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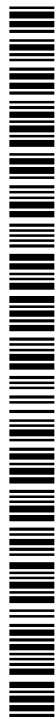
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(54) Title: METHOD FOR INHIBITING CANCER USING ARSENIC TRIOXIDE

(57) Abstract: The invention provides a method for treating cancers that are dependent on cyclin D1 for proliferation, survival, metastasis and differentiation, involving administering a composition containing an effective amount of arsenic trioxide to an affected patient. The arsenic trioxide can be administered orally, for example, as a solution, suspension, syrup, emulsion, tablet, or capsule. The composition can also contain one or more pharmaceutically acceptable carriers and/or excipients.

**METHOD FOR INHIBITING CANCER USING ARSENIC TRIOXIDE****Cross-Reference to Related Applications**

This application claims priority to U.S.S.N. 11/549,347 filed October 13, 2006.

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**Field of Invention**

This invention relates to methods of inhibiting cancer by affecting expression, translation, and biological activity of cancers overexpressing or dependent on cyclin D1 using arsenic trioxide.

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**Background of the Invention**

Mantle cell lymphoma (MCL) is a well-defined subtype of B cell lymphoma in the World Health Organization classification, and accounts for approximately 3-10% of all non-Hodgkin lymphomas. The chromosomal aberration t(11;14)(q13;q32) can be found in practically all cases of MCL. The translocation results in juxtaposition of the immunoglobulin heavy chain joining region on chromosome 14 to the *cyclin D1* gene on chromosome 11. The molecular consequence of the translocation is to place *cyclin D1* under the control of the immunoglobulin heavy chain gene enhancer, leading to over-expression of the cyclin D1 protein.

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Although MCL accounts for approximately 3 – 8% of B-cell lymphomas, it is difficult to manage. Initial treatment with rituximab plus combination chemotherapy or purine analogues results in complete remission (CR) rates varying from 34 – 87%. However, relapses occur in most patients with prolonged follow up. Treatment options for relapsed patients are limited. Several approaches have been adopted, including the use of the proteasome inhibitor bortezomib, thalidomide and the mammalian target of rapamycin (mTOR) inhibitor temsirolimus. The overall response (OR) rates of these agents varied from 38 – 81%, but the CR rate was only 3 – 31%. Therefore, there is an urgent need to define effective treatment strategies for MCL.

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It is an object of this invention to provide agents and methods for treating cancers such as MCL and other cancers over-expressing cyclin D1.

It is another object of this invention to provide methods, strategies, doses, and dosing schedules for the administration of  $As_2O_3$  in the clinical inhibition of cancers over-expressing cyclin D1.

### **Summary of the Invention**

It has been discovered that  $As_2O_3$  suppresses cyclin D1 and initiates down-regulation of cyclin D1 by activating GSK-3 $\beta$ , which phosphorylates cyclin D1. Activation of IKK $\beta$  leads to phosphorylation of cyclin D1, which is ubiquitinated. Ubiquitinated cyclin D1 is degraded in the proteasome. This is the basis for the discovery that MCL and other cancers over-expressing cyclin D1 can be treated with  $As_2O_3$ , preferably oral  $As_2O_3$ .

### **Brief Description of Drawings**

**Figure 1A** is a line graph showing  $As_2O_3$  (concentration in micromolar) percent induced apoptosis in MCL cells, based on a MTT test of Jeko-1 and Granta-519 cells treated for 72 hours with  $As_2O_3$ . There was a dose and time dependent suppression of cellular proliferation. Viability significantly decreased at or above 1  $\mu M$   $As_2O_3$  as compared with baseline (one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ) (triplicate experiments)

**Figure 1B** is a scatter plot of popidium iodide versus annexin expression in cells treated with  $As_2O_3$ . There was a significant increase in apoptotic cells after  $As_2O_3$  treatment. (#: apoptotic cells that were annexin V positive and popidium iodide negative).

**Figures 2A and 2B** show down-regulation of cyclin D1 by  $As_2O_3$  treatment. Figure 2A:  $As_2O_3$  (4  $\mu M$ ) induced a time dependent down-regulation of cyclin D1 in Jeko-1 and Granta-519 cells. Triplicate experiments and a representative Western blot demonstrate significant decrease in cyclin D1 level after 2 hours (one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ). Figure 2B:  $As_2O_3$  (treatment for 8 hours) induced a dose dependent down-regulation of cyclin D1 in Jeko-1 and Granta-519 cells. Triplicate experiments demonstrate

significant decrease in cyclin D1 level at or above 2  $\mu$ M (one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ).

**Figure 3** shows dephosphorylation of retinoblastoma (RB) by  $As_2O_3$  treatment in MCL lines.  $As_2O_3$  treatment resulted in dephosphorylation of RB (significant decrease of phosphor-Rb Ser-795 at or more that 8 hours of  $As_2O_3$  treatment, triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ).

**Figures 4A, 4B and 4C** show  $As_2O_3$  treatment induced phosphorylation of cyclin D1 and GSK-3. Figure 4A. Cell lysates immunoblotted with anti-phospho-cyclin D1 (Thr-286).  $As_2O_3$  treatment led to significantly increased phosphor-cyclin D1 (triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ). Figure 4B. Cell lysates immunoblotted with anti-phospho-cyclin GSK-3 $\beta$  (Try-216).  $As_2O_3$  treatment led to significantly increased phosphor-GSK-3 $\beta$  (triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ). Figure 4C. Pre-incubation with 6-bromoindirubin-3'-oxime (BIO; 10  $\mu$ M) before  $As_2O_3$  treatment (4  $\mu$ M, 8 hour, 37°C) prevented cyclin D1 down-regulation, showing that GSK-3b was involved. Result a significant reduction of cyclin D1 as compared with control (triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ).

**Figures 5A and 5B** show that IKK was involved in  $As_2O_3$ -induced down-regulation of cyclin D1. Figure 5A.  $As_2O_3$  treatment (4  $\mu$ M for 2 hours) led to a significant increase in phosphor-IKK $\alpha/\beta$  (Ser – 176/180) (triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ). Figure 5B. Pre-incubation with the IKK inhibitor BMS (10 mM, 30 minutes) successfully prevented  $As_2O_3$ -induced cyclin D1 down-regulation (triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ).

**Figure 6** shows  $As_2O_3$  induced ubiquitination of cyclin D1 in MCL. Cell lysates were immunoprecipitation with anti ubiquitin (Ub) or anti-cyclin D1 antibody. The immunoprecipitates and the crude lysates were immunoblotted with anti-cyclin D1 and anti-ubiquitin antisera.  $As_2O_3$  induced a significant increase in binding between cyclin D1 and ubiquitin (increase in ubiquitination

from 30 minutes to 2 hours after  $As_2O_3$  treatment as compared to the baseline, triplicate experiments, one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ).

**Figures 7A and 7B** show  $As_2O_3$ -induced cyclin D1 degradation involved the proteasome but not the lysosome in MCL. Figure 7A. Pre-  
5 incubation with the proteasome inhibitors MG132 (MG, 30  $\mu M$ ), bortezomib (bort, 10  $\mu g/ml$ ) and lactacystin (lact, 10  $\mu M$ ) successfully prevented  $As_2O_3$  induced cyclin D1 degradation. Figure 7B. Pre-incubation with the lysosomal inhibitor ammonium chloride ( $NH_4Cl$ , 2.5 mM) was ineffective in preventing  $As_2O_3$ -induced cyclin D1 degradation.

10 **Figure 8** is a schematic diagram showing the proposed mechanism of degradation of cyclin D1 mediated by  $As_2O_3$ .

### **Detailed Description of the Preferred Embodiments**

#### **I. Arsenic Trioxide Formulations**

##### **Arsenic Trioxide**

15 Arsenic trioxide is available from a number of different suppliers. Arsenic trioxide is an amphoteric oxide which is known for its acidic properties. It dissolves readily in alkaline solutions to give arsenites. It is much less soluble in acids, but will dissolve in hydrochloric acid to give arsenic trichloride or related species. It reacts with oxidizing agents such as ozone, hydrogen peroxide  
20 and nitric acid to give arsenic pentoxide,  $As_2O_5$ . It is also readily reduced to arsenic, and arsine ( $AsH_3$ ) may also be formed.

Arsenic trioxide has many uses including as: a starting material for arsenic-based pesticides; a starting material for arsenic-based pharmaceuticals, such as a neosalvarsan, a synthetic organoarsenic antibiotic; a decolorizing agent  
25 for glasses and enamels, a wood preservative, and a cytostatic in the treatment of refractory promyelocytic (M3) subtype of acute myeloid leukemia.

An oral arsenic trioxide ( $As_2O_3$ ) is highly efficacious for relapsed acute promyelocytic leukemia. Oral  $As_2O_3$  causes a smaller prolongation of QT intervals, and therefore is a much safer drug for treating leukemia.

### Formulations

The following delivery systems, which employ a number of routinely used pharmaceutical carriers, are only representative of the many embodiments envisioned for administering the instant compositions.

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#### *Parenteral Formulations*

Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprylactones and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylactone.

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#### *Enteral Formulations*

Oral delivery systems include solid dosage forms such as tablets (e.g., compressed tablets, sugar-coated tablets, film-coated tablets, and enteric coated tablets), capsules (e.g., hard or soft gelatin or non-gelatin capsules), blisters, and cachets. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc). The solid dosage forms can be coated using coatings and techniques well known in the art.

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Oral liquid dosage forms include solutions, syrups, suspensions, emulsions, elixirs (e.g., hydroalcoholic solutions), and powders for reconstitutable delivery systems. The formulations can contain one or more carriers or excipients, such as suspending agents (e.g., gums, xanthans, cellulose and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG, glycerin, and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), emulsifiers, preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating agents, chelating agents (e.g., EDTA), flavorants, colorants, and combinations thereof. The compositions can be formulated as a food or

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beverage (e.g., a shake) containing buffer salts, flavoring agents, coloring agents, sweetening agents, and combinations thereof.

#### *Topical Formulations*

Transmucosal delivery systems include patches, tablets, suppositories,  
5 pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Dermal delivery systems include, for example, aqueous and nonaqueous  
10 gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrrolidone). In one  
15 embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer.

## **II. Methods of Treatment**

Cyclin D1 is a D-type cyclin critically involved in the control of the cell cycle. It assembles with its catalytic partners cyclin-dependent kinase 4 (CDK4)  
20 and CDK6 to form an active holoenzyme complex, which controls G1 progression and G1/S transition. The active holoenzyme complex phosphorylates the retinoblastoma protein RB. Phosphorylated RB releases the E2F family of transcription factors from inhibition, enabling E2Fs to coordinately regulate genes necessary for DNA replication and hence  
25 progression into S phase. Over-expression of cyclin D1 is demonstrable in many cancers, including cancers of the digestive tract, cancers of the female genital tract, and malignant lymphomas.

Owing to its important influence on the cell cycle, cyclin D1 expression is carefully regulated. Cyclin D1 gene mRNA and transcription appears to be  
30 constant through the cell cycle. However, a decline in cyclin D1 level occurs during S phase, which has been attributed to its increased proteasomal

degradation. Cyclin D1 phosphorylation at a threonine residue 286 (Thr-286) positively regulates its proteasomal degradation. Thr-286 phosphorylation is mediated by glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). In addition to targeting cyclin D1 to proteosomes, GSK-3 $\beta$  -induced Thr-286 phosphorylation also  
5 promotes cyclin D1 nuclear export, by increasing the binding of cyclin D1 to a nuclear exportin CRM1. IkappaB kinase (IKK) alpha, IKK $\alpha$ , associates with and phosphorylates cyclin D1 also at Thr-286, thereby participating in the subcellular localization and turnover of cyclin D1.

As<sub>2</sub>O<sub>3</sub> induced apoptosis in MCL lines at 2 – 4  $\mu$ M, which is within the  
10 plasma levels achieved after As<sub>2</sub>O<sub>3</sub> therapy. As<sub>2</sub>O<sub>3</sub> induces a dose and time dependent suppression of cyclin D1. The suppression of cyclin D1 restores RB to a hypophosphorylated state, in parallel with a change in cell cycle. These biologic changes are consistent with the apoptosis observed upon As<sub>2</sub>O<sub>3</sub> treatment.

15 The down-regulation of cyclin D1 mediated by As<sub>2</sub>O<sub>3</sub> occurs at a post-transcriptional level since cyclin D1 is under the transcriptional control of the immunoglobulin heavy chain gene enhancer in MCL, which is unlikely to be affected by As<sub>2</sub>O<sub>3</sub>. Furthermore, in physiologic conditions, the control of cyclin D1 during the cell cycle is also mediated in part via alteration in the stability of  
20 cyclin D1. This process is controlled by phosphorylation of cyclin D1 at Thr-286, a process mediated by GSK-3 $\beta$ . GSK-3 $\beta$  is itself tightly regulated. Mitogens inactivate GSK-3 $\beta$  by a pathway involving Ras, phosphatidylinositol 3 kinase (PI3K), and protein kinaseB/Akt. Ras activates PI3K, which in turn activates Akt. Akt inactivates GSK-3 $\beta$  by phosphorylating it at serine residue 9.  
25 This removes the inhibition of GSK-3 $\beta$  on cyclin D1, allowing cyclin D1 to accumulate and thus activate cell cycling. GSK-3 $\beta$  can also be activated by phosphorylation at a tyrosine residue 216 (Try-216) in the kinase domain. As<sub>2</sub>O<sub>3</sub>-mediates an increase of GSK-3 $\beta$  Try-216 phosphorylation. The end result of As<sub>2</sub>O<sub>3</sub>-mediated increase in GSK-3 $\beta$  Try-216 phosphorylation is the increase  
30 in cyclin D1 Thr-286 phosphorylation, a key step in its degradation.



The IKK complex is the major regulatory component in the NF- $\kappa$ B pathway. It comprises the catalytic subunits IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit IKK $\gamma$ /NEMO. IKK $\alpha$  has been shown to phosphorylate cyclin D1 at Thr-286, the same site targeted by GSK-3 $\beta$ . IKK $\alpha$  needs to be activated by phosphorylation at a serine residue 176 (Ser-176) before participating in the regulation of NF- $\kappa$ B by phosphorylating I $\kappa$ B. IKK $\alpha$  Ser-176 phosphorylation is mediated by NF- $\kappa$ B inducing kinase (NIK). As<sub>2</sub>O<sub>3</sub>-induces an increase in IKK phosphorylation. As<sub>2</sub>O<sub>3</sub>-mediates an increase in physical interaction between IKK and cyclin D1, as shown in immunoprecipitation experiments. An IKK specific inhibitor BMS-345541 alleviated As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation. These results indicate that IKK is also an effector of As<sub>2</sub>O<sub>3</sub> treatment.

As<sub>2</sub>O<sub>3</sub>-mediated cyclin D1 Thr-286 phosphorylation increases its ubiquitination. The time course of ubiquitination is commensurate with the timing of the biologic functions of As<sub>2</sub>O<sub>3</sub> on the MCL lines. After As<sub>2</sub>O<sub>3</sub> treatment, increased ubiquitination is first detected at 30 minutes and continues to increase. At two hours, significant down-regulation of cyclin D1 is first observed, which is associated with a parallel hypophosphorylation of RB. Significant activation of caspase 3 is observed at four hours. These sequence of events are consistent with cyclin D1 down-regulation initiated by Thr-286 phosphorylation.

Cyclin D1 is a cytosolic and nuclear protein. Therefore, polyubiquitination is involved, which targets the protein to degrade in proteasomes. Inhibition of proteasomes successfully prevented As<sub>2</sub>O<sub>3</sub>-induced down-regulation of cyclin D1. Inhibition of lysosomes, the site of degradation of monoubiquitinated proteins, does not interfere with As<sub>2</sub>O<sub>3</sub>-induced down-regulation of cyclin D1. These results confirm that As<sub>2</sub>O<sub>3</sub> down-regulated cyclin D1 by promoting its proteasomal degradation.

Arsenic trioxide can be used for the treatment of cancers that are dependent on cyclin D1 for proliferation, survival, metastasis and differentiation.

Patients with cancers that overexpress cyclin D can be treated with As<sub>2</sub>O<sub>3</sub>. Mantle cell lymphoma is a cancer characterized by overexpression of

cyclin D, as are cancers of the digestive tract, cancers of the female genital tract, and malignant lymphomas.

The dose of oral  $As_2O_3$  is typically adjusted according to age and kidney function. In one embodiment, the dose range of  $As_2O_3$  varies from 1 to 10 mg, typically about 5 to 10 mg.

The present invention will be further understood by reference to the following non-limiting examples.

### Examples

**Example 1: In vitro studies show  $As_2O_3$  is effective in treatment of MCL by targeting cyclin D1.**

#### Materials and Methods

**Cell lines.** The MCL lines Jeko-1 and Granta-519 were obtained from German Collection of Microorganisms and Cell Cultures (ACC 553 and ACC 342, Braunschweig, Germany). Jeko-1 cells were cultured in RPMI 1640 with 20% fetal bovine serum (FBS), and Granta-519 cells in DMEM with 10% FBS; both with 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin, at 5%  $CO_2$ .

**Reagents and antibodies.** Reagents and antibodies used included cell culture reagents (Invitrogen, Carlsbad, CA, USA); kinase inhibitors and their inactive analogues (Calbiochem, Darmstadt, Germany); antiserum to phospho-GSK3 (tyrosine 216, Try-216) (Upstate, Lake Placid, NY, USA); antisera to cyclin D1, phospho-cyclin D1 (Thr-286), GSK3 $\beta$ , phospho-GSK3 $\beta$  (Tyr-216), I $\kappa$ B kinase (IKK) $\alpha/\beta$ , phospho-IKK $\alpha/\beta$  (serine 176/180, Ser-176/180), RB and phospho-RB (serine 795, Ser-795), caspase-3 and  $\beta$ -actin (Cell Signaling Technology, Beverly, MA, USA); protein G-agarose (Upstate); ECL kit (Amersham, Piscataway, NJ, USA); cell proliferation kit I (MTT) (Roche Applied Science, Indianapolis, IN, USA); annexin V-FITC Kit (Beckman Coulter, Fullerton, CA, USA); and RNeasy Kit and One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA).

**Cell viability assays.** Cells were seeded on 96-well microplates at  $2 \times 10^4$ /well in 100  $\mu$ l growth medium containing different concentration of  $As_2O_3$  as indicated at 37°C for 72 hours. MTT labeling reagent (10  $\mu$ l, 5 mg/ml)

(Roche Applied Science, Indianapolis, IN, USA) was added to each well at 37°C for 4 hours, followed by 100  $\mu$ l solubilization at 37°C overnight. Solubilized fomarzan crystals were quantified spectrophotometrically at 590 nm with a microplate ELISA reader.

5           **Apoptosis assay.** Cells were seeded at  $1 \times 10^6$ /ml in different concentrations of As<sub>2</sub>O<sub>3</sub> as indicated at 37°C for 24 hours, harvested, rinsed in ice-cold phosphate buffered saline (PBS), and resuspended in 500  $\mu$ l binding buffer containing annexin V- FITC and propidium iodide (PI) (Beckman Coulter, Fullerton, CA, USA) for 20 minutes on ice. The percentages of apoptotic cells  
10 (annexin-V positive, PI negative) were determined on a flow cytometer (Epics, Beckman Coulter) with appropriate color compensation.

**Cell Cycle Analysis.** Cells were seeded at  $1 \times 10^5$ /ml in different concentrations of As<sub>2</sub>O<sub>3</sub> as indicated at 37°C for 8 hours, harvested, washed in ice-cold PBS, resuspended in 500  $\mu$ l PBS, stained with PI for 10 minutes on ice.  
15 Cell cycle was determined by flow cytometry (Epics, Beckman Coulter).

**Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) for cyclin D1.** Cells were seeded at a density of  $1 \times 10^6$ /ml in different concentrations of As<sub>2</sub>O<sub>3</sub> at 37°C for 8 hours, washed with PBS buffer and lysed with RTL buffer. RNA was extracted with an RNeasy Kit, followed  
20 by cDNA synthesis and a 30-cycle PCR with a One-Step RT-PCR Kit with the forward primer 5'-CTG GCC ATG AAC TAC CTG GA-3' and the reverse primer 5'-GTC ACA CTT GAT CAC TCT GG-3'. Cycling conditions were denaturation (1 minute at 94°C, first cycle 5 minutes), annealing (2 minutes at 50°C) and extension (3 minutes at 72°C, last cycle 10 minutes).

25           **Western Blotting Analysis.** Cells were seeded at a density of  $1 \times 10^6$ /ml overnight. Where applicable, cells were pre-treated with various inhibitors for 30 minutes, and then incubated with 4  $\mu$ M As<sub>2</sub>O<sub>3</sub> for different time periods as indicated. Cells were lysed in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 40 mM NaP<sub>2</sub>O<sub>7</sub>, pH 7.5, 1% Triton X-100, 4  $\mu$ g/ml aprotinin, 1  
30 mM dithiothreitol, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 0.7  $\mu$ g/ml pepstatin, 100  $\mu$ M phenylmethylsulfonyl fluoride, and 2  $\mu$ g/ml leupeptin). Clarified lysates were

resolved on 12% SDS-phenylmethylsulfonyl fluoride and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk, washed, incubated with the appropriate antibodies followed by horseradish peroxidase-conjugated secondary antisera. Immuno-reactive bands were  
5 visualized by chemiluminescence with the ECL kit, detected on X-ray films and quantified by densitometric scanning (Eagle Eye II still video system, Stratagene, La Jolla, CA, USA).

**Coimmunoprecipitation Assays.** Cells were seeded at  $1 \times 10^6$ /ml overnight, treated with  $4 \mu\text{M As}_2\text{O}_3$  at  $37^\circ\text{C}$  for different time periods as  
10 indicated, and lysed in lysis buffer. Cell lysates were incubated with an anti-cyclin D1, anti-ubiquitin, anti-calpain 2 or anti-IKK $\alpha/\beta$  antibodies ( $4 \mu\text{g}/\text{sample}$ ) at  $4^\circ\text{C}$  for 1 hour, followed by incubation with  $30 \mu\text{l}$  of protein G-agarose (50% slurry) at  $4^\circ\text{C}$  for another 2 hour. Immunoprecipitates were washed four times with  $400 \mu\text{l}$  lysis buffer, resuspended in  $50 \mu\text{l}$  lysis buffer and 10 ml 6X sample  
15 buffer and boiled for 5 minutes. Immunoprecipitates were then analysed by Western blot analysis.

## **Results**

### **As<sub>2</sub>O<sub>3</sub> induced dose and time dependent apoptosis in MCL cells.**

The MTT test showed that As<sub>2</sub>O<sub>3</sub> induced a dose-dependent cytotoxicity  
20 in Jeko-1 and Granta-519 cells. Flow cytometric analysis showed that As<sub>2</sub>O<sub>3</sub> treatment led to induction of apoptosis. Western blot analysis showed that caspase 3 activation was involved in As<sub>2</sub>O<sub>3</sub>-induced apoptosis.

Figures 1A and 1B are graphs showing As<sub>2</sub>O<sub>3</sub> (concentration in microM) percent induced apoptosis in MCL cells measured using a MTT test of Jeko-1  
25 and Granta-519 cells treated for 72 hours with As<sub>2</sub>O<sub>3</sub>. There was a dose and time dependent suppression of cellular proliferation. Viability significantly decreased at or above  $1 \mu\text{M As}_2\text{O}_3$  as compared with baseline (one-way ANOVA with Dunnett's post-tests,  $p < 0.05$ ) (triplicate experiments). (. Significant increase in apoptotic cells after As<sub>2</sub>O<sub>3</sub> treatment. #: apoptotic cells that were annexin V  
30 positive and popidium iodide negative). Western Blotting showed activation of

caspase 3 by As<sub>2</sub>O<sub>3</sub> treatment, 0, 1.5 and 2.5 microM. Cleaved caspase 3 were detectable four hours after As<sub>2</sub>O<sub>3</sub> treatment.

**Cyclin D1 was down-regulated in MCL by As<sub>2</sub>O<sub>3</sub>.** To determine the molecular mechanisms of As<sub>2</sub>O<sub>3</sub>-induced apoptosis in MCL, the expression of cyclin D1 was examined. Western blot analysis showed that As<sub>2</sub>O<sub>3</sub>-induced a time and dose dependent suppression of cyclin D1 in both Jeko-1 and Granta-519 cell lines. Treatment with As<sub>2</sub>O<sub>3</sub> at 4 μM led to suppression of cyclin D1, first detectable at 2 hours and almost complete at 8 – 12 hours As<sub>2</sub>O<sub>3</sub> suppression of cyclin D1 was also dose-dependent. Triplicate experiments demonstrate significant decrease in cyclin D1 level after 2 hours (one-way ANOVA with Dunnett's post-tests, *p* < 0.05) Triplicate experiments demonstrate significant decrease in cyclin D1 level at or above 2 μM (one-way ANOVA with Dunnett's post-tests, *p* < 0.05). Semi-quantitative polymerase chain reaction showing that cyclin D1 gene transcription was unaffected by As<sub>2</sub>O<sub>3</sub> treatment.

**As<sub>2</sub>O<sub>3</sub> induced down-regulation of cyclin D1 disrupted its signaling.** To investigate if cyclin D1 down-regulation is biologically relevant, RB phosphorylation was investigated. As<sub>2</sub>O<sub>3</sub> treatment led to a time dependent decrease in RB phosphorylation, which occurred at a similar time-frame as compared with cyclin D1 down-regulation. Cell cycle analysis by flow cytometry showed that there was an increase in the proportion of apoptotic cells.

**Down-regulation of cyclin D1 by As<sub>2</sub>O<sub>3</sub> was post-transcriptional.** RT-PCR showed that cyclin-D1 gene transcription was unaffected by As<sub>2</sub>O<sub>3</sub> treatment of up to 8 μM, suggesting that the down-regulation of cyclin D1 was post-transcriptional.

**As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation was related to GSK3β activation.** Western blot analysis showed that As<sub>2</sub>O<sub>3</sub> treatment resulted in significant increases in cyclin D1 phosphorylation at Thr-286, a prerequisite for cyclin D1 degradation. Cyclin D1 phosphorylation by GSK-3β requires prior activation of GSK-3β by phosphorylation at Tyr-216. As<sub>2</sub>O<sub>3</sub> treatment significantly increased GSK-3β Tyr-216 phosphorylation, indicating that GSK-

3 $\beta$  might mediate As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 phosphorylation and hence degradation. To confirm the role of GSK-3 $\beta$  as a mediator of As<sub>2</sub>O<sub>3</sub>, Jeko-1 cells were pre-incubated with the GSK-3 $\beta$  inhibitor 6-bromoindirubin-3'-oxime (BIO; 10  $\mu$ M) before As<sub>2</sub>O<sub>3</sub> treatment. The results showed that BIO successfully prevented As<sub>2</sub>O<sub>3</sub>-induced down-regulation of cyclin D1. Collectively, these observations indicate that As<sub>2</sub>O<sub>3</sub> down-regulated cyclin D1 post-transcriptionally, probably by increasing its degradation.

**As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation was also dependent on IKK $\alpha$ / $\beta$ .** To determine if IKK was involved in As<sub>2</sub>O<sub>3</sub>-induced down-regulation of cyclin D1, IKK $\alpha$ / $\beta$  phosphorylation at Ser-178/180 was examined. As<sub>2</sub>O<sub>3</sub> significantly increased IKK $\alpha$ / $\beta$  Ser-178/180 phosphorylation, which was required for activation of IKK $\alpha$ / $\beta$  (Figure 5A). Pre-treatment with the IKK $\alpha$ / $\beta$  inhibitor BMS-345541 (BMS; 10  $\mu$ M) significantly prevented As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation, suggesting that IKK $\alpha$ / $\beta$  was a molecular mediator of As<sub>2</sub>O<sub>3</sub> (Figure 5B). Immunoprecipitation with an anti-IKK $\alpha$ / $\beta$  antibody showed that cyclin D1 bound IKK $\alpha$ / $\beta$ . Similarly, when cyclin D1 was immunoprecipitated, IKK $\alpha$ / $\beta$  was also confirmed to co-immunoprecipitate. These results confirmed that As<sub>2</sub>O<sub>3</sub> activated IKK $\alpha$ / $\beta$ , which participated in the down-regulation of cyclin D1.

**As<sub>2</sub>O<sub>3</sub> promoted cyclin D1 ubiquitination.** To study if As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation was mediated via ubiquitination, immunoprecipitation experiments were performed on lysates from Jeko-1 cells treated with As<sub>2</sub>O<sub>3</sub>. Immunoprecipitation with an anti-ubiquitin antibody showed a time-dependent increase in bound cyclin D1 (Figure 6). Similarly, lysates immunoprecipitated with an anti-cyclin D1 antibody also showed a time dependent increase in bound ubiquitin. These results showed that As<sub>2</sub>O<sub>3</sub> promoted cyclin D1 ubiquitination, confirming that As<sub>2</sub>O<sub>3</sub>-induced GSK-3 $\beta$  and IKK $\alpha$ / $\beta$  activation was biologically relevant.

**As<sub>2</sub>O<sub>3</sub> induced cyclin D1 degradation in 26S and 20S proteasomes but not lysosomes.** Pre-incubation of Jeko-1 cells with the 26S and 20S

proteasome inhibitors MG132 (30  $\mu$ M), bortezomib (10  $\mu$ g/ml) and lactacystin (10  $\mu$ M) attenuated As<sub>2</sub>O<sub>3</sub>-induced cyclin D1 down-regulation (Figure 7A).

However, pre-incubation with the lysosomal inhibitor ammonium chloride (NH<sub>4</sub>Cl) had no effect on As<sub>2</sub>O<sub>3</sub>-induced down-regulation of cyclin D1 (Figure 7B). The results confirmed that As<sub>2</sub>O<sub>3</sub> down-regulated cyclin D1 by promoting its ubiquitination, hence targeting it to the proteasome for degradation.

**Overall model.** An overall model of the action of As<sub>2</sub>O<sub>3</sub> on MCL is shown in Figure 8.

**Example 2: Clinical study of oral-As<sub>2</sub>O<sub>3</sub> in the treatment of patients with refractory and relapsed MCL that over-expressed cyclin D1.**

#### Materials and Methods

**Patients.** Consenting patients with relapsed or refractory B-cell lymphomas, and an ECOG performance status of < 2 were recruited. All patients gave informed consent, and the treatment was approved by the institute review board of Queen Mary Hospital.

**Treatment.** Treatment was initiated with oral-As<sub>2</sub>O<sub>3</sub> (10 mg/day for patients below 70 years old with normal renal function; 5 mg/day for patients over 70 years old, or with impaired renal function), ascorbic acid (AA, 1 g/day) and chlorambucil (4 mg/day) as outpatients until disease response or progression was documented. In patients with bulky disease, debulking with VPP (vincristine 2 mg/day x 1, prednisolone 30 mg/day x 14 and procarbazine 50 - 100 mg/day x 14) was used. After maximum response was achieved, chlorambucil was taken off and a maintenance regimen of As<sub>2</sub>O<sub>3</sub> (5 - 10 mg/day) and AA (1 g/day) was given for two weeks every 2 months for a planned two years. Responses were classified according to standard NCI criteria, and monitored by regular physical examination, marrow and blood assessment, and computerized tomographic scans.

#### Results

**Characteristics of patients with MCL.** Table 1 shows the results of the clinical use of oral-As<sub>2</sub>O<sub>3</sub> in patients with refractory or relapsed mantle cell lymphoma that over-expressed cyclin D1. The results showed an overall

response rate of 64%. Four patients achieved complete remission (CR), whereas two patients achieved complete remission unconfirmed. Of the fourteen patients treated (Table 1), eleven had advanced relapses (R) (R2, n=5; R3, n=4; R4, n=2). Three patients treated in R1 had advanced age (76, 77 and 90 years). All but two  
5 patients had received an anthracycline based multi-agent chemotherapy. Other previous treatment included rituximab (n=8), autologous hematopoietic stem cell transplantation (HSCT) (n=3), and bortezomib (n=1). Other poor prognostic indicators included marrow infiltration (n=11) and extensive extranodal involvement (n=9), so that 12/14 (86%) cases had stage IV disease. The median  
10 time from initial diagnosis to As<sub>2</sub>O<sub>3</sub> treatment was 33 (8 – 85) months.



**Table 1. Clinicopathologic features and treatment outcome of 14 patients with relapsed or refractory MCL**

	Initial disease sites		Previous treatment	Current relapse		Total As <sub>2</sub> O <sub>3</sub> response	Outcome and survival
	stage	sites		Time*No	Sites		
1	M / 69	III Colon, abdomen	FND x 6, COPP x 6	56 m 2	Cervical	140 mg	CR off Rx, 28 mt.
2	M / 63	IV BM, generalized LN	R-CEOP x 6, IMVP x 6	11 m 2	BM, cervical	160 mg	CR on Rx, 13 mt.
3	M / 65	IV BM, mesentery, generalized LN	FND x 7, IMVP x 2, R-DHAP x 8	85 m 3	Eye	120 mg	CR on Rx, 17 mt.
4	F / 77	IV Pleura, generalized LN	Cilb	33 m 1	Groin, jaw	140 mg	CR R2 at 16 m, CR again with As <sub>2</sub> O <sub>3</sub> + Cilb
5	M / 70	III Generalized LN	COPP x2, IMVP x 6, Cilb	85 m 4	Cervical, abdomen	250 mg	CRu R5 at 20 m, on As <sub>2</sub> O <sub>3</sub> + Cilb
6	M / 76	IV BM, generalized LN	CEOP x 7	19 m 1	BM, leukemic, eyes, generalized LN	210 mg*	CRu on Rx, 8 mt+
7	M / 58	IV BM, generalized LN	CEOP x 6, R-ESHAP x 6	18 m 2	Generalized skin	140 mg	PR On Rx, 3 mt+
8	M / 81	IV BM, leukemic, liver, spleen	CHOP x 6, ChlVPP x 2	18 m 2	BM, LN, liver, spleen, leukemic	300 mg*	PR died at 16 m
9	M / 51	IV Generalized LN, spleen, BM, scalp, eye	CVAD x 7, CEOP x 2, R-DHAP x 3, Thal	25 m 4	BM, LN, scalp	NA*	Static on Rx, 8 mt+
10	F / 76	IV General LN, BM, scalp	R-COPP x 6	12 m 2	BM, LN	160 mg*	PR died at 6 m
1	M / 90	IV BM, leukemic	Cilb	8 m 1	BM, leukemic	NA	NR died at 4 m
2	M / 54	IV Generalized LN, BM, gut, liver, spleen, leukemic	CEOP x 6, DHAP x 1, NOPP x 5, Cilb	36 m 3	BM, generalized LN, spleen	NA	Static died at 17 m
3	F / 57	IV Generalized LN, BM, spleen	CEOP x 6, DHAP x 4, R-BVP x 3	36 m 3	BM, LN	NA	NR died at 1 m
4	M / 63	IV Generalized LN, pleura, BM	CEOP x 6, AHSCT, R-DHAP x 6, Thal, velcade, FND	72 m 3	BM, generalized LN	NA	NR died at 1 m

M: male; F: female; LN: lymphadenopathy; BM: bone marrow; m: months; R: rituximab; CEOP: cyclophosphamide, epirubicin, vincristine, prednisolone  
 FND: fludarabine, mitoxantrone, dexamethasone; DHAP: cisplatin, cytosine arabinoside, dexamethasone; Thal: thalidomide  
 ChlVPP: chlorambucil, vincristine, procarbazine, prednisolone; COPP: cyclophosphamide, vincristine, procarbazine, prednisolone;  
 NOPP: mitoxantrone; vincristine, procarbazine, prednisolone; BVP; bleomycin, vinblastine, prednisolone; AHSCT: autologous hematopoietic stem cell transplantation  
 Cilb: chlorambucil; NA: not available; CR: complete remission; CRu: complete remission (unconfirmed); PR: partial remission, NR: no response

**Treatment response.** Nine patients responded, giving an OR rate of 64%. Four patients (cases 1 – 4) achieved CR. Two patients (cases 5, 6) achieved unconfirmed CR (CRu). They had become asymptomatic without any detectable superficial diseases. Marrow and peripheral blood involvement was also cleared. However, small residual internal lymph nodes remained. These lymph nodes were negative on gallium scan and had remained static in size. Three patients had partial responses (PR) with > 50% reduction in the size of assessable lymph nodes.

Case 6 had bilateral orbital infiltration at relapse that completely resolved after 4 months of oral As<sub>2</sub>O<sub>3</sub> treatment and ascorbic acid. Case 8 who was relapsing in leukemic phase with massive splenomegaly showed partial remission after 8 months of treatment with oral As<sub>2</sub>O<sub>3</sub> and ascorbic acid as determined by MRI scans. Histological analysis revealed that case 8 had dense marrow infiltration that resolved after 8 months of treatment with oral-As<sub>2</sub>O<sub>3</sub> and ascorbic acid.

**Outcome.** Of the four patients with CR, one had relapsed at 16 months. She achieved a CR3 again with daily As<sub>2</sub>O<sub>3</sub> and resumption of chlorambucil. Two patients were still on maintenance As<sub>2</sub>O<sub>3</sub> + AA treatment, while one had completed the planned two years of treatment. Of the two patients with CRu, one patient had relapsed at 20 months. He achieved CR5 again with As<sub>2</sub>O<sub>3</sub> and chlorambucil therapy. For the three patients with PR, one patient developed progressive disease while on maintenance therapy 12 months later and died of refractory lymphoma. Two defaulted treatment and both relapsed.

**Toxicity.** Significant (W.H.O grade 3 – 4) neutropenia and thrombocytopenia was observed in 7 patients. These patients had previously received multiple chemotherapy, or autologous HSCT. The neutropenia responded to hematopoietic growth factors. No significant sepsis or bleeding were observed. Other side effects included fever (n=7), herpes zoster reactivation (n=3), fluid accumulation (n=2), nausea (n=3) and headache (n=2).

No significant QT prolongation or arrhythmia was observed. Five patients did not report any side effects at all.

As<sub>2</sub>O<sub>3</sub> suppresses MCL cell growth by targeting cyclin D1. As<sub>2</sub>O<sub>3</sub> induces the phosphorylation of GSK-3β and IKK. Cyclin D1 over-expression is pathogenetically important in a vast diversity of cancers. Oral-As<sub>2</sub>O<sub>3</sub> inhibited refractory or relapsed MCL in 14 patients, which over-expressed cyclin D1, with an overall response in 9 patients (64%). Four patients achieved complete remission, two patients complete remission unconfirmed, and three patients with partial remissions. These results were very good, given that these patients had refractory or relapsed disease.

Taken together, the evidence demonstrates that As<sub>2</sub>O<sub>3</sub> decreases cyclin D1 and that the decrease in cyclin D1 was post-transcriptional. As<sub>2</sub>O<sub>3</sub> induces GSK-3β and IKK activation and hence phosphorylation of cyclin D1. Phosphorylated cyclin D1 is degraded in the proteasome. Oral As<sub>2</sub>O<sub>3</sub> induces a high response rate clinically in patients with refractory or relapsed MCL, a cancer that over-expresses cyclin D1.

We claim:

1. A method for inhibiting cyclin D1 production in a cell, comprising contacting the cell with an amount of arsenic trioxide effective to inhibit cyclin D1 production therein.
2. The method according to claim 1, wherein the cell is a cancer cell.
3. The method according to claim 2, wherein the cell is from a cancer of the digestive tract, cancer of the female genital tract, and malignant lymphomas.
4. The method according to claim 2, wherein the cancer cells are in a patient and the arsenic trioxide is administered orally.
5. The method according to claim 4, wherein the cancer cell is from a human female genital tract cancer, a digestive tract cancer, or a malignant lymphoma.
6. The method according to claim 4 wherein the cell is a human mantle cell lymphoma (MCL).
7. A unit dosage form for oral administration comprising arsenic trioxide in a pharmaceutically acceptable carrier for enteral administration.
8. The unit dosage form of claim 7, further comprising one or more pharmaceutically acceptable excipients.
9. The unit dosage form of claim 7, wherein the arsenic trioxide is present in an amount from 5 to 10 mg.
10. The unit dosage form of claim 7, wherein the unit dosage form is selected from the group consisting of solutions, suspensions, emulsions, syrups, tablets, and capsules.
11. A unit dosage form for oral administration comprising arsenic trioxide in a pharmaceutically acceptable carrier for enteral administration, wherein the dosage form contains a sufficient amount of arsenic trioxide to deliver a dose in the range of 5 to 10 mg.
12. The unit dosage form of claim 11, further comprising one or more pharmaceutically acceptable excipients.

13. The unit dosage form of claim 11, wherein the unit dosage form is selected from the group consisting of solutions, suspensions, emulsions, syrups, tablets, and capsules.

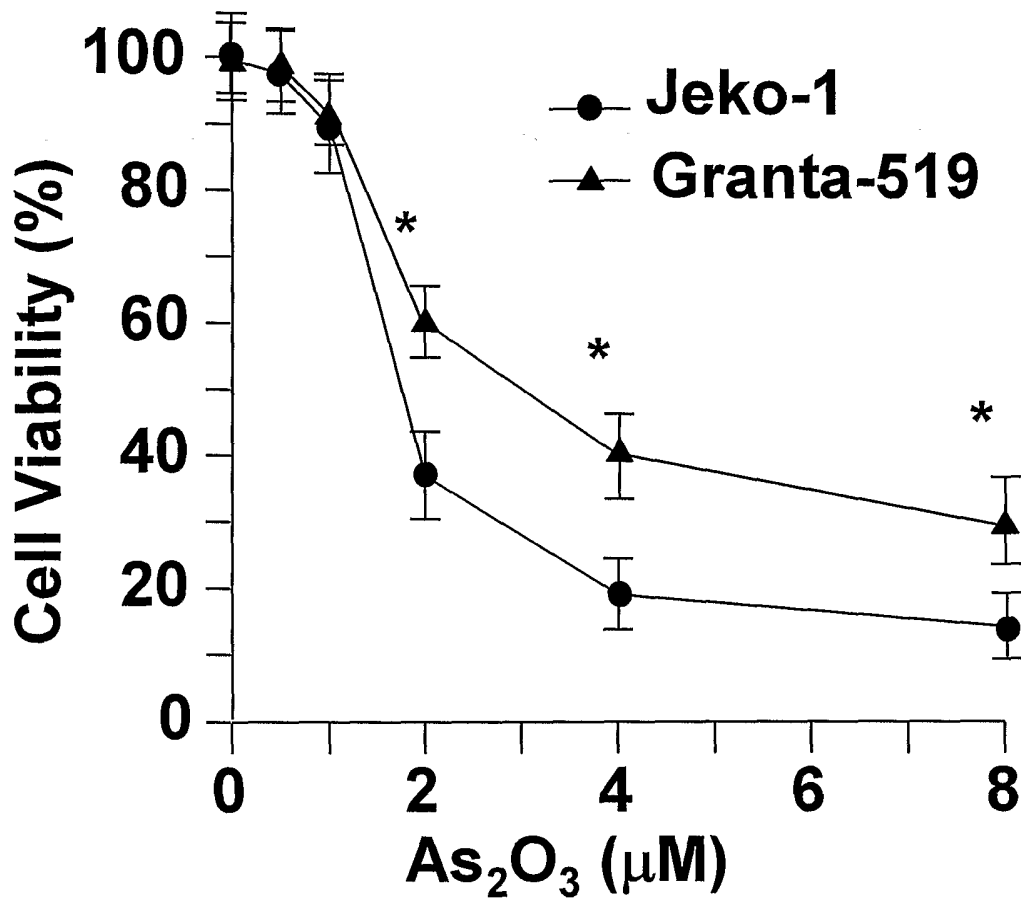


Fig. 1A

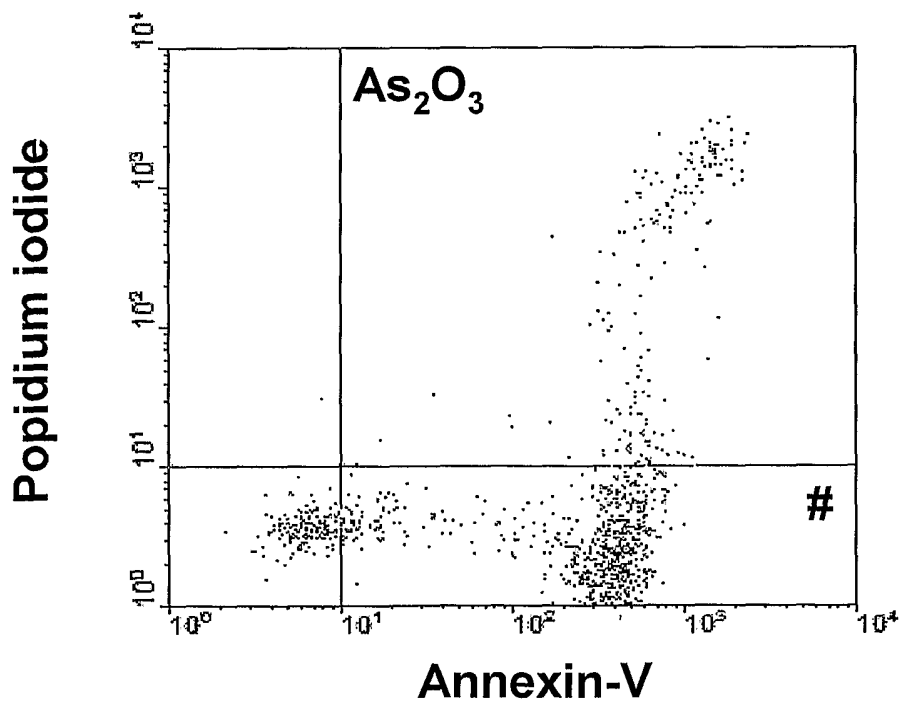


Fig. 1B

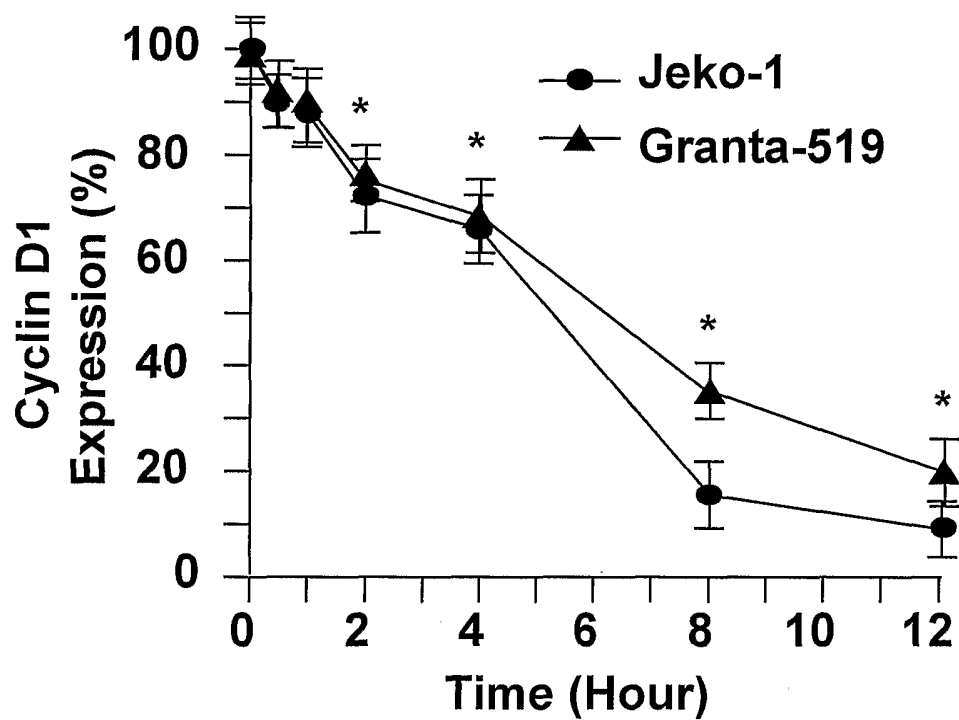


Fig. 2A

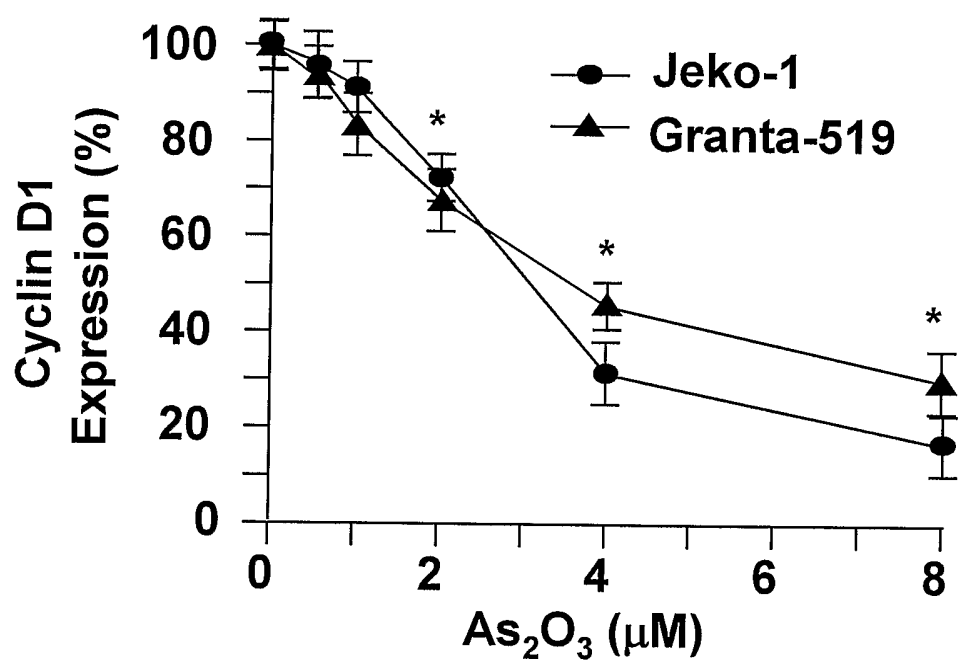


Fig. 2B



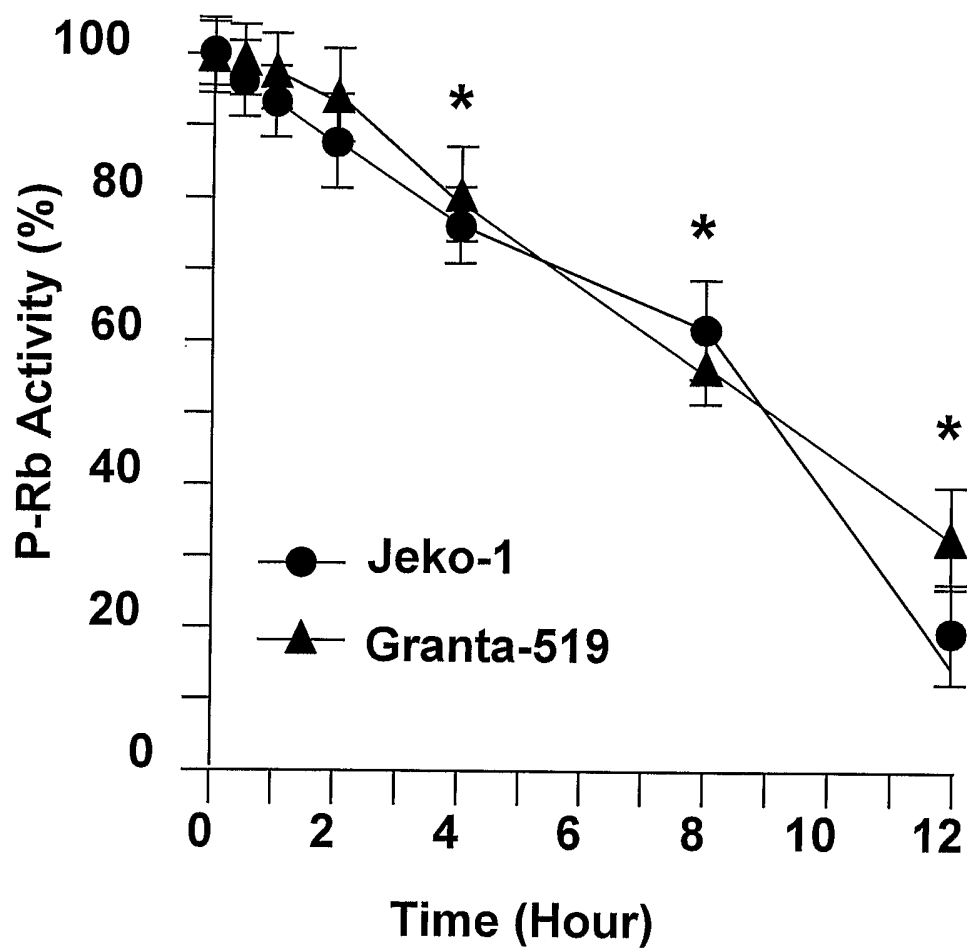


Fig. 3

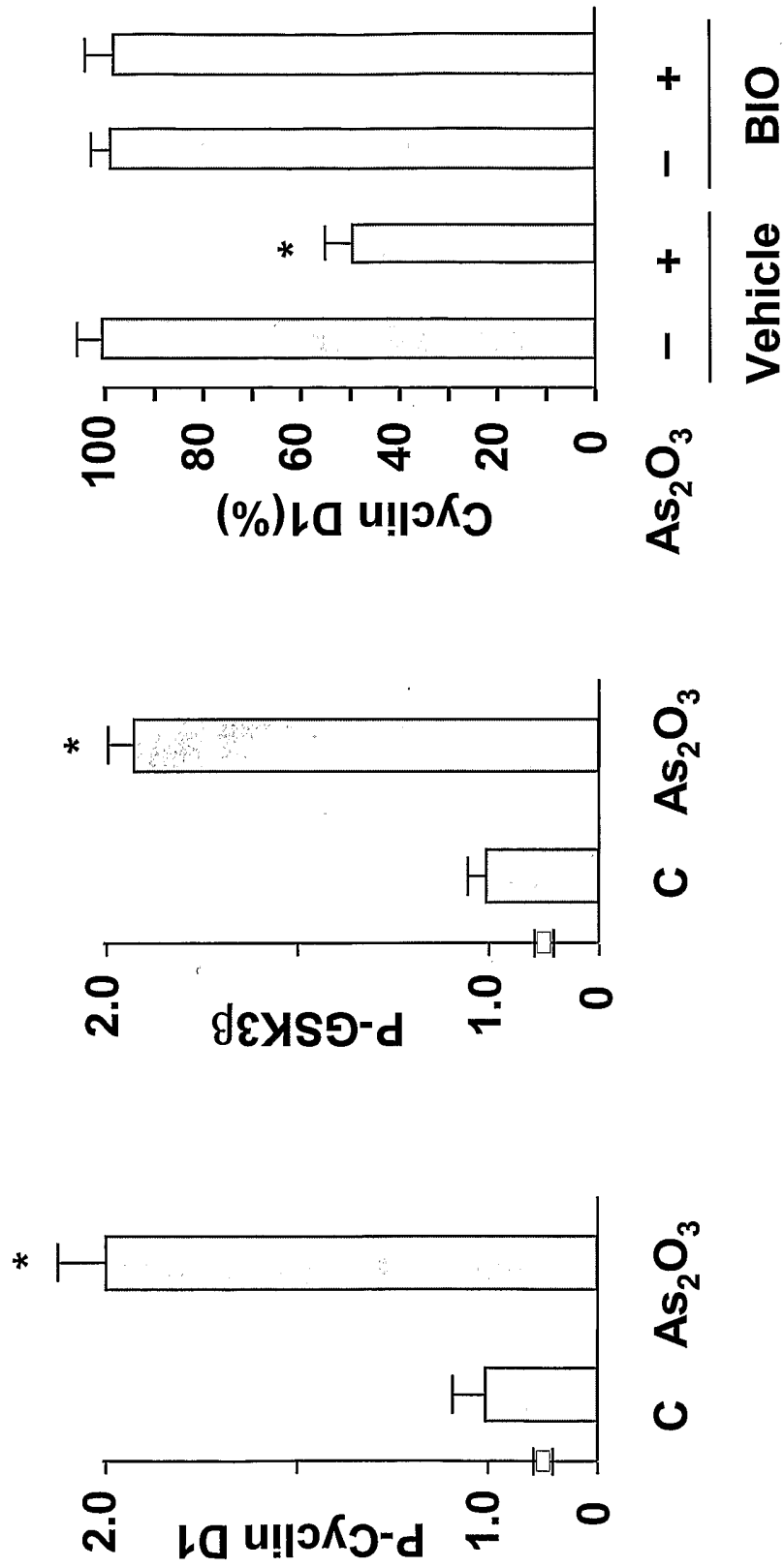


Fig. 4C

Fig. 4B

Fig. 4A

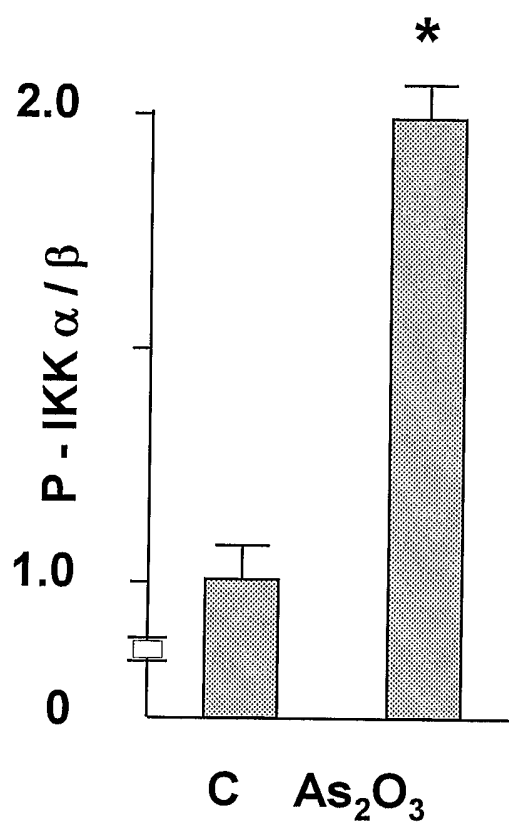


Fig. 5A

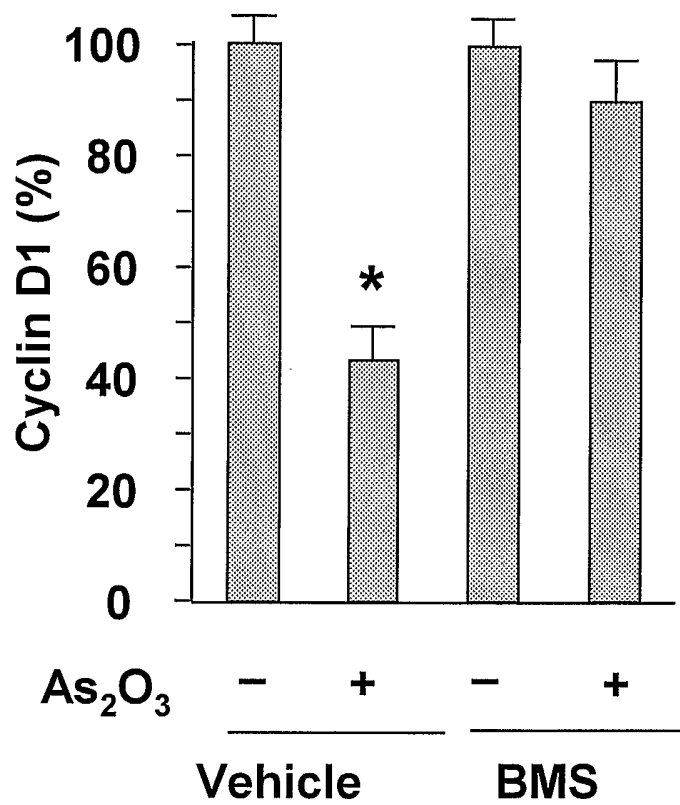


Fig. 5B

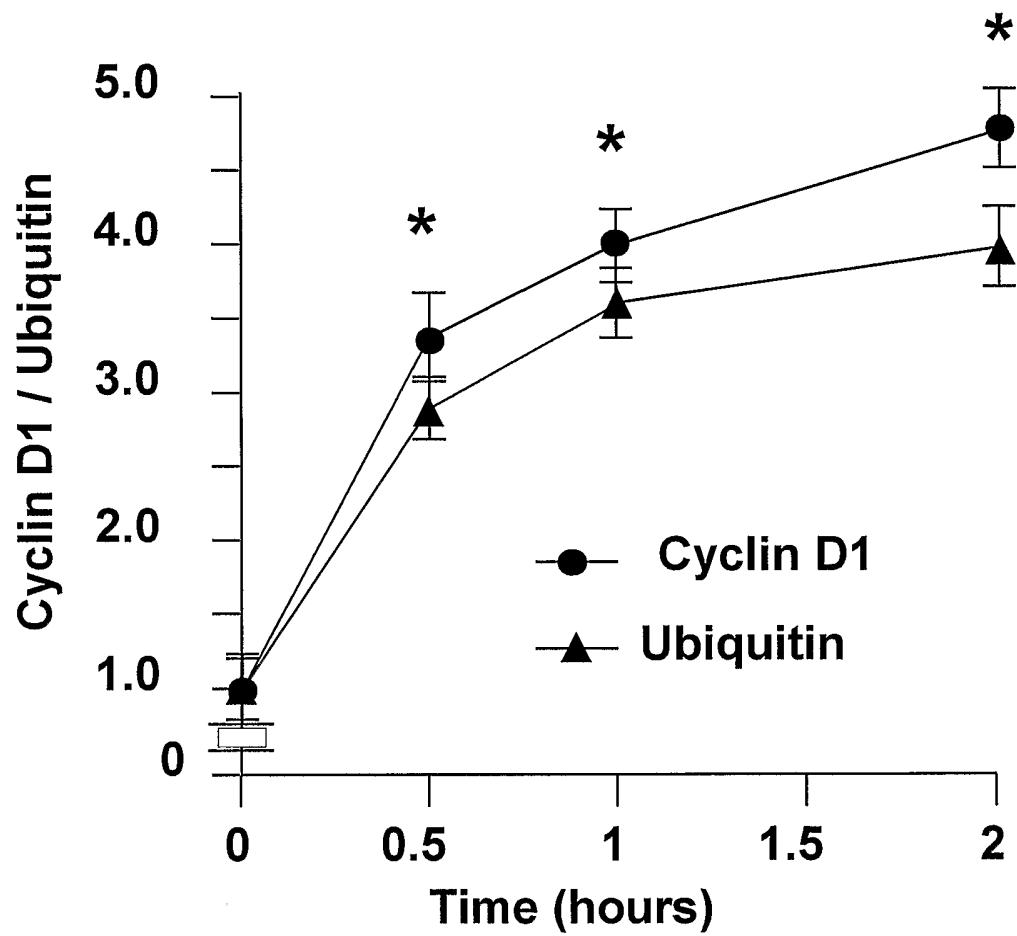


Fig. 6

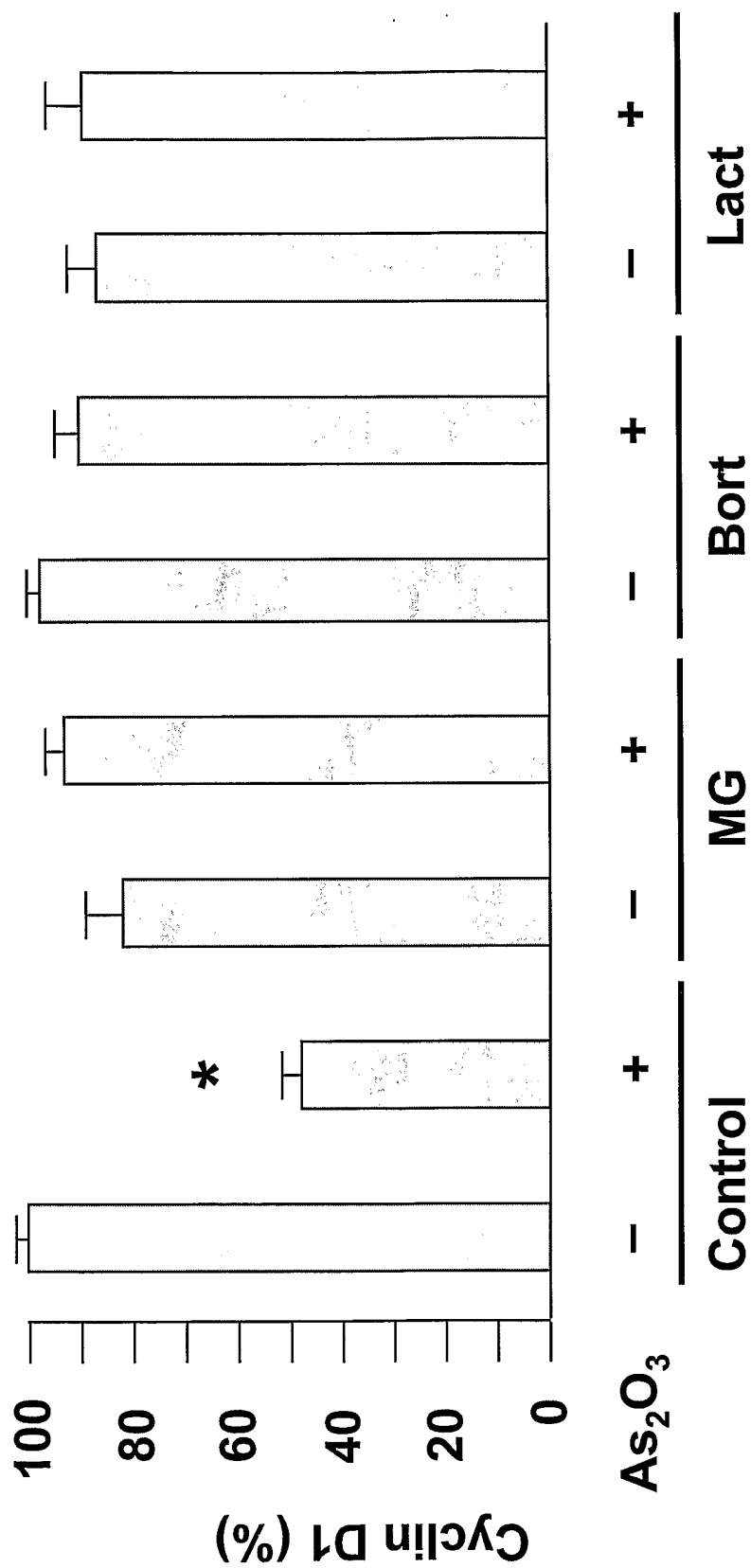


Fig. 7A

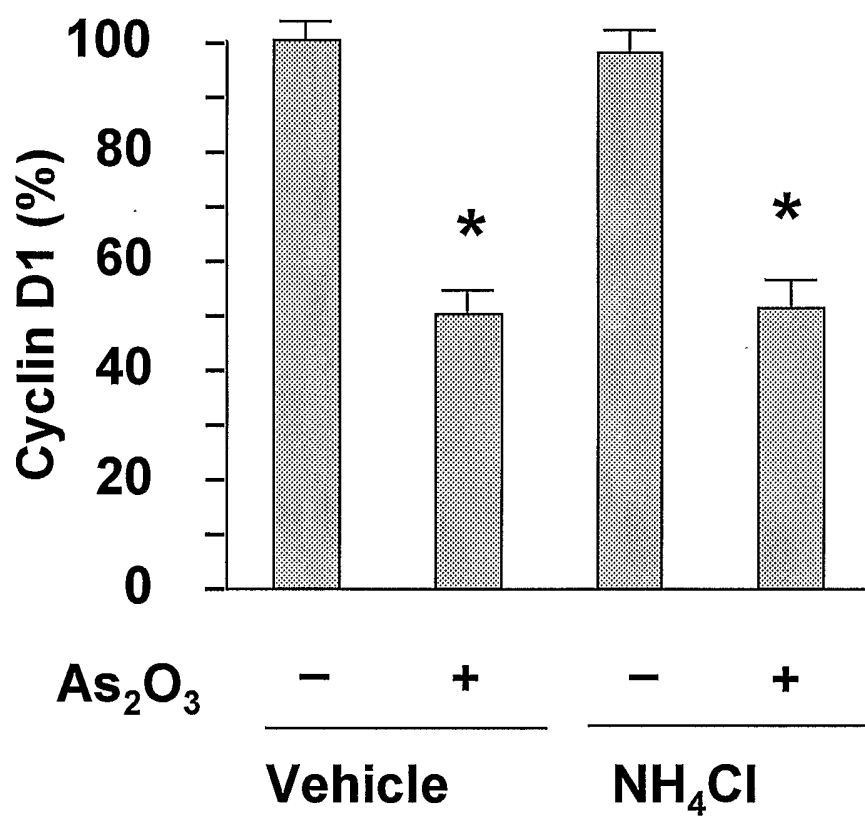


Fig. 7B

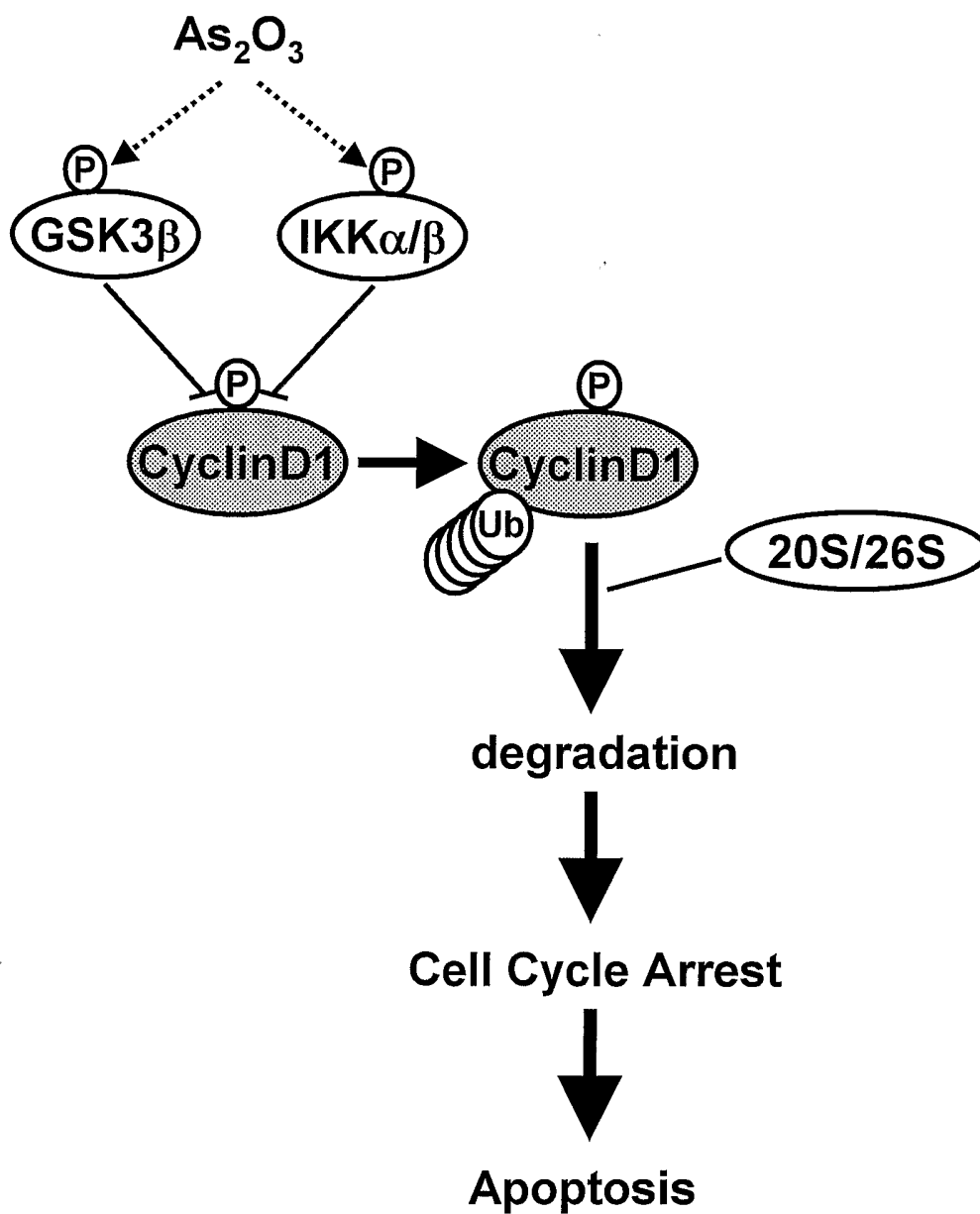


Fig. 8



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CN2007/002938

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>  <p style="text-align: center;">See the extra sheet</p> <p>According to International Patent Classification (IPC) or to both national classification and IPC</p>				
<b>B. FIELDS SEARCHED</b>  <p>Minimum documentation searched (classification system followed by classification symbols)</p> <p style="text-align: center;">IPC: A61K, A61P</p> <p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p> <p>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)</p> <p style="text-align: center;">DATABASE: WPI, EPODOC, PAJ, CNPAT(CN), CNKI(CN), CA, MEDLINE, EMBASE; SEARCH TERMS: arsenic trioxide, AS<sub>2</sub>O<sub>3</sub>, white arsenic, arsenic acid, cancer, carcinoma, tumor, TM, new growth, leukemia, leucocythemia, mantle cell lymphoma, MCL, malignant lymphoma, cyclin d1, cyclin, arsenous acid, oral administration, solution, suspension, emulsion, syrups, tablet, capsule</p>				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	Yin-Hua Tang et al., Inhibitory effect of arsenic trioxide on proliferation of hepatoma cells in mice. World Chin. J. Dig. September 2005, vol.13, No. 17, pages 2074-2077, ISSN 1009-3079	1-2		
A		3-6		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <p>* Special categories of cited documents:</p> <p>“A” document defining the general state of the art which is not considered to be of particular relevance</p> <p>“E” earlier application or patent but published on or after the international filing date</p> <p>“L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>“O” document referring to an oral disclosure, use, exhibition or other means</p> <p>“P” document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; border: none; vertical-align: top;"> <p>“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>“&amp;” document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents:</p> <p>“A” document defining the general state of the art which is not considered to be of particular relevance</p> <p>“E” earlier application or patent but published on or after the international filing date</p> <p>“L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>“O” document referring to an oral disclosure, use, exhibition or other means</p> <p>“P” document published prior to the international filing date but later than the priority date claimed</p>	<p>“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>“&amp;” document member of the same patent family</p>
<p>* Special categories of cited documents:</p> <p>“A” document defining the general state of the art which is not considered to be of particular relevance</p> <p>“E” earlier application or patent but published on or after the international filing date</p> <p>“L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>“O” document referring to an oral disclosure, use, exhibition or other means</p> <p>“P” document published prior to the international filing date but later than the priority date claimed</p>	<p>“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>“&amp;” document member of the same patent family</p>			
Date of the actual completion of the international search <p style="text-align: center;"><b>10 December 2007(10.12.2007)</b></p>		Date of mailing of the international search report <p style="text-align: center;"><b>24 Jan. 2008 (24.01.2008)</b></p>		
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451		Authorized officer <p style="text-align: center;"><b>LIANG, Jingchen</b></p> Telephone No. (86-10)62411134		

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2007/002938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Woo Hyun Park et al., Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochem.Biophys.Res.Comm. 2003, pages 230-235, ISSN 0006-291X	1-2
A		3-6
X	ZHAO Shi-guang et al., Effect of AS2O3 on cyclins D1 and B1 expression in two glioblastoma cell lines differing in p53 status. Chin J Neurosurg, May 2004, vol.20, No.3, pages 202-206, ISSN 1001-2346	1-2
A		3-6
X	CN 1546059 A (CHONGQING WEITERUI MEDICINE DEV CO LT) 17 November 2004(17.11.2004), see the whole document	7-13
A	NIE Lin et al., Studies of Apoptosis induced in malignatlymphoma cell-lines by arsenic trioxide. TUMOR (Shanghai), March 2001, Vol.21, No.2, pages79-82, ISSN 1000-7431	1-6
A	SHUI Ruohong et al., Cyclin D1 and mantle cell lymphoma. J Clin Exp Pathol. Feb 2002, Vol.18, No.1, pages86-87, ISSN 1001-7399	1-6

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2007/002938

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 1-6  
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-6 relate to methods for inhibiting cyclin D1 production in a cell, belonging to subject-matters which do not need to be searched (Rule 39.1(iv) PCT). The search has still been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/CN2007/002938

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 1546059 A	17.11.2004	none	

# INTERNATIONAL SEARCH REPORT

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## Continuation of classification of subject matter:

A61K33/36 (2006. 01) i

A61K 9/00 (2006. 01) i

A61K 9/08 (2006. 01) i

A61K 9/10 (2006. 01) i

A61K 9/20 (2006. 01) i

A61K 9/48 (2006. 01) i

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