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

(57) Abstract: It has been discovered that $As_2O_3$ suppresses tyrosine kinase receptors, in particular EGFR and IL-6R, by targeting RTKs to lysosomes and/or proteasome for degradation. This is the basis for the discovery that cancers dependent on RTKs for signaling, proliferation, survival, metastasis and differentiation can be treated with $As_2O_3$, preferably oral $As_2O_3$. Representative cancers include EGFR and cytokine dependent cancers, for example, head and neck squamous sarcomas and multiple myelomas, respectively.

# METHOD FOR INHIBITING CANCER USING ARSENIC TRIOXIDE

## Cross-Reference to Related Applications

This application claims priority to and benefit of U.S. Provisional Patent Application 60/829,126 filed on October 11, 2006 and is a continuation-in-part of U.S.S.N. 11/867,834 filed October 5, 2007, and where permitted, all of which are incorporated by reference in their entirety.

## Field of Invention

This invention relates to methods of inhibiting certain cancers by affecting expression, translation, and biological activity of cancers expressing receptor tyrosine kinases using arsenic trioxide.

## Background of the Invention

Receptor tyrosine kinases (RTK) are of pathogenic significance in many types of cancer. RTKs regulate multiple signaling pathways and contribute to cancer development, progression, proliferation, differentiation and metastasis. There are slightly ferer than 100 tyrosine kinases known. Derangement of many of these tyrosine kinases has been shown to lead to tumorigenesis in a large number of cancers, including leukemias, cancers of the head and neck, lung and breast. Therefore, targeting RTK would be an important therapeutic advance in cancer treatment. Representative cancers with RTK components include head and neck squamous cell carcinoma (HNSCC) and multiple myeloma.

Head and neck squamous cell carcinoma (HNSCC) and the RTK epidermal growth factor receptor.

Head and neck squamous cell carcinoma (HNSCC) is a common malignancy and a worldwide health problem (Jemal, et al., *CA Cancer J Clin.*, 55(1):10-30 (2005)). Despite recent advances in surgical and chemotherapeutic treatment, about 40% of patients will still develop incurable or disseminated diseases. Palliative systemic therapy with single or combination chemotherapy leads to a response in approximately 30% to 40% of cases. However, the response is often brief, and the median survival is only 6 to 9 months (Cohen, et al., *J Clin Oncol.*, 22(9):1743-52 (2004)).

The epidermal growth factor receptor (EGFR), a transmembrane protein with tyrosine kinase activity, is expressed at high levels in HNSCC, with the degree of expression correlating negatively with clinical outcome (Grandis, et al., *J Natl Cancer Inst.*, 90:824-32 (1998)). Aberrant signaling involving the EGFR plays an important pathogenetic role in HNSCC. Accordingly, EGFR represents a therapeutic target in HNSCC (Pomerantz and Grandis, *Semin Oncol.*, 31(6):734-43 (2004)). At the extracellular level, EGFR can be blocked by monoclonal antibodies or toxin conjugates directed against the ligand binding site. The intracellular part of the EGFR can be inhibited by specific tyrosine kinase inhibitors. Antisense strategies against EGFR or proteins involved in EGFR signaling have also been reported (Pomerantz and Grandis, *Semin Oncol.*, 31(6):734-43 (2004)).

Because of the extensive biologic consequences of EGFR activation, EGFR signaling is tightly regulated. Binding of EGF to EGFR results in the phosphorylation of several tyrosine residues in the intracellular portion of EGFR, including tyrosine 1045. Phosphorylated tyrosine 1045 becomes a docking site for Cbl, a protein that contains a tyrosine kinase binding domain that binds phosphorylated tyrosine, and a RING finger domain that mediates E3 ubiquitin ligase activity (Marmor and Yarden, *Oncogene*, 23(11):2057-70 (2004)). Binding of Cbl to EGFR leads to ubiquitination of intracellular lysine residues of the receptor through the attachment of multiple monoubiquitin moieties (Mosesson, et al., *J Biol Chem.*, 278(24):21323-6 (2003)). Ubiquitinated EGFR is endocytosed and targeted to degradation in lysosomes.

Arsenic trioxide ($As_2O_3$) is highly efficacious in the treatment of acute promyelocytic leukemia (APL), and has shown promise in other malignancies, notably myeloma (Kwong, *Expert Opin Drug Saf.*, 3(6):589-97 (2004)). Interestingly, one of the molecular mechanisms of $As_2O_3$ in APL is arsenic-augmented attachment of the ubiquitin-related peptide SUMO-1 to the leukemogenic PML/RARA protein, leading it to degrade in the proteosome (Lalemand-Breitenbach, et al., *J Exp Med.*, 193(12):1361-71 (2001)). Therefore, $As_2O_3$ may act by targeting oncoproteins for enhanced degradation.

Multiple myeloma and the interleukin-6 receptor (which contains a component related to RTK).

Another malignancy, multiple myeloma (MM), is a clonal plasma cell neoplasm. Conventional treatment strategies including standard chemotherapy or high-dose therapy followed by autologous stem cell transplantation are not curative in most instances. The majority of patients eventually relapse with the development of chemoresistant diseases. With conventional chemotherapeutic regimens, the median survival rate of patients with MM is about 3-5 years (Catley, et al., *Drug Resist Updat.*, 8(4):205-18 (2005)). Recent advances in the treatment of MM have focused on the development of molecular-based therapeutic agents.

A critical molecule involved in MM survival and proliferation is interleukin-6 (IL-6). IL-6 may be produced by MM cells in an autocrine fashion, and by bone marrow stromal cells. Furthermore, adhesion of MM cells to stromal cells, and other cytokines secreted by MM cells including Tumour Necrosis Factor-α, vascular endothelial growth factor and Transforming Growth Factor, can further augment IL-6 production (Van de Donk, et al., *Leukemia*, 19(12):2177-85 (2005)).

IL-6 signal transduction is mediated via the IL-6 receptor (IL-6R) complex, which comprises two subunits, IL-6Rα and gp130. IL-6Rα binds IL-6 but possesses no intrinsic activity. Formation of the IL-6/IL-6Rα complex recruits the gp130 subunit and induces gp130 homodimerisation. The cytoplasmic region of gp130 is associated with Janus kinase (JAK), which is activated upon gp130 dimerization. Phosphorylation of gp130 by JAK on specific tyrosine residues exposes a docking site for signaling molecules that contain the Src-homology-2 (SH2) domain. The three major signaling cascades thus activated include the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, the Ras/mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol-3 kinase (PI3K)/Akt pathway. While the JAK/STAT3 pathway is implicated in MM cell survival, the

Ras/MAPK and PI3K/Akt pathways are known to promote MM cell proliferation. In addition, JAK/STAT3 and PI3K/Akt signaling has been shown to protect MM cells from Fas- and dexamethasone-induced apoptosis (Cohen, et al., *J Clin Oncol.*, 22(9):1743-52 (2004)). IL-6 also plays important role in MM bone disease by stimulating osteoclastogenic factors production and osteoclast formation (Roodman, *Blood Cells Mol Dis.*, 32(2):290-2 (2004)).

Another cytokine implicated in MM pathogenesis is insulin-like growth factor-1 (IGF-1), a multifunctional protein produced by stromal and endothelial cells. IGF-1 signals through binding to the IGF-1 receptor (IGF-1R), which comprises α- and β- subunits (Tai, et al., *Cancer Res.*, 63(18):5850-8 (2003)). The β-subunit, which possesses tyrosine kinase activity, is activated upon ligand binding. IGF-1 induces MM cell proliferation and survival independent of IL-6 via activation of the MAPK and PI3K/Akt signaling pathways (Ferlin, et al., *Br J Haematol.*, 111(2):626-34 (2000); Qiang, et al., *Blood*, 99(11):4138-46 (2002)). It may collaborate with IL-6 by augmenting cell responsiveness to the latter cytokine (Abroun, et al., *Blood*, 103(6):2291-8 (2004)).

It is an object of this invention to provide agents and methods for treating cancers that are dependent on tyrosine receptor kinases, particularly EGFR and IL-6R for signaling, proliferation, survival, metastasis, or differentiation.

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 dependent on tyrosine receptor kinases, particularly EGFR and IL-6R for signaling, proliferation, survival, metastasis, or differentiation.

## Summary of the Invention

It has been discovered that $As_2O_3$ suppresses tyrosine kinase receptors, in particular EGFR and IL-6R, by targeting RTKs to lysosomes and/or proteasome for degradation. This is the basis for the discovery that cancers dependent on RTKs for signaling, proliferation, survival, metastasis and differentiation can be treated with $As_2O_3$, preferably oral $As_2O_3$. Representative

cancers include EGFR and cytokine dependent cancers, for example, head and neck squamous sarcomas and multiple myelomas, respectively.

**Brief Description of Drawings**

Figure 1A is a line graph of percent proliferating HNSCC FaDu cells versus concentration of $As_2O_3$ (μM) incubated for two days. Figure 1B is a bar graph of caspase activity (arbitrary units) versus concentration of $As_2O_3$ (μM).

Figure 2A is a bar graph of EGFR expression determined by Western blot analysis (arbitrary units) versus concentration of $As_2O_3$ (μM) in HNSCC FaDu cells incubated for a period of two days. Figure 2B is a bar graph of constitutive phosphorylation of STAT3 determined by Western blot analysis (arbitrary units) versus concentration of $As_2O_3$ (μM) in HNSCC FaDu cells incubated for a period of two days. Figure 2C is a bar graph of constitutive phosphorylation of STAT3 determined by Western blot analysis (arbitrary units) (arbitrary units) as a function of control or 10 μM anti-EGFR antibodyin HNSCC FaDu cells incubated for a period of two days. Figure 2D is a bar graph of percent proliferating HNSCC FaDu cells versus control, 2.5 μM $As_2O_3$, or 10 μM anti-EGFR antibody incubated for two days.

Figure 3 is a bar graph of percent expression of EGFR mRNA determinative quantitative reverse transcription polymerase chain reaction versus control or 2.5 μM $As_2O_3$ in HNSCC FaDu cells incubated for a period of two days.

Figure 4 is a bar graph of $As_2O_3$-induced down-regulation of EGFR determined by Western blot analysis (percent control) versus concentration of $NH_4Cl$ pretreatment of HNSCC FaDu cells incubated for a period of two days in 2.5 μM $As_2O_3$.

Figure 5 is a bar graph of percent proliferating HNSCC FaDu cells versus DMSO or UO126 in HNSCC FaDu cells incubated in 2.5 μM $As_2O_3$ for two days.

Figure 6A is a line graph of percent proliferating cells versus concentration of $As_2O_3$ (μM) in U266 myeloma cells incubated for 24 hours (▲), 48 hours (■), or 72 hours (●). Figure 6B is a line graph of percent proliferating

cells versus concentration of of $As_2O_3$ (μM) in U266 myeloma cells incubated with Z-VAD-FMK (■), or control (●). Figure 6C is a bar graph of percent stimulation of control versus concentration of $As_2O_3$ (μM) in U266 cells treated with interleukin-6 (IL-6) or insulin-like growth factor 1 (IGF-1).

Figure 7A is a bar graph of STAT-3 phosphorylation determined by Western blot analysis versus incubation of U266 cells with IL-6 (50 ng/ml) in the presence or absence of 4 μM $As_2O_3$. Figure 7B is a bar graph of Akt phosphorylation determined by Western blot analysis versus incubation of U266 cells with IL-6 (50 ng/ml) in the presence or absence of 4 μM $As_2O_3$.. Figure 7C of Erk1/2 phosphorylation determined by Western blot analysis versus incubation of U266 cells with IL-6 (50 ng/ml) in the presence or absence of 4 μM $As_2O_3$.

Figure 8 shows IL-6 internalization determined by Western blot analysis versus time in hours in U266 cells transfected with pcDNA3.1-IL-6Rα (U266-IL-6Rα) versus log signal in the presence of 4 μM $As_2O_3$.

Figure 9A is a line graph of down regulation of gp130 determined by band density in Western blots versus time in hours in U266 cells treated 4 μM $As_2O_3$ with or without 1 hour pre-treatment with the lysosomal inhibitor ammonium chloride ($NH_4Cl$, 40 mM). Figure 9B is a line graph of down regulation of IL-6α determined by band density in Western blots versus time in hours in U266 cells treated with 4 μM $As_2O_3$ with or without 1 hour pre-treatment with the lysosomal inhibitor ammonium chloride ($NH_4Cl$, 40 mM). Figure 9C is a line graph of down regulation of gp130 determined by band density in Western blots versus time in hours in U266 cells treated 4 μM $As_2O_3$ with or without 1 hour pre-treatment with MB-132. Figure 9D is a line graph of down regulation of IL-6α determined by band density in Western blots versus time in hours in U266 cells treated with 4 μM $As_2O_3$ with or without 1 hour pre-treatment withMB-132. Figure 9E is a line graph of down regulation of gp130 determined by band density in Western blots versus time in hours in U266 cells treated 4 μM $As_2O_3$ with or without 1 hour pre-treatment with the pan-caspase inhibitor Z-VAD-FMK. Figure 9F is a line graph of down regulation of IL-6α

determined by band density in Western blots versus time in hours in U266 cells treated 4 μM $As_2O_3$ with or without 1 hour pre-treatment with the pan-caspase inhibitor Z-VAD-FMK.

## Detailed Description of the Preferred Embodiments

### I. Arsenic Trioxide Formulations

Arsenic trioxide is a standard treatment for acute promyelocytic leukemia. Studies *in vitro* have shown that $As_2O_3$ may be effective in MM. $As_2O_3$ induces MM cell apoptosis by manipulating the cellular redox state, leading to decreased intracellular GSH levels, disruption of mitochondria potential and cleavage of caspases 3, 8, 9 (Gartenhaus, et al., *Clin Cancer Res.*, 8(2):566-72 (2002); Liu, et al., *Blood*, 101(10):4078-87(2003)). $As_2O_3$-induced apoptosis may also be associated with the TNF-related apoptosis inducing ligand (APO/TRAIL) pathway, which induces caspase 8-dependent apoptosis (Akay and Gazitt, *Cell Cycle*, 2(4):358-68 (2003)). Furthermore, $As_2O_3$ induces G1/G2 cell cycle arrest by upregulating the expression of the cyclin-dependent kinase (CDK) inhibitor p21, with concomitant increase in p21 binding to and hence inhibition of cell cycle-regulatory proteins CDK6, cyclin A and cyclin E, resulting in inhibition of the activities of these proteins (Park, et al., *Cancer Res.*, 60(11):3065-71 (2000)).

In preliminary clinical studies, $As_2O_3$ has also shown significant activities in MM. In a phase II study involving 14 patients with relapsed and refractory MM, response was observed in three patients, with prolonged stable disease observed in a fourth patient (Munshi, *Oncologist*, 6 Suppl 2:17-21 (2001)). In another multicenter, phase II trial of 24 similar patients, $As_2O_3$ achieved a response rate of 33%, and stable disease in an additional 25% of patients (Hussein, et al., *Br J Haematol.*, 125(4):470-6 (2004)). Furthermore, satisfactory response rates were also observed in trials using $As_2O_3$ in combination with ascorbic acid or melphalan (Bahlis, et al., *Clin Cancer Res.*, 8(12):3658-68 (2002); Berenson, et al., *Clin Lymphoma*, 5(2):130-4 (2004)).

It has been shown previously that oral arsenic trioxide can be successfully formulated for the treatment of acute promyelocytic leukemia (Kumana, et al., *Eur J Clin Pharmacol.*, 58(8):521-6 (2002)). Furthermore, oral arsenic trioxide is highly efficacious for this leukemia and results in very few side effects (Siu, et al., *Blood*, 108(1):103-6 (2006)). Recently, oral arsenic

trioxide has been shown to produce minimal QT prolongation in the heart, meaning that it is very safe for prolonged use (Siu, et al., *Blood*, 108(1):103-6 (2006)). For the treatment of other cancers, it is expected that prolonged use of arsenic trioxide is needed. Therefore, oral arsenic trioxide is preferred for treatment of patients.

**Arsenic Trioxide**

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 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 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.

*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.

*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 pyrilodone, 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.

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

## II. Methods of Treatment

Tyrosine kinase receptors play an important role in multiple signaling pathways and contribute to cancer development, progression, proliferation, differentiation and metastasis. Representative cancers that depend on TKRs include, but are not limited to, head and neck squamous cell carcinoma (HNSCC) and multiple myeloma. Representative TKRs that are involved in cancer include, but are not limited to, EGFR and IL 6R (which contains a component related to RTK). In the method described herein, an effective amount of arsenic trioxide

to downregulate the TKR in a cancer cell is administered to an individual for treating cancers that are dependent on TKRs.

### A. EGFR Dependent Cancers

Owing to the protean biologic consequences of EGFR signaling in cellular proliferation, differentiation, survival, angiogenesis and inhibition of apoptosis, suppression of EGFR holds much promise in the control of EGFR-dependent malignancies. Currently available strategies offer a two-prong extracellular / intracellular approach (Choong and Cohen, *Crit Rev Oncol Hematol.*, 57(1):25-43 (2006)). The extracellular domain of EGFR can be blocked by anti-EGFR antibodies, which prevent ligand binding to EGFR. Antibody-dependent cellular cytotoxicity may also be involved. Molecular determinants control the sensitivity to anti-EGFR antibodies, including the level of EGFR expression that correlate positively with efficacy. However, anti-EGFR antibodies possess relatively poor clinical efficacy when used as single agents, with a response rate of less that 10%. The intracellular EGFR tyrosine kinase domain of EGFR can be blocked by small-molecule tyrosine kinase inhibitors (TKI). Administered singly, TKIs showed a response rate varying between 10-20%. The response is correlated with somatic mutations in the receptor kinase domain and/or EGFR gene amplification (Jane, *Semin Oncol.*, 32(6 Suppl 10):S9-15 (2005)). implying that the efficacy of TKI is limited in cancers with wildtype EGFR.

The data presented in the Examples show that the EGFR signaling pathway was constitutively activated in the HNSCC line FaDu. FaDu was critically dependent on EGFR signaling for cellular survival and proliferation, as treatment with a blocking EGFR antibody led to apoptosis. $As_2O_3$ treatment also resulted in down-regulation of EGFR, which produced biologic effects mimicking those caused by antibody blockade of EGFR.

EGF gene transcription was active in FaDu cells. $As_2O_3$ treatment did not affect EGF transcription, ruling out disruption of EGFR signaling owing to down-regulation of EGF. Therefore, $As_2O_3$ targeted EGFR but not EGF in the

EGFR signaling pathway, suggesting that EGFR down-regulation played an important part in mediating cytotoxicity.

The data further demonstrated that EGFR down-regulation occurred at a post-transcriptional level. EGFR gene transcription was unaffected by $As_2O_3$, as shown by semi-quantitative and quantitative polymerase chain reaction. These results might be expected, as arsenic is not known to directly bind DNA or transcription factors. Therefore, $As_2O_3$ likely down-regulates EGFR by increasing its degradation.

Normal ligand dependent degradation of EGFR starts with stimulation by EGF, leading to EGFR phosphorylation at tyrosine 1045 (Marmor and Yarden, *Oncogene*, 23(11):2057-70 (2004)). The data show that $As_2O_3$ also mediated an apparent EGF independent EGFR phosphorylation at tyrosine 1045. Once this had occurred, the molecular consequences were similar to those due to ligation of EGF. Cbl was recruited to phosphorylated tyrosine 1045. By virtue of its RING figure domain that mediated E3 ubiquitin ligase activity, Cbl promoted EGFR mono-ubiquitination, targeting EGFR to lysosomal degradation (Mosesson, et al., *J Biol Chem.*, 278(24):21323-6 (2003)).

EGFR is known to be involved in signaling networks activated by stimuli that are not ligands of the receptor. Participation in these signaling pathways also entails EGFR phosphorylation similar to that induced by EGF. Non-EGF stimuli that activate EGFR include specific agonists of other membrane receptors, membrane depolarizing agents, and environmental stressors (Carpenter, *J Cell Biol.*, 146(4):697-702 (1999)). $As_2O_3$ falls into the last category, as previous studies have shown that $As_2O_3$ induces many stress proteins (Del Razo, et al., *Toxicol Appl Pharmacol.*, 177(2):132-48 (2001)). Important stress proteins include heat shock protein and the non-tyrosine kinase protein Src. Interestingly, arsenic-induced Src expression leading to phosphorylation of downstream target proteins has been well recognized. Src is in fact known to phosphorylate EGFR at all five sites that autophosphorylate upon stimulation by EGF (Hubbard and Till, *Annu Rev Biochem.*, 69:373-98 (2000); Simeonova, et al., *J Biol Chem.*, 277(4):2945-50 (2002)). The data

disclosed here showed that Src was activated by $As_2O_3$, and that inhibitors of Src abolished $As_2O_3$ induced phosphorylation of and hence Cbl binding to EGFR. These findings implicate Src as a downstream effector of $As_2O_3$.

In carcinogenicity studies, arsenic induced activation of other signaling pathways is regarded to promote cellular proliferation (Yang and Frenkel, *Environ Pathol Toxicol Oncol.*, 21(4):331-42 (2002)). The data also showed that only ERK1/2 phosphorylation was activated. However, this did not lead to a demonstrable biologic effect, as ERK inhibitor neither increased or decreased $As_2O_3$ induced cytotoxicity. JNK was activated as well, although the biologic effects are not immediately obvious in the current cell model. In leukemia cells, activation of $As_2O_3$-mediated JNK activation has been reported to contribute to induction of apoptosis (Davison, et al., *Blood*, 103(9):3496-502 (2004)).

Collectively the data show a new biological and therapeutic mechanism of controlling the EGFR signaling pathway. $As_2O_3$-induced EGFR degradation is reminiscent of the action of arsenic on another fusion oncoprotein PML-RARA in APL (Sternsdorf, et al., *Mol Cell Biol.*, 19(7):5170-8 (1999); Lallemand-Breitenbach, et al., *J Exp Med.*, 193(12):1361-71 (2001)). Previous studies had shown that $As_2O_3$ triggered conjugation of the ubiquitin-related peptide SUMO-1 to the PML part of PML-RARA. Sumolated PML-RARA is then sorted to the proteosome for degradation. In fact, $As_2O_3$-induced oncoprotein degradation had been proposed to be one of the important anti-cancer effects of $As_2O_3$. The data in the Examples show that $As_2O_3$ increased the ubiquitination of a growth factor receptor related to cancer cell survival and proliferation. These data provide a new paradigm whereby arsenic might mediate its anti-proliferative effect in cancer cells.

$As_2O_3$ is in general inhibitory to the growth of most cell lines *in vitro*. However, with cell lines commonly used in carcinogenicity studies (Qian, et al., *J Inorg Biochem.*, 96(2-3):271-8 (2003)). Arsenic has been found to be stimulatory and might therefore contribute to carcinogenesis. As shown herein, activation of Src and EGFR might be construed biochemically to be

carcinogenic. However, the ultimate biologic effect was suppression of cellular proliferation.

$As_2O_3$ is a new strategy of EGFR targeting. The therapeutic use of $As_2O_3$ is remarkably safe, side effects being usually minor. With the development of oral $As_2O_3$, cardiac QT prolongation is much reduced, so that arrhythmias are no longer a serious threat (Siu, et al., *Blood*, 108(1):103-6 (2006)). Treatment doses of $As_2O_3$ lead to plasma concentrations of 2 – 5 μM, well within the $IC_{50}$ of most cell lines tested *in vitro* (Kumana, et al., *Eur J Clin Pharmacol.*, 58(8):521-6 (2002)). $As_2O_3$ is not ligand specific, so that it does not have the tissue precision of anti-EGFR antibodies. However, it is relatively inexpensive and can be used for prolonged durations.

**B. Cytokine Dependent Cancers**

Another embodiment provides a method for treating cancer dependent on cytokines or activation of cytokine receptors by administering an effective amount of $As_2O_3$ to down regulate the cytokine receptor, preferably IL-6R, in the cancer cell. Preferably, the cytokine receptor is down regulated post-translationally. The data provided herein demonstrate that $As_2O_3$ suppressed the growth of the multiple myeloma (MM) cell line U266 by inducing apoptosis. Furthermore, $As_2O_3$ totally abrogated the stimulatory effects of IL-6 and IGF-1 on U266. $As_2O_3$ inhibited the major effector pathways of IL-6, including JAK/STAT, Akt and Erk1/2. The simultaneous suppression of several different pathways meant that a common upstream component of IL-6 signaling cascade was involved, suggesting that the IL-6R might be targeted.

It was further established that $As_2O_3$ treatment led to a dose and time dependent suppression of the IL-6 complex, including the IL-6Rα and gp130. Not only was IL-6Rα and gp130 down-regulated, the interaction of the two proteins was also decreased. The transcription of the IL-6Rα and gp130 genes was unaffected. These results implied that down-regulation of the IL-6R (IL-6Rα and gp130) occurred at the post-transcription level.

$As_2O_3$ induced down-regulation of the surface IL-6Rα expression. The down-regulation was shown to be due to an internalization of the IL-6Rα protein

in the myeloma cells. This was demonstrated by immunofluorescence studies and confirmed by flow cytometry. These results therefore suggest that $As_2O_3$ induced down-regulation of the surface IL-6Rα might be mediated by internalization of the IL-6R complex, leading to degradation in the lysosome or proteasome.

$As_2O_3$ induced IL-6Rα and gp130 down-regulation and internalization by increasing the binding of ubiquitin to these two proteins. The increase in ubiquitin binding to these proteins formed the first important step in their internalization and degradation (Marmor and Yarden, *Oncogene*, 23(11):2057-70 (2004)). This was an important novel finding, as the ubiquitination of IL-6Rα and gp130 has not been previously examined. Furthermore, pharmacological means of increasing IL-6Rα and gp130 ubiquitination has never been described before.

$As_2O_3$-induced down-regulation of IL-6Rα was alleviated by the lysosomal inhibitor $NH_4Cl$. Furthermore, the proteasome inhibitor MG132 partly alleviated the suppressive effect of $As_2O_3$ on IL-6Rα. These results confirmed that $As_2O_3$ targeted the IL-6R complex to degradation in the lysosome and maybe also the proteasome.

$As_2O_3$ kills myeloma cells by targeting and down-regulating the interleukin-6 receptor. The destruction of the cancer cells is a consequence of degradation of a growth factor receptor, by a common mechanism of increased ubiquitination, leading to degradation in the lysosome and proteasome.

Importantly, both growth factor receptors (epidermal growth factor receptor and interleukin-6 receptor) are receptor tyrosine kinases. Thus, $As_2O_3$ targets receptor tyrosine kinases by increasing their ubiquitination and therefore degradation in the lysosome and proteasome.

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

Patients with cancers that are dependent on TRKs for proliferation, survival, metastasis and differentiation can be treated with $As_2O_3$.

Representative cancers include, but are not limited to head and neck squamous cell carcinoma (HNSCC) and multiple myeloma. .

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 Related to EGFR**

**Methods and Materials**

*Cancer cell lines.*

The hypopharyngeal SCC cell line FaDu (Catley, et al., *Drug Resist Updat.*, 8(4):205-18 (2005)) was maintained in Eagle's minimum essential medium (MEM; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in 5% $CO_2$ at 37°C.

*MTT assay.*

Cellular proliferation was assessed by an MTT assay (GE Healthcare, Piscataway, NJ, USA). Briefly, cells (5,000 cells/well in 90-well plates) were incubated for 48 hours with control medium or reagents. MTT assays were then performed by incubating the treated cells with the MTT labeling solution (0.5 mg/ml). After 4 hours of incubation, cells were lysed, formazan crystals solubilized and detected at 570 nm. All experiments were performed in triplicate.

*Annexin V apoptosis assay.*

Cells were trypsinized, washed and incubated on ice for 10 minutes with FITC-conjugated annexin-V and propidium iodide (PI) (Immunotech; Fullerton, CA, USA). Apoptotic cells (annexin-V-positive, PI-negative) were enumerated by flow cytometry (Epics, Beckman Coulter, Fullerton, CA, USA) after appropriate color compensation.

*Western blot analysis.*

Western blot analysis was performed as previously reported. Briefly, cell lysis and collection of protein was conducted according to standard protocols. Protein samples (typically 20 μg) were separated by SDS-PAGE in

9% or 12% resolving gel and electro-transferred to nitrocellulose membranes. Incubation with the primary antibody was performed overnight at 4°C, followed by reaction with 1:1,500 horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia Biotechnology, Piscataway, NJ, USA). Immunoreactive bands were detected with either the Western Lightning Chemiluminescence reagent (PE Biosystems, Foster City, CA, USA), or the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL, USA), and visualized on X-ray film. Integrated density of immunoreactive bands was quantified by the BioChemi gel documentation system (UVP Inc., Upland, CA, USA). All experiments were performed in triplicates.

*Immunoprecipitation.*

Immunoprecipitation was performed by standard procedures. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) supplemented with 1mM sodium orthovanadate and Complete protease inhibitor cocktail (Complete; Roche Molecular Biochemicals), lysed with lysis buffer (50mM Tris-Cl, pH: 8, 150mM NaCl, 1% TX, 1x aprotinin (Sigma, St Louis, MO, USA), 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, and 1x Complete) at 4°C for 15 minutes. Lysates were collected and centrifuged. Protein assays were performed with the Bio-Rad Protein Assay Kit (Philadelphia, PA, USA), and adjusted to 1 μg/ μL (typically 800 to 1000 μg). Immunoprecipitation was preformed by incubation of protein samples with the appropriate antibodies (typically 4 μg) at 4°C overnight with general shaking. For each reaction, the antibody/protein complex was precipitated by incubation of 30 μL of Protein G beads (Zymed, San Francisco, CA, USA) at 4°C for 2 hour with general shaking. Protein G beads were then washed 3 times with 400 μL of lysis buffer. The supernatant was aspirated, and 50 μL of 2X Laemmli buffer was added. The antibody/protein complex was released from the beads by heating at 95°C for 10 minutes. The immunoprecipates were then analyzed by Western blotting.

*Reagents and antibodies.*

Reagents used included $As_2O_3$ and dimethylsulfoxide (Sigma, St Louis, MO, USA); EGFR inhibitor AG1478 (Biosource, Camarillo, CA, USA); Src inhibitors PP1, PP2 and PP3 (Calbiochem, Merck, Darmstadt, Germany). Antibodies used included anti-EGFR neutralizing antibody (clone LA1, Upstate Biotechnology, Lake Placid, NY, USA); rabbit anti-EGFR anti-sera and rabbit anti-cbl anti-sera (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-ubiquitin anti-sera, mouse monoclonal anti-phosphorylated tyrosine anti-sera, mouse monoclonal anti-STAT3 antibody, rabbit anti-phosphorylated EGFR (tyrosine 1045) (pEGFR-1045) anti-sera, rabbit anti-phosphorylated STAT3 (tyrosine 705) (Py-STAT3) anti-sera, mouse monoclonal anti-caspase-3 antisera, rabbit polyclonal anti-phosphorlyated Erk1/2 (p-Erk1/2) anti-sera, mouse monoclonal anti-Erk-2 anti-sera, rabbit polyclonal anti-phosphorylated p54/p46 JNK anti-sera, rabbit polyclonal anti-Src anti-sera, rabbit polyclonal anti-phosphorylated Src (tyrosine 416, p-Src-416) (Cell Signaling Technology, Beverly, Massachusetts, USA); rabbit polyclonal anti-phosphorylated Akt (p-Akt) and anti-Akt anti-sera (Pharmingen, BD Biosciences, San Jose, CA USA), mouse anti-actin antibody, (Sigma); mouse monoclonal anti-hemaglutinin (HA) antibody (Zymed).

*Plasmids and Primers.*

Plasmid encoding the full-length wild type *EGFR* in pUSEamp vector (pUSEamp-EGFR) was obtained from Upstate. Primers for reverse-transcription polymerase chain reaction (RT-PCR) of EGFR were $F_1$ (forward: 5'-TGA CTC CTT CAC ACA TAC TC-3' (SEQ ID NO:1) corresponding to bp 1320–1339 in exon 10, accession number: AF288738) and $R_1$ (reverse: 5'-TTC TCA AAG GCA TGG AGG TC-3' (SEQ ID NO:2) corresponding to bp 1432–1451 in exon 11). Primers and probes for quantification of EGFR mRNA by quantitative PCR (Q-PCR) were $Q_F$ (forward: 5'-GAC AGC ATA GAC GAC ACC TTC CT-3' (SEQ ID NO:3), corresponding to bp 3238-3260 in exon 27), $Q_R$ (5'-GCC AGC GGG CCT TTT G-3' (SEQ ID NO:4), corresponding to bp 3294-3309 in exon 28) and the TaqMan probe $Q_T$ (5'-CCA GTG CCT GAA TAC ATA AAC

CAG TCC GTT C-3' (SEQ ID NO:5), corresponding to bp 3262-3292 crossing exon 27 and 28, and dual-labeled at the 5' end with 6-carboxyfluorsecein, FAM, and the 3' end with 6-carboxytetramethylrhodamine, TAMRA). Primers and TaqMan probe were designed by the Primer Express software (PE Biosystems).

The following plasmids were be used in transfection experiments: pUSEamp-EGFR (containing the full length EGFR); p-HA-Ub (containing wildtype ubiquitin tagged to hemagglutinin); p-HA-Ub-GG (containing a mutant ubiquitin that precluded substrate binding); and p-GFP (containing green fluorescence protein).

*RNA and PCR.*

Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA). Quantitative-PCR was performed with the ABI Prism 7700 Sequence Detector (PE Biosystems) as reported (Roodman, *Blood Cells Mol Dis.*, 32(2):290-2 (2004)). Q-PCR was set up in a reaction volume of 50 μL using the TaqMan Universal PCR Master Mix (PE Biosystems), containing 10 μL cDNA, 200 and 400 nM of $Q_F$ and $Q_R$, and 50 nM QT. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Q-PCR data were collected and analysed by the ABI Sequence Detection System.

*Transfection.*

Transfection of cancer cells was performed by using Lipofectamine 2000 reagent (Invitrogen). Target cells were seeded at about 80% to 90% confluence in 60 mm tissue culture plate and incubated for 24 hours. On the day of transfection, 6 μg of each plasmid was diluted with 500 μL of serum-free MEM in separated tubes. For each transfection, 40 μL of Lipofectamine 2000 reagent was diluted to 500 μL serum-free MEM in a separated tube and incubated at room temperature for 5 minutes. After 5 minutes of incubation, each diluted plasmid was mixed with 500 μL diluted Lipofectamine 2000 reagent and incubated in room temperature for 20 minutes, leading to the formation of a DNA/Lipofectamine 2000 complex. After 20 minutes of incubation, the

complex was added to cells and incubated at 37°C for 48 hours. Cells were then selected with 50 μg/ml G418.

**Example 1: $As_2O_3$ induced apoptosis in FaDu cells by activation of caspase-3.**

MTT results showed that $As_2O_3$ inhibited FaDu cell proliferation in a dose- and time-dependent manner (Figure 1A). After 2 days of $As_2O_3$ treatment at 2.5 μM, about 60% of FaDu cells were apoptotic, as determined by flow cytometric analysis of annexin-V/PI. Western blot analysis showed that activated (cleaved) caspase-3 was progressively up-regulated with increasing concentrations of $As_2O_3$ (Figure 1B), suggesting that the apoptosis was mediated via the caspase 3 pathway.

**Example 2: EGFR signaling was necessary for FaDu cell proliferation and targeted by $As_2O_3$.**

Western blot analysis showed that FaDu cells expressed high levels of EGFR (Figure 2A). Therefore, EGFR signaling might be involved in FaDu cell proliferation. Indeed, there was constitutive phosphorylation of STAT3, a downstream effector of EGFR signaling. Western blot analysis showed that EGFR was targeted by $As_2O_3$. Treatment of FaDu cells with $As_2O_3$ led to down-regulation of EGFR and hence decreased phosphorylation of STAT3 (Figure 2B). To show that EGFR signaling was critical for cellular proliferation, FaDu cells were treated with a neutralizing anti-EGFR antibody. This led to suppression of STAT3 phosphorylation (Figure 2C), which resulted in a decrease of cellular proliferation to an extent comparable with $As_2O_3$ treatment (Figure 2D). These results suggested that $As_2O_3$ inhibited FaDu cell proliferation by targeting EGFR. RT-PCR showed that FaDu cells constitutively expressed EGF. However, the EGF transcription was not affected by $As_2O_3$ treatment.

**Example 3: $As_2O_3$ did not affect EGFR gene transcription.**

Semi-quantitative RT-PCR showed that $As_2O_3$ treatment did not affect EGFR gene transcription. The results were confirmed by Q-PCR, which showed practically no change in EGFR mRNA before and after $As_2O_3$ treatment (Figure

3). Therefore, $As_2O_3$ down-regulated EGFR by post-transcriptional mechanisms, possibly by increasing its degradation.

To investigate the reason for $As_2O_3$ induced down-regulation of EGFR, Western blot analysis was performed. Triplicate experiments showed a dose dependent increase of EGFR phosphorylation at tyrosine 1045 with As2O3 treatment (1 hour). There was yet no change in EGFR at this time point. Immunoprecipitation with an anti-EGFR antibody followed by immunoblotting with the appropriate antibodies was performed. There was a dose dependent increase in Cbl binding with $As_2O_3$ treatment (1 hour), in parallel with increase in EGFR 1045 phosphorylation. Immunoprecipitation with an anti-EGFR antibody was followed by immunoblotting with the appropriate antibodies. $As_2O_3$ (100 μM for 1 hour) led to increased EGFR phosphorylation and Cbl binding, which was totally abrogated by pre-treatment with AG 1478 (AG) (for 1 hour). These results were comparable with those observed after treatment with EGF (10 μM for 1 hour). Further experiments of the effects of $As_2O_3$ on EGFR ubiquitintion were performed. Iummunoprecipitation with an anti-EGFR antibody followed by immunoblotting with appropriate antibodies was performed. Treatment with $As_2O_3$ (100 μM for 1 hour) led to a significant increase in ubiquitination of EGFR as compared with control, suggesting that $As_2O_3$ acts by increasing EGFR ubiquitination.

To further demonstrate that Src may be involved in $As_2O_3$ actions, $As_2O_3$ induced EGFR phosphorylation and Cbl binding was investigated by Western blot analysis for the relationship of Src and its inactivation by pretreatment (for 1 hour) with Src-specific inhibitors PP1 and PP2 (but not the inactive analogue PP3), on $As_2O_3$ (100 μM for 1 hour)-induced EGFR tyrosine 1045 phosphorylation. Inhibition of Src prevented $As_2O_3$ induced EGFR phosphorylation. Immunoprecipitation with an anti-EGFR antibody was followed by immunoblotting with appropriate antibodies. Src inhibitors PP1 and PP2 decreased $As_2O_3$-induced EGFR tyrosine phosphorylation and abrogated Cbl binding. The inactive PP3 analogue did not suppress EGFR phosphorylation, but abrogated Cbl binding because of an inherent EGFR inhibiting activity.

**Example 4: $As_2O_3$ induced EGFR tyrosine 1045 phosphorylation and consequently c-cbl binding.**

More than 50% of EGFR had been down-regulated 48 hours post-$As_2O_3$ treatment. To evaluate the initial events leading to EGFR down-regulation, early time points had to be investigated. FaDu cells were therefore examined one hour after $As_2O_3$ treatment. EGFR phosphorylation at tyrosine 1045 is the first step in its degradation. Indeed, $As_2O_3$ treatment resulted in a dose-dependent increase in EGFR phosphorylation at tyrosine 1045. EGFR tyrosine 1045 phosphorylation provided a docking site for Cbl binding, which was confirmed by immunoprecipitation showing a dose dependent increase in Cbl binding to EGFR with $As_2O_3$ treatment.

**Example 5: $As_2O_3$ induced Cbl binding to EGFR is phosphorylation dependent.**

Treatment of FaDu cells with the tyrosine kinase inhibitor AG 1478 abolished $As_2O_3$ induced EGFR tyrosine phosphorylation. Expectedly, $As_2O_3$ induced Cbl binding to EGFR was also abrogated. The molecular events of $As_2O_3$ treatment were comparable with those consequent to stimulation of EGFR by EGF, which would initiate its degradation. These observations suggested that $As_2O_3$-induced EGFR phosphorylation and hence Cbl binding might be a process critical for the down-regulation of EGFR.

**Example 6: $As_2O_3$ induced EGFR degradation by ubiquitination.**

To demonstrate that $As_2O_3$-enhanced Cbl binding to EGFR resulted in ubiquitination, FaDu cells were transiently transfected with the plasmid p-HA-Ub, and then treated with $As_2O_3$. Immunoprecipitation showed that $As_2O_3$ treatment led to a significant increase in EGFR ubiquitination.

**Example 7: $As_2O_3$ suppression of EGFR was lysosome dependent.**

To show that ubiquitinated EGFR was degraded by the lysosome, FaDu cells were pre-incubated with the lysosome inhibitor ammonium chloride ($NH_4Cl$), and then treated with $As_2O_3$. The results showed that $NH_4Cl$ treatment reversed $As_2O_3$-mediated suppression of EGFR, confirming that EGFR down-regulation was lysosomal dependent (Figure 4).

**Example 8: $As_2O_3$ induced EGFR phosphorylation was related to Src activation.**

To explore the mechanism of $As_2O_3$-induced EGFR phosphorylation, mediators of EGF-independent signaling pathways were examined. The non-receptor tyrosine kinase Src has been shown to mediate EGFR phosphorylation. In FaDu cells, Src was constitutively phosphorylated. $As_2O_3$ treatment led to a further increase in Src phosphorylation. Treatment with the Src inhibitors PP1 and PP2 suppressed Src phosphorylation. Interestingly, this was associated with abrogation of $As_2O_3$-induced EGFR tyrosine 1045 phosphorylation. The inactive analogue PP3 was ineffective in suppressing Src phosphorylation, which resulted in preservation of $As_2O_3$-induced EGFR tyrosine 1045 phosphorylation. Immunoprecipitation confirmed that inhibition of Src by PP1 and PP2 led to inhibition of $As_2O_3$-induced EGFR-1045 phosphorylation, which expectedly resulted in inhibition of Cbl binding to EGFR. The inactive analogue PP3 was ineffective in suppressing $As_2O_3$-induced EGFR tyrosine 1045 phosphorylation. However, PP3 is itself a known EGFR inhibitor, which might account for the inhibition of Cbl binding despite preservation of EGFR tyrosine 1045 phosphorylation.

**Example 9: $As_2O_3$ did not target other EGFR signaling pathway effectors.**

To examine if $As_2O_3$ affected other components of EGFR signaling, downstream effectors of the pathway were studied. $As_2O_3$ did not affect Akt phosphorylation. Erk1/2 phosphorylation was increased with $As_2O_3$ treatment. However, treatment with the Erk inhibitor U0126 had no effect on $As_2O_3$ cytotoxicity (Figure 5), suggesting that activation of Erk was not a major downstream mechanism of $As_2O_3$ cytotoxicity. $As_2O_3$ also induced JNK phosphorylation, which was not associated with EGFR signaling. In contrast to EGFR, the increase phosphorylation of Erk1/2 and JNK was unrelated to Src.

## Examples Related to IL-6R

**Methods and Materials**

*Cell line.*

The human myeloma cell line U266 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovin serum (Invitrogen).

*Reagents and antibodies.*

Reagents and antibodies used included $As_2O_3$ and ATRA (Sigma, St Louis, MO, USA); bortezomib (Millennium, Cambridge, MA, USA); lysosome inhibitor ammonium chloride ($NH_4Cl$, AMRESCO, Solon, OH, USA); proteosome inhibitor MG132, p38 inhibitor SB203580, JNK inhibitor SP600125, MEK inhibitor U0126 and pan-caspase inhibitor Z-VAD-FMK (Calbiochem, La Jolla, CA, USA); rabbit polyclonal antibodies to total (and phosphorylated) JAK2 (Tyrosine-1007/1008), STAT3 (Tyrosine-705), ERK1/2 (Threonine-202/Tyrosine-204), Akt (Serine-743), p38 (Threonine-180/Tyrosine-182) and JNK (Threonine-183/Tyrosine-185) (Cell signaling Technology, Beverly, MA, USA); rabbit polyclonal antibodies to caspase-3, NF-κB, cyclin D1 and phosphorylated tyrosine (Cell signaling Technology); rabbit polyclonal antibodies to IL-6Rα (C20) and gp130 (Santa-Cruz, Sant Cruz, CA, USA); rabbit polyclonal antibody to survivin (Calbiochem); mouse monoclonal neutralising antibody against IL-6Rα, R & D Systems, Minneapolis, MN, USA); mouse monoclonal antibodies to ubiquitin, clone FK1 and FK2 (Biomol, Plymouth Meeting, PA, USA) and to actin (Sigma); horse raddish peroxide (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) and rabbit anti-mouse IgG secondary antibodies (Zymed, South San Francisco, CA, USA); and fluorescent-conjugated secondary antibody, Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA).

*Cell proliferation assay.*

Cell proliferation was analyzed by the MTT assay. Cells ($3 \times 10^4$) were cultured in 96-well plates with the tested drugs, after which 10 μl of 5 mg/ml

MTT per well was added. Four hours later, solubilizing buffer (100 μl/well) was added to dissolve the formazan crystal, followed by determination of absorbance at 570 nm. All experiments were performed in quadriplicates.

*Flow cytometric analyses.*

For evaluation of apoptosis, 1 x $10^6$ cells were treated with $As_2O_3$ (4 μM, 0 – 8 hours), washed with ice-cold phosphate-buffered-saline (PBS) and resuspended in binding buffer containing annexin V-FITC and propidium iodide (PI) (Beckman Coulter, Paris, France) for 15 minutes in dark. Apoptotic cells (annexin-V positive, popidium iodide negative) were then analyzed. For expression of IL-6Rα , 1 x $10^6$ cells were suspended in 100 μl of PBS containing 20 μl of PE-conjugated anti-CD126 (IL-6Rα ) antibody, incubated in dark for 20 minutes, and then analyzed for CD126 expression. All analyses were performed on an Epics flow cytometer (Beckman Coulter).

*Western blot analysis.*

Cells were harvested by centrifugation, washed with ice-cold PBS, and lysed with radioimmunoprecipitation buffer (RIPA) supplemented with protease inhibitors (1 mg/ml aproptinin, lupeptin, and pepstatin, 1 mM phenylmethysulfony fluoride), phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium fluoride), and protease inhibitor mix (Complete; Roche Diagnostic, Alameda, CA, USA) at 4°C for 20 minutes. Lysates were vortexed for 20 seconds thrice on ice and centrifuged at 14,000 r.p.m. for 10 minutes. Protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-rad, Hercules, CA, USA). Total cell lysate was mixed with one-forth volume of 5 x Laemmli sample buffer and heated at 95°C for 10 minutes. Proteins were resolved by sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred to nitrocellulose membrane (PE Biosystems, Foster City, CA, USA) (100V, 2 hours), and blocked by 5% non-fat milk in TBS-Tween for 1 hour. Membranes were incubated with primary antibody at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (Zymed) for 90 minutes. After washing, immuno-reactive protein bands were detected using Western Lightning Chemiluminescence

reagent (PE Biosystems), and visualised after exposure on X-ray film (Fuji, Tokyo, Japan).

*Immunoprecipitation.*

To immunoprecipitate IL-6Rα and gp130, total cell lysate was prepared from $1 \times 10^7$ cells. Cell lysate was incubated at 4°C with 4 μg of specific antibody to IL-6Rα or gp130 in 500 μl of RIPA buffer/inhibitors for 2 hours. Rec-Protein G sepharose 4B solution (40 μl) (Zymed) was added after 3 washes with 500 μl of ice-cold RIPA buffer/inhibitors. Following further incubation for 90 minutes at 4°C, beads were collected by centrifugation and washed four times with 1 ml of ice-cold RIPA buffer/inhibitors. Washed beads were then resuspended in 30 μl of 2 x Laemmli sample buffer and immunoprecipitated protein was eluted by heating at 95°C for 5 minutes.

*Semi-quantitative and quantitative polymerase chain reaction (PCR).*

Total mRNA was prepared with TRIZOL™ (Invitrogen) by standard procedures. Reverse transcription was performed with 1 μg RNA by M-MLV reverse transcriptase (Invitrogen). PCR was performed with one tenth of the cDNA with 2.5 U of Fast-Taq polymerase (Roche) in 1 x PCR Buffer, 2.5 mM $MgCl_2$, 0.2 μM of forward/reverse primers, and 250 μM of each of the four dNTP, at a final volume of 25 μl in a thermal cycler (9700, PE Biosystems). Real-time quantitative PCR (Q-PCR) was performed with the Assays-on-Demand™ Gene Expression Products (AssayID: Hs00794121_m1) (PE Biosystems) according to the manufacturer's instructions (ABI Prism 7700 Sequence Detector, PE Biosystems). The β-actin was used as an internal control for cDNA input. Sequence of primers and reaction conditions were shown in table 1. All experiments were performed in triplicates.

*Cloning of IL-6Rα expression plasmid.*

The complete coding sequence of IL-6Rα (Gene Bank accession: NM000565) was amplified from total RNA of peripheral blood cells from a normal donor (Table 1), and cloned into the *Bam*HI and *Eco*RI sites of the pcDNA3.1(+) mammalian expression vector (Invitrogen) to give pcDNA3.1-IL-

6Rα . Nucleotide sequence of the cloned IL-6Rα fragment was confirmed by DNA sequencing.

*Transfection.*

pc-DNA3.1-IL-6Rα was transfected into U266 cells by electroporation. Cells ($5 \times 10^6$) were mixed with 20 μg of vector in 300 μl of serum-free medium, and subjected to electroporation (250V, 960 μF) (Bio-Rad Gene Pulser X-Cell™, Bio-Rad). U266 cells stably over-expressing IL-6Rα (U266-IL-6Rα ) was selected and maintained in G418 (1 mg/ml).

*Immunofluorence staining and confocal microscopy.*

Sterilised coverslips were immersed in poly-L-Lysine (PLL) solution (10 mg/ml) for 20 minutes, rinsed with PBS, and placed into 6-well culture plate (1 coverslip/well). U266-IL-6Rα cells were cultured onto PLL-coated coverslips at a density of $5 \times 10^5$ cells/ml overnight. Cells were then treated with $As_2O_3$ (4 μM, 0 – 8 hours). Culture medium was removed, coverslips rinsed once with ice-cold PBS, cells fixed with 100% methanol (–20°C) for 10 minutes and air-dried. After blocking with 10% goat serum and washing, coverslips were incubated overnight at 4°C with primary antibody to IL-6Rα. Coverslips were then washed with PBS, and incubated at room temperature for 45 minutes in the dark with the Alexa Fluor® 488 $F(ab')_2$ antibody. After thorough washing, the coverslips were mounted onto glass slides with Clearmount mounting solution (Zymed) and examined under confocal microscopy.

**Example 10: $As_2O_3$ decreased U266 cellular proliferation in response to IL-6 / IGF-1 and induced apoptosis.**

$As_2O_3$ treatment of U266 led to a dose and time dependent suppression of cellular proliferation (Figure 6A). Although U266 cells grew autonomously, they still responded to additional stimulation by IL-6 and IGF-1. However, $As_2O_3$ totally negated the stimulatory effects of IL-6 and IGF-1. Flow cytometric analysis showed that $As_2O_3$ treatment resulted in apoptosis. Western blot analysis showed a dose and time dependent up-regulation of activated caspase 3 after $As_2O_3$ treatment. However, pre-treatment with the pan-caspase inhibitor Z-VAD-FMK only partly rescued U266 cells from $As_2O_3$ cytotoxicity

(Figure 6B). These results suggested that $As_2O_3$ inhibited U266 cell growth by both caspase dependent and independent mechanisms.

**Example 11: $As_2O_3$ suppressed the IL-6 signaling pathway.**

The effects of $As_2O_3$ on major signaling pathways in U266 cells as induced by IL-6 were examined. Treatment with IL-6 (50 ng/ml) induced the phosphorylation of JAK2, STAT3, Akt and ERK1/2, which were known downstream effectors of IL-6. Pre-treatment of U266 cells with $As_2O_3$ (4 μM) significantly suppressed subsequent IL-6 enhanced phosphorylation of JAK2, STAT3, Akt and ERK1/2 (Figures 6C, 7A-C). These results implied that $As_2O_3$ might inhibit IL-6 signaling by blocking components upstream of these pathways, possibly the IL-6R.

**Example 12: $As_2O_3$ targeted IL-6R.**

To test whether $As_2O_3$ targeted the IL-6R, U266 cells were pre-treated with either an IL-6R neutralizing antibody (10 μg/mL) or $As_2O_3$ (4 μuM) for 4 hours, and then stimulated with IL-6. Both the anti-IL-6R neutralizing antibody and $As_2O_3$ totally suppressed IL-6 induced STAT3 phosphorylation, implying that $As_2O_3$ might target the IL-6 receptor. Western blot analysis showed that $As_2O_3$ induced a dose and time dependent down-regulation of gp130 and IL-6Rα. At 4 μM, $As_2O_3$ decreased gp130 and IL-6Rα at as early as 2 hours, and after 8 hours, both proteins were almost completely down-regulated. The results suggested that $As_2O_3$ suppressed IL-6 signaling at the IL-6 receptor level. This was further confirmed by investigating the effect of $As_2O_3$ on IL-6R complex formation. U266 cells were treated with IL-6 with or without pre-incubation with $As_2O_3$. Cell lysates were then immunoprecipitated with an anti-gp130 antibody. Subsequent immunoblotting showed that $As_2O_3$ inhibited IL-6-induced binding of IL-6Rα to gp130, leading to decreased phosphorylation of gp130. The results showed that $As_2O_3$ targeted both IL-6Rα and gp130.

**Example 13: IL-6R was suppressed by $As_2O_3$ post-transcriptionally.**

Semi-quantitative PCR showed no change in IL-6Rα and gp130 gene transcription. This was confirmed by real-time quantitative PCR, showing that $As_2O_3$ treatment did not lead to demonstrable change in IL-6Rα and gp130

mRNA. Therefore, $As_2O_3$ suppressed IL-6Rα and gp130 at a post-transcriptional level.

**Example 14: $As_2O_3$ induced internalization of IL-6Rα.**

To examine the mechanism of down-regulation of IL-6R, U266 cells stably transfected pc-DNA3.1-IL-6Rα and hence over-expressing IL-6Rα (U266-IL-6Rα) were studied. Western blot analysis confirmed that U266-IL-6Rα over-expressed IL-6Rα by about 2,000 fold. Immunofluorescence studies under confocal microscopy showed that in untreated U266-IL-6Rα cells, IL-6Rα was detected predominantly at the cell surface. On treatment with $As_2O_3$ (4 μM), there was a time-dependent reduction in plasma membrane staining, with a redistribution of IL-6Rα intracellularly. Internalization of IL-6Rα was almost complete at 8 hours. These results were confirmed by flow cytometry, which also showed a time-dependent decrease in surface IL-6Rα expression with $As_2O_3$ treatment (Figure 8). These observations suggested that $As_2O_3$ induced internalization of IL-6Rα .

**Example 15: $As_2O_3$ induced IL-6Rα and gp130 ubiquination.**

The internalization of the IL-6R complex is partly mediated by ubiquitination. To study whether ubiquitination was involved in $As_2O_3$-induced down-regulation of IL-6R, U266 cells were incubated with $0-4$ μM of $As_2O_3$, lysed, and subjected to immunoprecipitation with anti-IL-6Rα or anti-gp130 antibodies. Immunoblotting of the immunoprecipitates with an anti-ubiquitin antibody FK2, which recognized both mono- and poly-ubiquitin moieties, showed a dose dependent increase in ubiquitination of both IL-6Rα and gp130. These results confirmed that $As_2O_3$ induced the ubiquitination of the IL-6R complex, leading to its internalization and down-regulation. Internalization of IL-6Rα occurs after ligation with IL-6, but the molecular mechanisms controlling this process and the subsequent ubiquitination of IL-6α remains undefined (Hussein, et al., Br J Haematol., 125(4):470-6 (2004)). Internalization of gp130, however, may be related to phosphorylation of a serine residue at 782 (Ser-782), which is adjacent to a di-leucine internalization motif. Western blot

analysis, however, showed that $As_2O_3$ treatment did not lead to an increase in gp130 Ser-782 phosphorylation.

**Example 16: $As_2O_3$ induced suppression of IL-6R was blocked by lysosomal inhibition.**

To investigate if $As_2O_3$-induced internalization and hence down-regulation of IL-6R was due to increased degradation, U266 cells were incubated with the lysosomal inhibitor ammonium chloride ($NH_4Cl$, 40 mM) or the proteosome inhibitor MG132 (25 μM) for 1 hour before $As_2O_3$ (4 μM) treatment. $NH_4Cl$ prevented $As_2O_3$-induced suppression of IL-6Rα and gp130 (Figures 9A-B), whereas MG132 was ineffective (Figures 9C-D). As apoptosis was induced by $As_2O_3$ treatment, and caspase-3 might also mediate protein degradation, the effect of the pan-caspase inhibitor Z-VAD-FMK on $As_2O_3$-mediated IL-6Rα degradation was examined. The results showed that inhibition of caspase-3 had no effect on $As_2O_3$-mediated IL-6Rα and gp130 degradation (Figures 9E-F). These results confirmed that $As_2O_3$ suppressed the IL-6R complex by increasing its ubiquitination, hence targeting it to degradation in the lysosome.

**Example 17: $As_2O_3$ did not affect IGF-1 signaling.**

Treatment with IGF-1 induced the activation (phosphorylation) of IGFR and Akt. $As_2O_3$ treatment exerted no effect on IGFR and Akt phosphorylation, suggesting $As_2O_3$ might not target IGF-1 signaling. Finally, $As_2O_3$ treatment had no effect on IGF-1 receptor (IGF-1R) level. These results showed that although $As_2O_3$ suppressed IGF-1 induced stimulation of U266 cells, the inhibition was not mediated via targeting of the IGF-1 signaling pathway.

We claim:

1. A method for promoting cytokine receptor or receptor tyrosine kinase degradation in cancer cells comprising contacting the cells with an amount of arsenic trioxide effective to down regulate a cytokine receptor therein.

2. The method of claim 1 wherein the cell is from a cancer dependent on the receptor tyrosine kinase for development, progression, proliferation, differentiation or metastasis.

3. The method of claim 1, wherein the cancer is an EGFR dependent cancer.

4. The method of claim 3 wherein the cancer is a head and neck squamous cell carcinoma (HNSCC).

5. The method of claim 1 wherein the cancer is dependent on the cytokine receptor for development, progression, proliferation, differentiation or metastasis.

6. The method of claim 5 wherein the cancer is a multiple myeloma.

7. The method of claim 1, wherein the cytokine receptor is IL-6R.

8. The method of claim 1 wherein the arsenic trioxide is administered orally.

9. The method of claim 1 wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for enteral administration.

10. The method of claim 1 wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for parenteral administration..

11. The method of claim 1 wherein the arsenic trioxide is present in an amount from 5 to 10 mg.

12. The method of claim 10 wherein the arsenic trioxide is in a unit dosage form selected from the group consisting of solutions, suspensions, emulsions, syrups, tablets, and capsules.

13. The method of claim 1 wherein the arsenic trioxide is administered in an amount effective to produce a plasma concentration of between 2 and 5 μM.

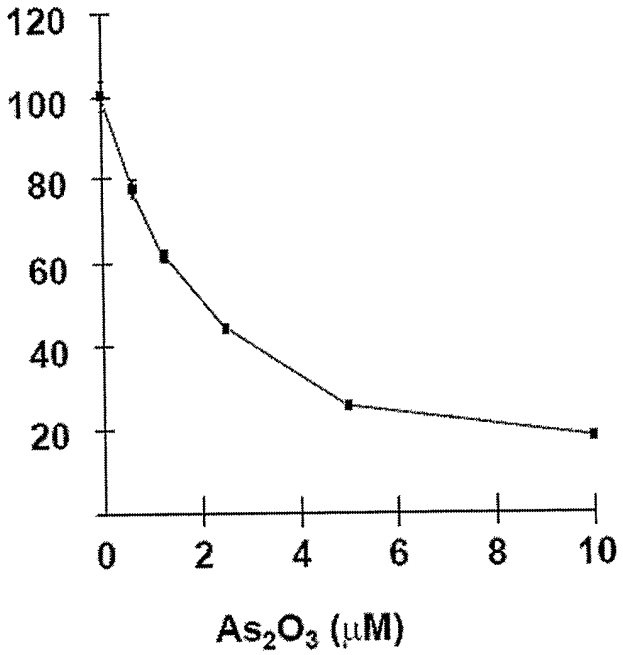



FIGURE 1A

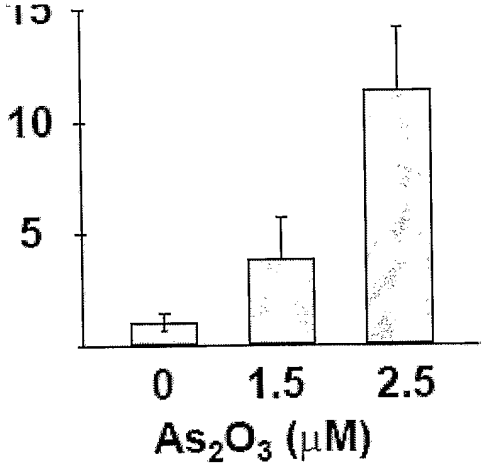



FIGURE 1B

FIGURE 2A

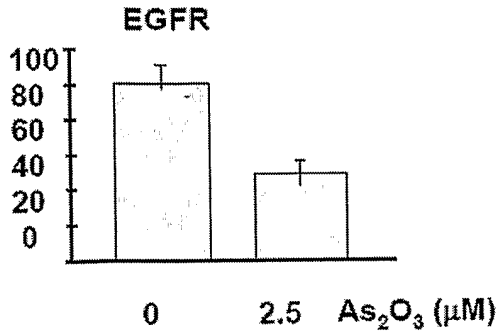



FIGURE 2B

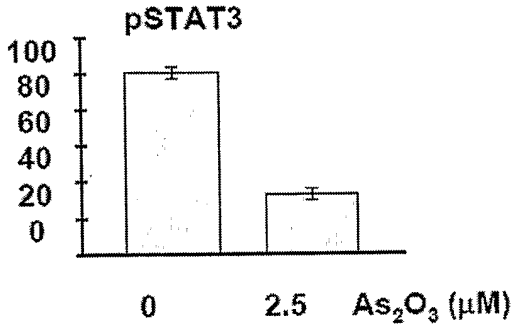


FIGURE 2C

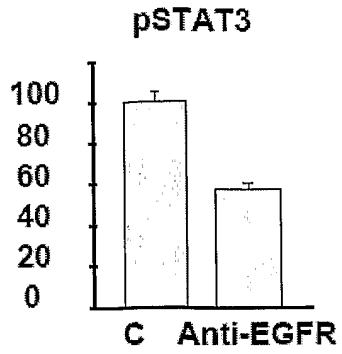
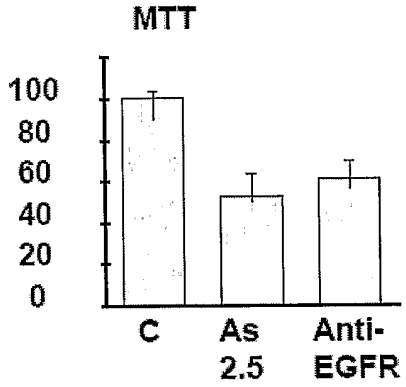



FIGURE 2D



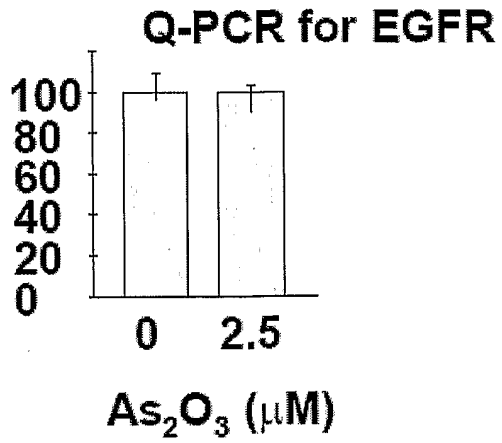



FIGURE 3

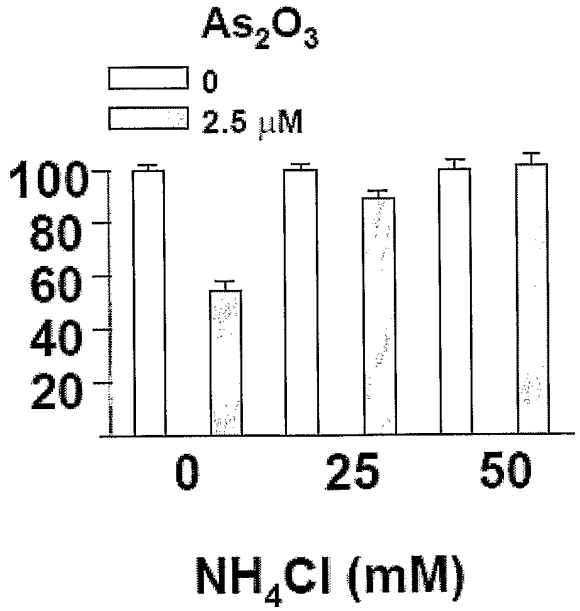



FIGURE 4

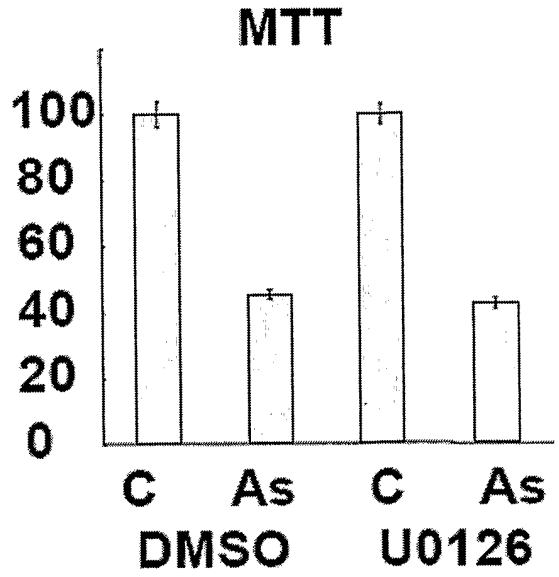



FIGURE 5

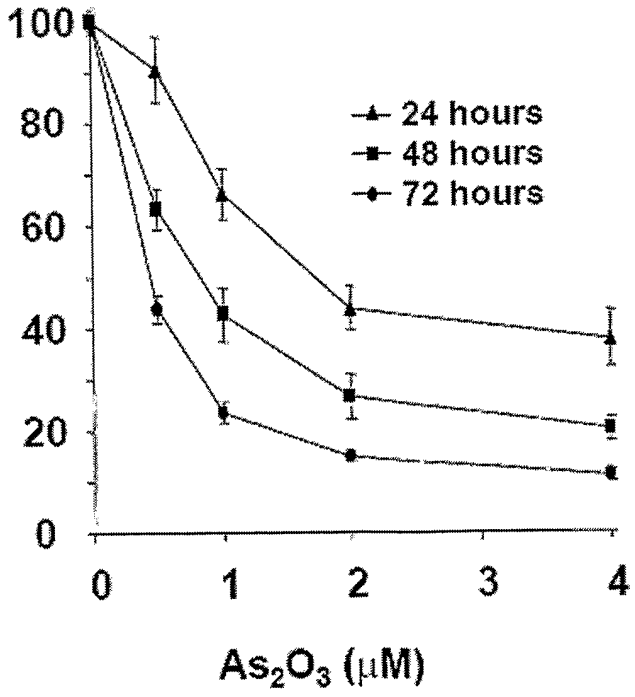



FIGURE 6A

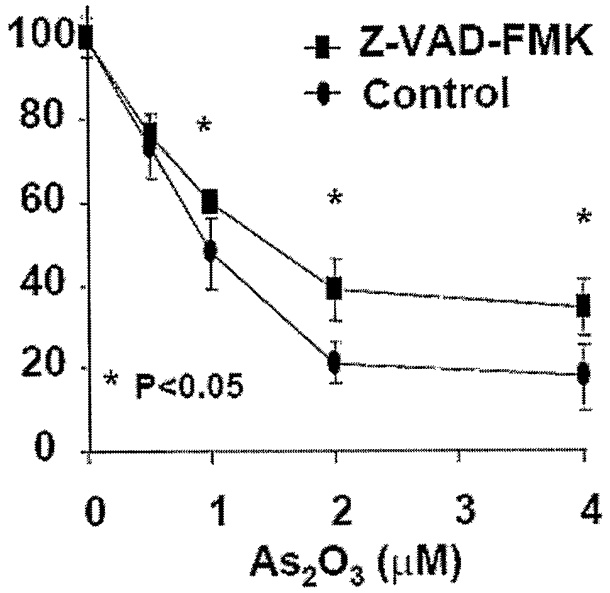



FIGURE 6B

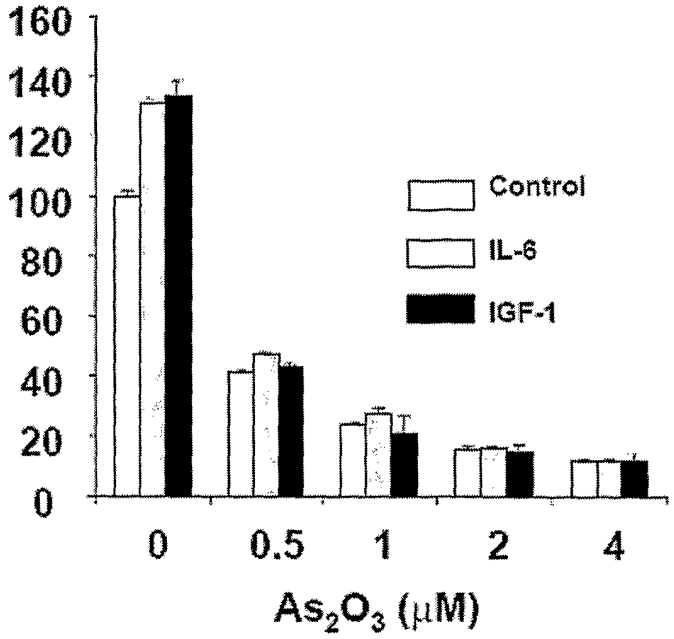



FIGURE 6C

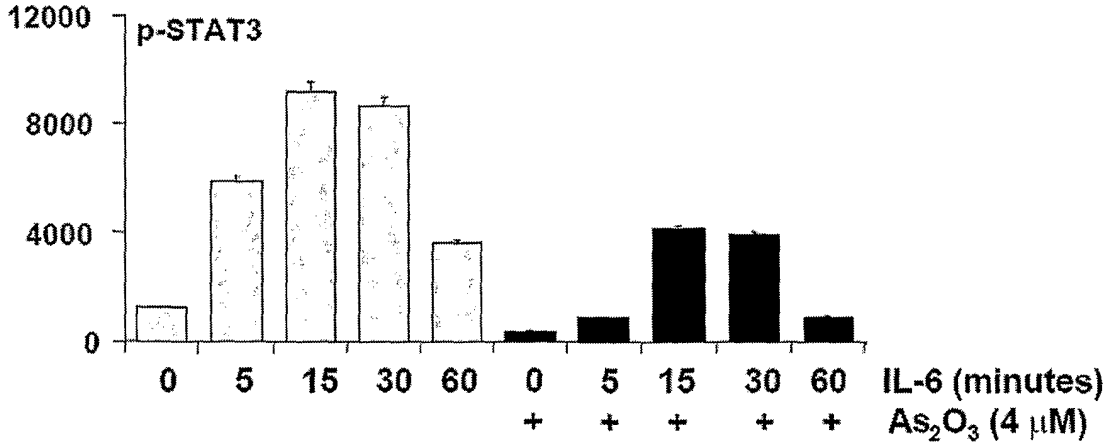



FIGURE 7A

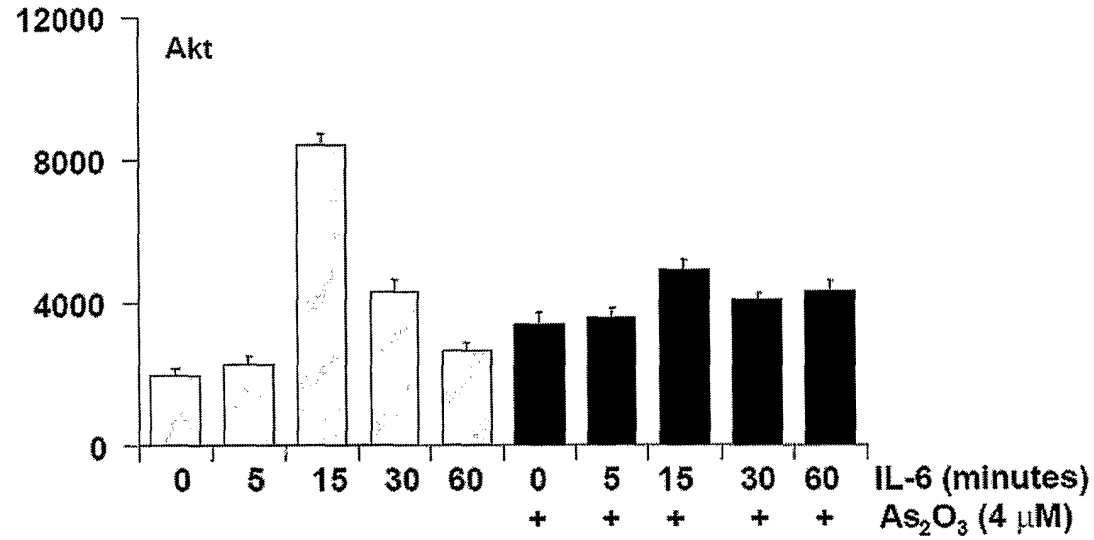



FIGURE 7B

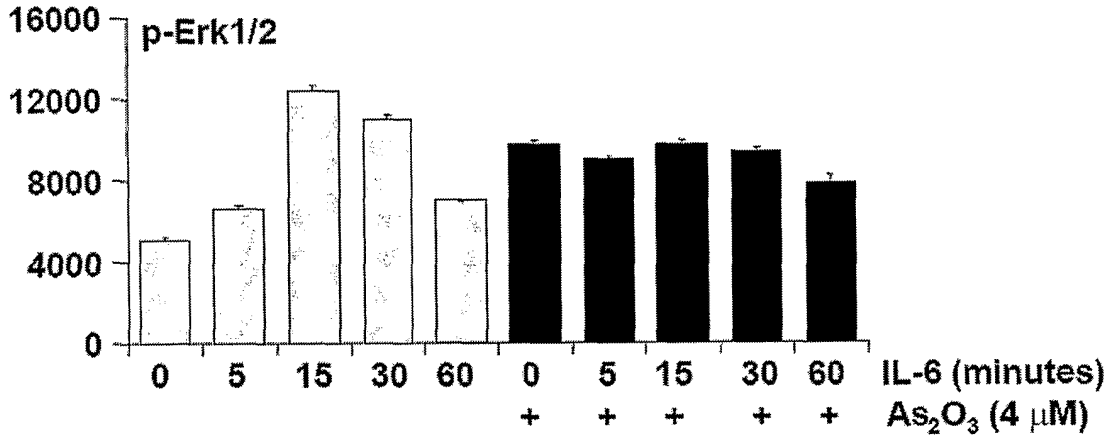



FIGURE 7C

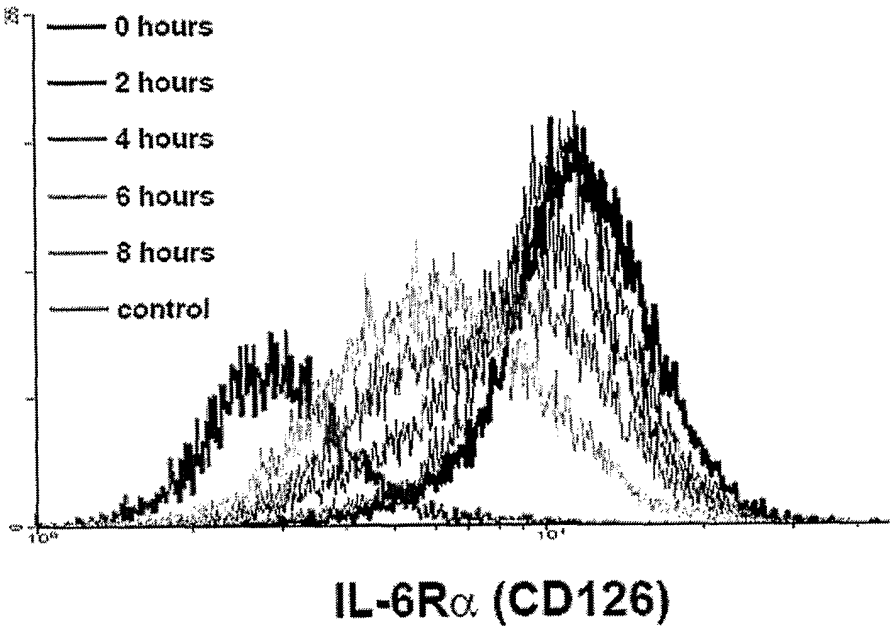
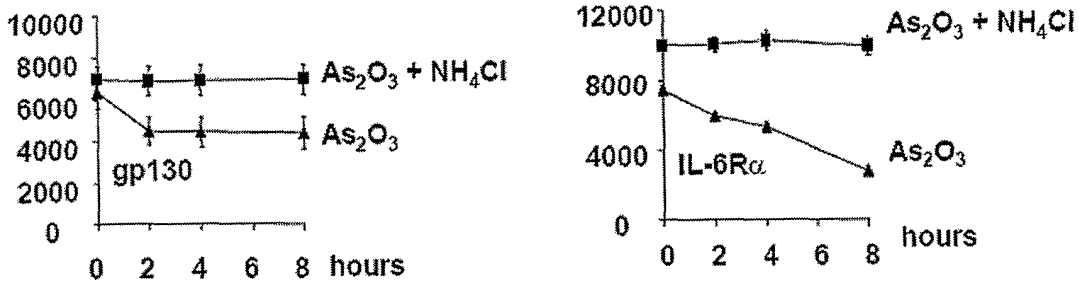
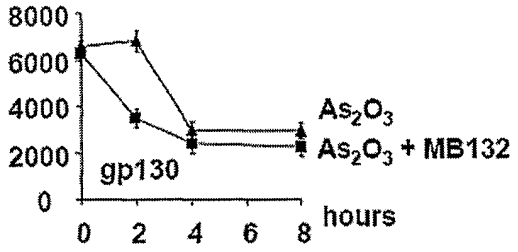
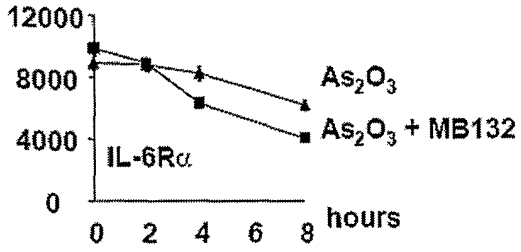



FIGURE 8

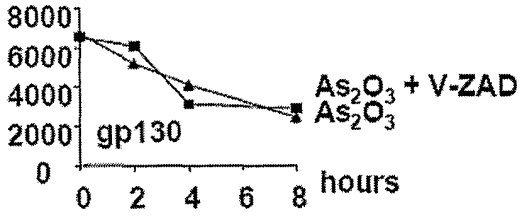


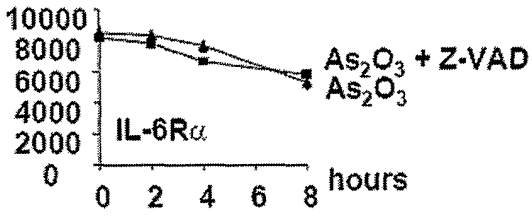

FIGURES 9A AND 9B




FIGURES 9C AND 9D




FIGURES 9E AND 9F

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2007/002919

A. CLASSIFICATION OF SUBJECT MATTER

See the extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNPAT(Cprs), CTCMPD , CNKI Full-Text database, Chinese Medicine Abstract, WPI, EPODOC, PAJ, CA, Embase, Medline; arsenic, arsenous, cytokine, tyrosine, epidermal growth factor, interleukin, receptor, acceptor, ceptor, donee, degradation, decomposition, degrade, decompose, disintegrate, disintegration,head, neck, squamous carcinoma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | CN1723029A(THE UNIVERSITY OF HONG KONG ) 18 January 2006(18. 01. 2006), see the descriptions, page 7, line 5-page 11, line 30; claims 28-40 | 1-2, 5-13 |
| X | Munshi. Arsenic Trioxide: An Emerging Therapy for Multiple Myeloma. The Oncologist,2001, 6(suppl 2), pages 17-21 | 1-2, 5-13 |
| X | CHEMICAL ABSTRACTS, abstract No. 135:116672, see CA abstract & Seol, Jae Goo, et al. Potential role of caspase-3 and –9 in arsenic trioxide-mediated apoptosis in PCI-1 head and neck cancer cells. International Journal of Oncology, 2001, Vol.18, No.2, pages 249-255 | 1-4, 8-13 |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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

"&"document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 06 January 2008(06. 01. 2008) | **24 Jan. 2008 (24.01.2008)** |
| Name and mailing address of the ISA/CN<br>The State Intellectual Property Office, the P.R.China<br>6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088<br>Facsimile No. 86-10-62019451 | Authorized officer<br>LEIYAOLONG<br>Telephone No. (86-10)62411119 |

Form PCT/ISA/210 (second sheet) (April 2007)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2007/002919

**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.: 1-13
because they relate to subject matter not required to be searched by this Authority, namely:
claims 1-13 are directed to a method of treatment of the human/animal body( Article 17 (2) (a) (i) and Rule 39. 1(iv) PCT), however, the search has been carried out and based on the alleged effects of the compounds in claims 1-13

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/CN2007/002919

| Patent Documents referred in the Report | Publication Date | Patent Family | Publication Date |
|---|---|---|---|
| CN 1723029A | 18. 01. 2006 | WO2004032822 A2 | 22. 04. 2004 |
| | | US2004126434 A1 | 01. 07. 2004 |
| | | AU2003271510 A1 | 04. 05. 2004 |
| | | EP1562616 A2 | 18. 08. 2005 |
| | | JP2006503109T | 26. 01. 2006 |
| | | AU2003271510A8 | 03. 11. 2005 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2007/002919

CLASSIFICATION OF SUBJECT MATTER:

A61K 33/36(2006. 01) i

A61P 35/00(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