DNA damaging agents such as radiation or chemotherapy. This activation constitutes one of multiple pathways responding to these insults (Gudkov and Komarova, 2007). The time course of p53 activation by different agents, especially by multi-agent chemotherapy, is not well understood. This may explain somewhat higher (10 out of 50 screened patients) than expected (less than 5%) screen failure rate due to baseline p53 activation in peripheral lymphocytes in our patient population, as all of our patients carry the diagnosis of cancer with about half of them having history of previous treatment with chemotherapy or radiation therapy. During the first stage of this clinical trial to determine the lowest safe dose of arsenic trioxide that suppresses p53 activation as measured by western blotting of patients' lymphocytes that were radiated *ex-vivo*, our starting dose of 0.005 mg/kg for 3 consecutive days turned out to suppress p53 activation at least for 4 days in all 5 patients in the first cohort. Though our

demonstrate in the trial the direct relationship between suppression of p53 upon pretreatment with LDA and protection of the normal bone marrow during treatment with radioimmunotherapy

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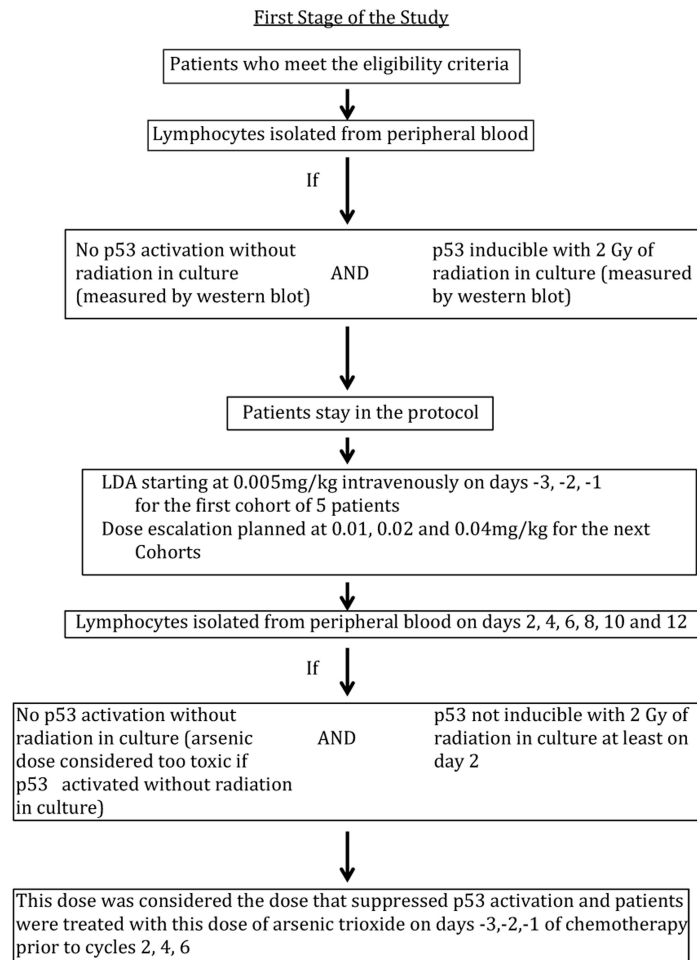
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Abbreviations

LDA	low dose arsenic
CBC	complete blood count
WBC	white blood cell
ANC	absolute neutrophil count
HgB	hemoglobin

P53 activation is a major pathway for pathological response to DNA damaging agents.
This p53 pathway is independent of tumor suppressor pathway of p53.
Suppression of this pathway selectively protects normal tissue, not cancer, in vivo.
We found arsenic dose that temporarily and reversibly suppresses this pathway in man.
Successful suppression of p53 leads to protection of bone marrow from chemotherapy.



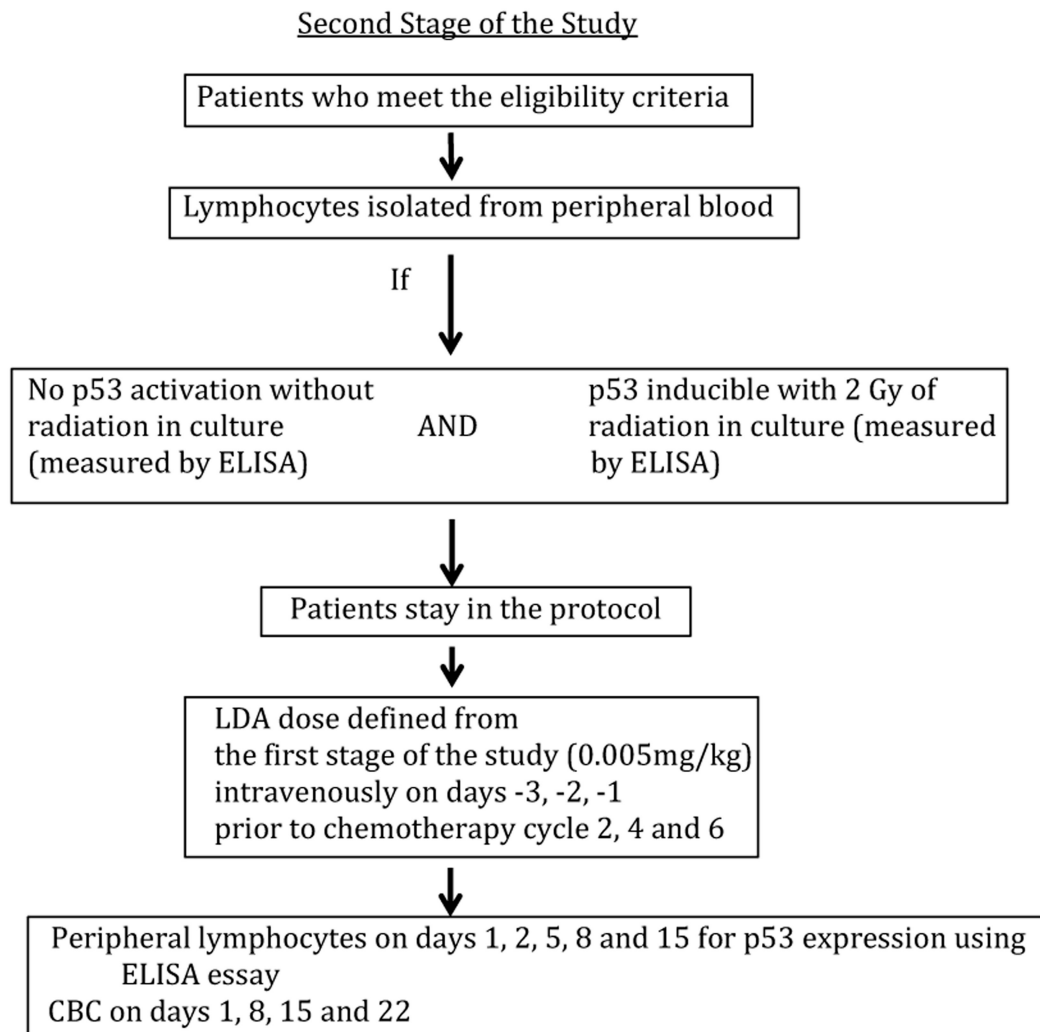


Figure 1. Schematic illustration of the study design

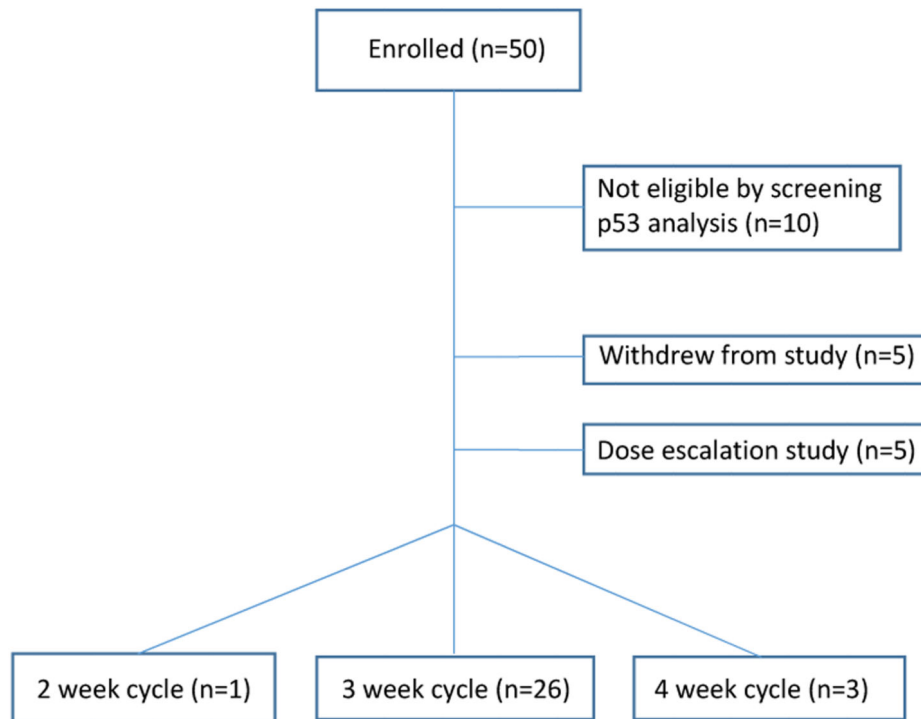


Figure 2. CONSORT diagram for the study

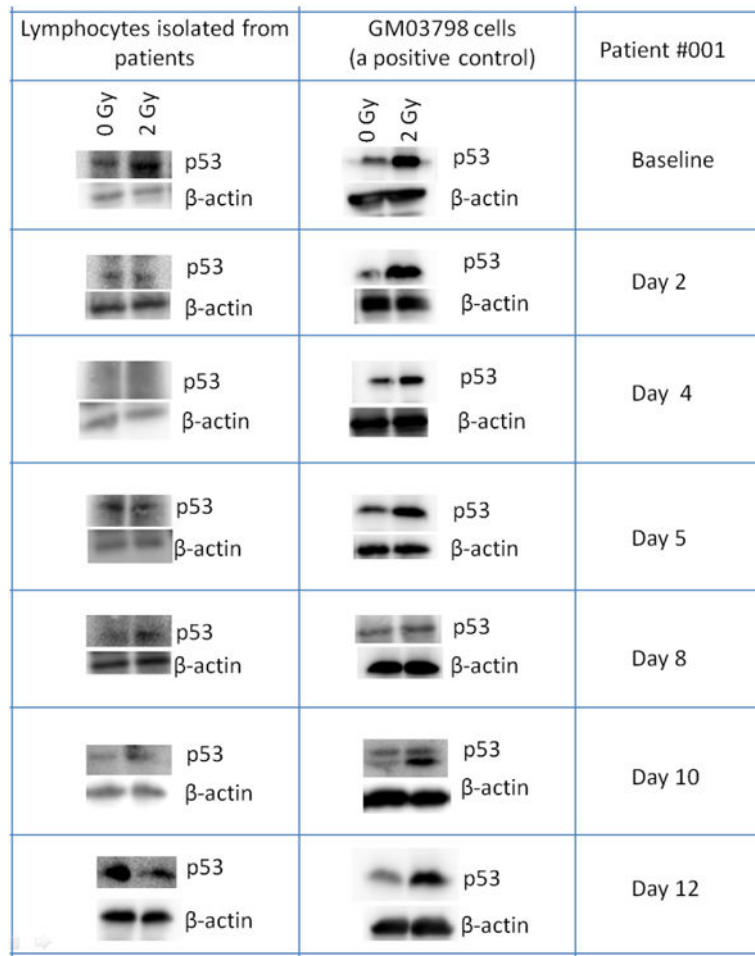
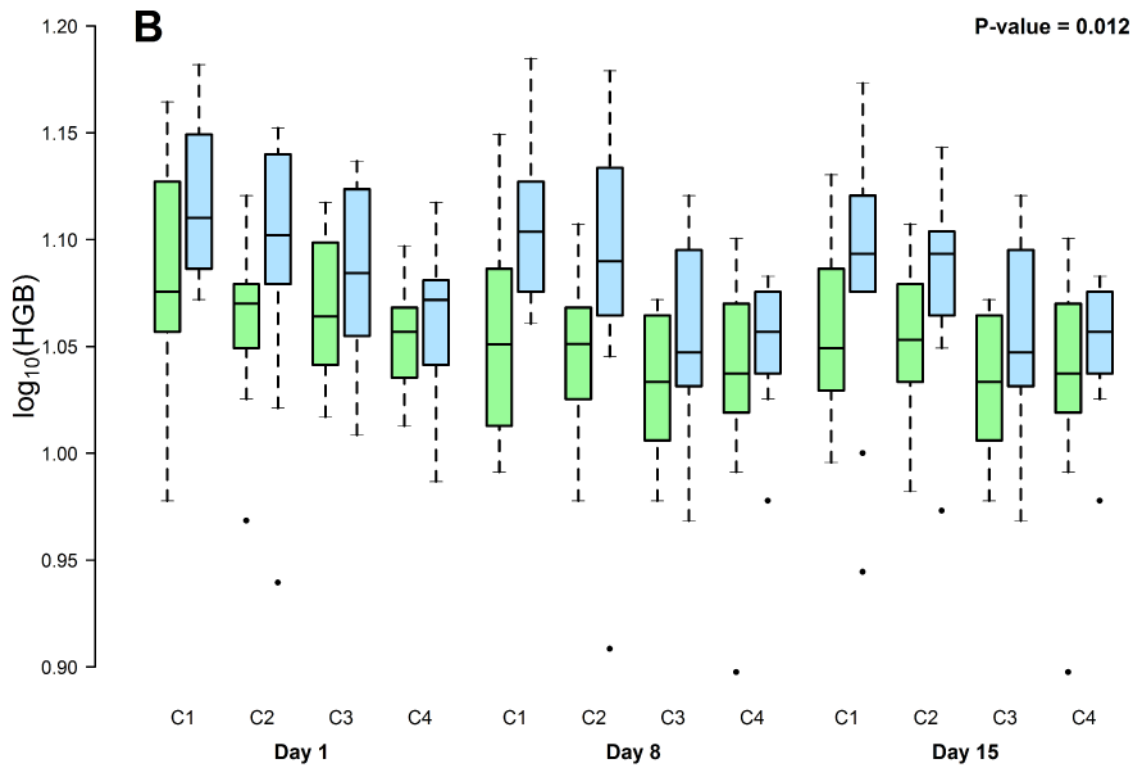
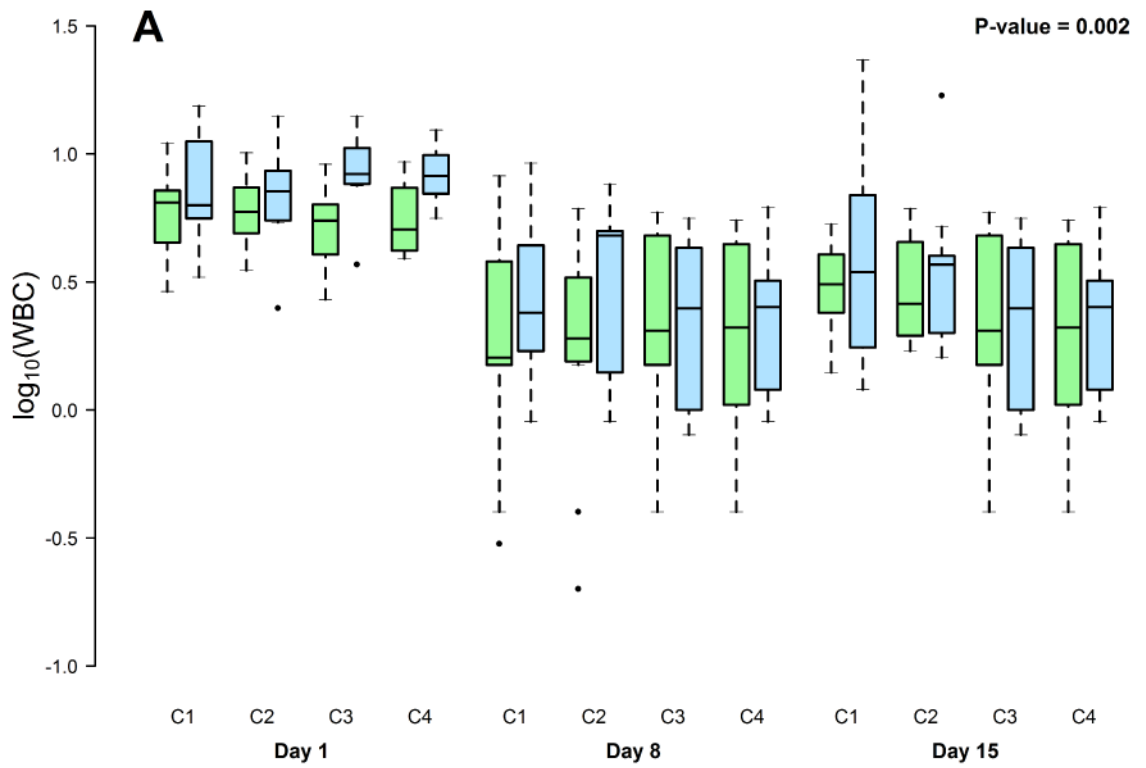


Figure 3. p53 expression level from peripheral lymphocytes measured by western blot for patient #1. This patient's p53 activation is suppressed on days 2, 4 and 5 in spite of 2 Gy of radiation ex-vivo. Beta-actin was used as a loading control. GM03798 cells were used as positive control for p53 expression.



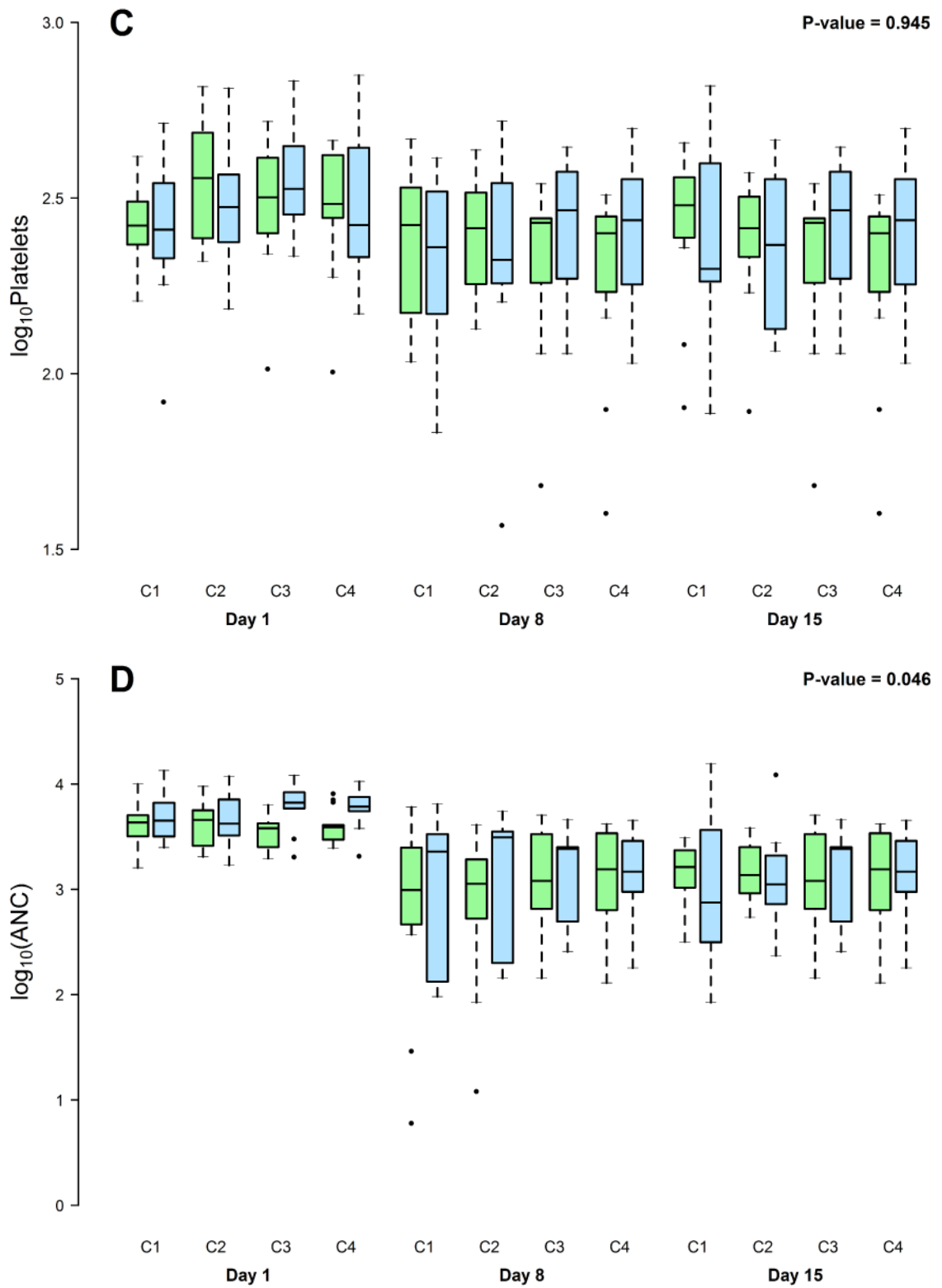


Figure 4.

Blood cell counts in log units by cycle (1, 2, 3, 4), day (1, 8, 15) and p53 expression [green: activated, blue: suppressed]; Panel A: WBC, B: HgB, C: Platelets, D: ANC. P-values indicate the significance of differences in the mean count as a function of p53 expression.

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Table 1
Baseline Patient Characteristics (n=26)

Characteristic	Total (N = 26)
<u>Age (years)</u>	
Mean±SD	52.7±11.3
<u>Gender</u>	
Female	17 (65.4%)
Male	9 (34.6%)
Total	26
<u>Current Diagnoses</u>	
Breast cancer	14 (53.9%)
Diffuse large B cell lymphoma	1 (3.9 %)
Lung cancer	5 (19.2%)
Multiple myeloma	1 (3.9%)
Prostate cancer	4 (15.4%)
Rhabdomyosarcoma	1 (3.9%)
Total	26
<u>Previous Treatments</u>	
Chemotherapy alone	2 (7.7%)
Chemotherapy and radiation therapy	6 (23.1%)
Radiation therapy alone	4 (15.4%)
None	14 (53.9%)
Total	26

Sample sizes by cycle, and P53 expression at day 1 among the 26 patients treated with 3-week cycle chemotherapy regimens. (NA: not available)

Table 2

Cycle	P53 Expression			Total
	Activated	Suppressed	NA	
1	14	11	0	26
2	14	11	0	26
3	13	9	2	25
4	13	9	1	24
5	3	0	4	7
6	3	0	1	4

Table 3
Means by cell count and p53 expression in all subjects treated with 3 week cycle chemotherapy regimens (n=26)

Count		Activated	Suppressed	Contrast
WBC	Mean±SE	1.13±0.055	1.41±0.068	-0.28±0.088
	95% CI			-0.454,-0.106
	p-value			0.002
HgB	Mean±SE	2.43±0.013	2.48±0.015	-0.051 ±0.02
	95% CI			-0.091,-0.012
	p-value			0.012
Platelets	Mean±SE	5.53±0.053	5.53±0.064	0.006±0.084
	95% CI			-0.161,0.172
	p-value			0.945
ANC	Mean±SE	7.36±0.08	7.61±0.1	-0.26±0.13
	95% CI			-0.512,-0.004
	p-value			0.046