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ARSENIC TRIOXIDE AND NEUROBLASTOMA CYTOTOXICITY

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ABSTRACT

The majority of aggressive forms of the childhood tumor neuroblastoma can with current treatment protocols not be cured and possess a major challenge in pediatric oncology. After initial rounds of chemotherapy, surgery and irradiation, which in most cases result in tumor regression, these aggressive neuroblastomas relapse and frequently develop drug resistance. As approximately 50% of the children with neuroblastoma have an aggressive form, there is a compelling demand for new treatment strategies. Arsenic trioxide has the capacity to kill multidrug-resistant neuroblastoma cells *in vitro* and *in vivo* and the drug is currently evaluated in clinical trials. In this report we discuss the background to the use of arsenic trioxide in cancer therapy and the currently known mechanisms by which arsenic trioxide kills human neuroblastoma cells.

Key Words: Neuroblastoma, arsenic trioxide, cell death, Bax, APL

ARSENIC TRIOXIDE – AN INTRODUCTION

Arsenic-containing substances occur naturally and different arsenical compounds have been used as medical agents for more than 2400 years (Waxman and Andersson, 2001). In traditional Chinese medicine, it was used in treatment of diseases like syphilis, psoriasis and arthritis. Fowler's solution (arsenic trioxide (As_2O_3) dissolved in potassium bicarbonate) was developed in the 18th century and this agent was used in the Western world for over 150 years to treat malignancies such as leukemia and Hodgkin's disease, as well as non-malignant diseases like eczema, psoriasis and asthma. In the mid-20th century Fowler's solution was replaced by radiation- and chemotherapy during primary treatment of leukemia (Waxman and Andersson, 2001). Besides introduction of modern treatment modalities, concerns about toxicity and carcinogenesis also contributed to the reduced use of arsenic compounds for therapeutic purposes, fostered by a growing awareness

that arsenic poisoning due to contamination of drinking water and industrial pollutants were serious public health problems in different parts of the world (Ratnaïke, 2003). However, the clinical use of arsenic trioxide was revived in the 1990s when Chinese studies demonstrated that this drug is highly effective in treatment of acute promyelocytic leukemia (APL). Those and additional studies demonstrated that low doses of the drug can induce complete remission in patients with relapsed APL with minimal general toxicity (Cohen et al., 2001; Niu et al., 1999; Shen et al., 1997; Soignet et al., 2001; Sun et al., 1992). As a result of these studies, arsenic trioxide is now an approved first-line treatment modality for these APL patients.

ARSENIC TRIOXIDE IN THE TREATMENT OF APL AND OTHER MALIGNANT DISEASES

Recently, arsenic trioxide has shown to be highly effective also in treating newly diagnosed APL (Mathews et al., 2002; Shen et al., 2004). The successful trials with untreated, newly diagnosed APL patients as well as patients with relapsed and refractory APL have contributed to an increased interest in investigating the effects of arsenic trioxide in other tumor forms. *In vitro* data have indicated that arsenic trioxide induces cell death at clinically tolerable doses in a variety of cancer cells, including non-APL leukemias like acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), multiple myeloma (MM) and chronic myelogenous leukemia (CML), reviewed in (Amadori et al., 2005), as well as lymphoma cells (Zhu et al., 1999). Effectiveness of arsenic trioxide has also been demonstrated in cancer cells from solid tumors like neuroblastoma (Akao et al., 1999; Karlsson et al., 2004; Øra et al., 2000), glioma (Kanzawa et al., 2003) and tumors originating from liver, prostate, kidney, cervix and bladder, reviewed in (Rojewski et al., 2004). Phase I and II clinical trials have been initiated to evaluate the potential effects of arsenic trioxide in various cancer types. Ongoing clinical trials in the USA are listed in Table 1.

MECHANISMS OF ACTION

The mechanisms of arsenic trioxide-induced cytotoxicity are complex and are not known in detail, but several preclinical investigations have provided insight into processes involved. In a majority of studies, the activity of arsenic trioxide has been investigated in APL cells, which carry the t(15:17) translocation generating the PML-RAR α (promyelocytic leukaemia gene product-retinoic acid receptor α) fusion protein. Induction of apoptotic mechanisms, growth inhibition as well as differentiation by arsenic trioxide have been observed in these cells (Fig. 1A) (Cai et al., 2000; Chen et al., 1997; Chen et al., 1996). Arsenic trioxide-induced differentiation of APL is most likely mediated by direct targeting of PML, which in turn induces degradation of the t(15;17)-specific fusion protein PML-RAR α (Chen et al., 1997; Chen et al., 1996). The apoptotic effect of arsenic trioxide occurs mainly through direct or indirect effects on mitochondria. For instance, arsenic trioxide induces collapse of the inner mitochondrial transmembrane potential ($\Delta\psi_m$) (Jing et al., 1999; Larochette et al., 1999), voltage-dependent anion channel (VDAC)-mediated release of cytochrome c and other apoptotic proteins, which eventually leads to the activation of caspases (Jiang et al., 2001; Kitamura et al., 2000; Soignet et al., 1998; Zheng et al., 2004), downregulation of Bcl-2 (Chen et al., 1996), modulation of p53 (Jiang et al., 2001), generation of reactive oxygen species (ROS) (Jing et al., 1999) as well as changes in the cellular glutathione level (Dai et al., 1999).

In cells other than APL, caspase-independent pathways are also involved in arsenic trioxide-induced cell death, as will be discussed in more detail below (Karlsson et al., 2004; McCafferty-Grad et al., 2003). Moreover, inhibition of angiogenesis has also been implicated in arsenic trioxide-induced cytotoxicity (Roboz et al., 2000). In addition to induction of cell death, arsenic trioxide has been shown to induce growth inhibition in various cell lines of the lympho-hematopoietic system, as well as in solid tumor cell lines (Fig. 1A),

reviewed in (Amadori et al., 2005; Rojewski et al., 2002). There is also evidence of an immunological mechanism behind the therapeutic effects of arsenic trioxide on myeloma cells (Deaglio et al., 2001), a similar immune mechanism has not been demonstrated in arsenic trioxide-treated APL.

NEUROBLASTOMA

The childhood tumor neuroblastoma is derived from precursor or immature cells of the sympathetic nervous system (Hoehner et al., 1996). Depending on tumor spread, neuroblastomas are staged from 1 to 4, where stage 4 tumors show tumor spread to distant organs, mostly to the bone marrow (Brodeur et al., 1993). Children with low stage (localized) neuroblastomas are treated by surgery alone, unless the tumor harbors an amplified MYCN gene, while children with high risk or MYCN-amplified tumors are aggressively treated with a regimen of cytotoxic drugs including etoposide, carboplatin, doxorubicin and vincristine. Despite intensive treatment and that the initial tumor response is very good in most cases, more than 60 % of the children diagnosed with high risk neuroblastoma will experience a non-treatable relapse and die from the disease (Matthay et al., 1999; Philip et al., 1997). Relapsing neuroblastomas have frequently gained multidrug-resistance and the underlying mechanisms are poorly understood, but inhibition of apoptotic pathways, due to for instance methylation of caspase 8, as well as acquired mutations of TP53 have been observed (Keshelava et al., 2001; Teitz et al., 2000; Tweddle et al., 2001). Many established neuroblastoma cell lines are derived from such multidrug-resistant tumors and these cell lines have kept the drug resistance also during *in vitro* growth (Keshelava et al., 1998). As discussed below, arsenic trioxide has the capacity to kill multidrug-resistant neuroblastoma cells both *in vitro* and *in vivo*, which forms the basis for our interest in arsenic trioxide-induced neuroblastoma cell death and the mechanisms involved.

ARSENIC TRIOXIDE-INDUCED DEATH OF MULTIDRUG-RESISTANT NEUROBLASTOMA CELLS

For unknown reasons, cultured human neuroblastoma cells are comparatively sensitive to arsenic trioxide, and concentrations needed to kill 50% of the tumor cells within 3 days of treatment are well below clinically tolerable doses (Akao et al., 1999; Øra et al., 2000). Most neuroblastoma cell lines appear to be as sensitive as many APL cell lines (Chen et al., 1996; Karlsson et al., 2004; Øra et al., 2000), and the *in vivo* effects of arsenic trioxide on multidrug-resistant neuroblastoma cells xenotransplanted in mice appear to be in the same order as those on APL-xenograft tumor growth (Lallemand-Breitenbach et al., 1999; Øra et al., 2000). Using a panel of human neuroblastoma cell lines, we could interestingly show that there was no apparent difference in sensitivity correlating to multidrug-resistance. Thus, while the four cytotoxic drugs used for induction therapy of high risk neuroblastoma patients, i.e. etoposide, doxorubicin, carboplatin and vincristine, failed to kill multidrug-resistant neuroblastoma cells at clinically relevant concentrations, arsenic trioxide efficiently killed neuroblastoma cells irrespective of their drug-resistance status (Karlsson et al., 2004). The combined effects of arsenic trioxide and either of these four cytotoxic drugs in drug sensitive cells were at least additive, and with some drug combinations we observed synergistic effects (Fig. 1B), further lending support to the hypothesis that arsenic trioxide acts via different pathway(s) than the four other cytotoxic drugs tested.

Drug sensitivity in neuroblastoma at low oxygen levels. Previously published results indicate that hypoxia (shortage of oxygen) may lead to increased treatment resistance towards certain cytotoxic drugs and ionizing radiation (Shannon et al., 2003; Teicher, 1994). Different underlying mechanisms have been suggested for these observations, such as direct effects as a result of the absence of oxygen and oxygen radicals required for the efficacy of certain cytotoxic drugs and ionizing radiation. There are also indirect effects through changes

in metabolism that decrease the drugs' cytotoxicity, and increased genetic instability that may more rapidly lead to the development of drug resistant tumor cells (Brizel et al., 1996; Schlappack et al., 1991; Shannon et al., 2003). It has also been shown that tumors with pronounced areas of hypoxia metastasize more effectively (Brizel et al., 1996; Pennacchiotti et al., 2003; Zhong et al., 1999). We therefore investigated whether a low oxygen level (1% O₂) results in deteriorated cytotoxic effects of arsenic trioxide in different neuroblastoma cells. Our data show that the cytotoxic effect is retained under hypoxic conditions, even in multidrug-resistant neuroblastoma cells such as SK-N-BE(2)c and SK-N-FI (Karlsson et al., 2005). Despite that the neuroblastoma cells subjected to hypoxia is dividing somewhat more slowly, the cytotoxic effect of arsenic trioxide is maintained. In contrast, the cell death induced by the conventionally used drug etoposide was significantly impaired in etoposide-sensitive neuroblastoma cell lines cultivated at hypoxia (Karlsson et al., 2005). Since hypoxic areas are frequent in solid tumors, these results highlight arsenic trioxide as a potential drug in treatment of patients with relapsed neuroblastomas that have ceased to respond to established therapy.

ARSENIC TRIOXIDE-INDUCED DEATH MECHANISMS IN NEUROBLASTOMA CELLS

The role of caspases. The mechanisms by which arsenic trioxide kills human neuroblastoma cells are far from understood. However, Akao et al. report that the sensitivity of cultured neuroblastoma cells to arsenic trioxide exposure correlates negatively to glutathione levels and thus is affected by redox processes (Akao et al., 1999). In agreement with these results, buthionine sulfoxide (BSO) reduces glutathione levels and makes cells more sensitive to arsenic trioxide (Dai et al., 1999). Akao et al. further found that caspase 3 was activated followed by DNA fragmentation in arsenic trioxide-treated neuroblastoma cells,

supporting their conclusion that arsenic trioxide induces cell death via an apoptotic death mechanism (Akao et al., 1999). We could confirm that caspase 3 was activated by arsenic trioxide, although the induction was weak, less than three-fold at best and in one well-characterized neuroblastoma cell line, IMR-32, no caspase 3 activation was observed (Øra et al., 2000). Furthermore, the arsenic trioxide-induced caspase 3 activation was a late event with no induction at 6 h and a slight increase at 24 h when tested in SK-N-BE(2)c cells (Karlsson et al., 2004). Furthermore, we could show that the pan-caspase inhibitor zVAD-fmk used at concentrations specifically inhibiting only caspases, did not block arsenic trioxide-induced neuroblastoma cell death and we concluded that caspase activation is not of central importance in the mechanism(s) leading to arsenic trioxide-induced cell death. We also found that arsenic trioxide does not induce neuroblastoma cells to differentiate (Øra et al., 2000) as has been reported for APL cells (Cai et al., 2000; Chen et al., 1997), a finding that exclude differentiation-induced growth arrest as an explanation for the reduced proliferation seen in arsenic trioxide-treated neuroblastoma cultures (Fig. 1A) (Karlsson et al., 2004).

The comparatively slow death response to arsenic trioxide might suggest that neuroblastoma cells need a triggering phase of arsenic trioxide exposure before the drug confers its toxic effect. To test this assumption, we treated neuroblastoma cells with arsenic trioxide for 12 and 24 h, washed the cultures and added fresh medium without the drug and cultured the cells for an additional 60 and 48 h, respectively (Fig. 1C). As shown in this figure, removal of arsenic trioxide after 12 h of exposure had a limited reducing effect on the number of cells after a total of 72 h of growth as compared to cultures either grown with or without the drug for the entire culture period, i.e. 72 h. We conclude that in neuroblastoma cells there seems to be an activation phase of arsenic trioxide that the cells need to go through before they can die. This concept is further investigated and discussed in a recently submitted

study on arsenic trioxide-induced death mechanisms in neuroblastoma (Karlsson J., Pietras A., Beckman S., Pettersson H.M., Larsson C., and Pålman S. To be published).

Bax-induced cell death. Caspase-independent cell death has previously been described and can involve activation of the proapoptotic Bcl-2 family member Bax (Miller et al., 1997; Quignon et al., 1998; Xiang et al., 1996). We therefore investigated how Bax expression in neuroblastoma cells was affected by arsenic trioxide. Although we observed a slight increase in total Bax levels after arsenic trioxide treatment that appeared to be p53-independent as the increase also occurred in p53-mutated neuroblastoma cells, the most striking effect was the induced proteolytic cleavage of full-length, p21 Bax, to a truncated p18 form (Karlsson et al., 2004). Proteolytic modification of Bax can be executed by calpains and as the calpain inhibitor calpeptin could block arsenic trioxide-induced cleavage of Bax in neuroblastoma cells we postulate that calpains become activated by arsenic trioxide, and that this process probably involves mobilization of free cellular Ca^{2+} as calpains are Ca^{2+} -dependent proteases (Karlsson et al., 2004). Calpeptin is partially toxic in itself to neuroblastoma cells, hence, a direct test of the effect on arsenic trioxide-induced neuroblastoma cell death, when calpain activity and p18 Bax formation were inhibited, could not be performed. Instead we took advantage of the facts that z-VAD-fmk at high concentrations (c:a 100 μM) also inhibits calpains and that neuroblastoma cells tolerate high zVAD-fmk concentrations. Thus, in a series of experiments we could demonstrate in arsenic trioxide-treated neuroblastoma cells that a) at a modest zVAD-fmk concentration (20 μM) caspase 3 activity was blocked but p18 Bax formation was only slightly affected, b) at 100 μM zVAD-fmk, caspase 3 activity was still blocked and so was the arsenic trioxide-induced p18 Bax formation and c) treatment with 100, but not 20 μM zVAD-fmk did block arsenic trioxide-induced cell death (Karlsson et al., 2004). Thus, we postulate that proteolytic

activation of Bax is a key event in arsenic trioxide neuroblastoma cell death and that intact p53 is not needed for this process to occur.

The calpain-dependent proteolytic truncation of p21 Bax to a p18 form has in other cell systems been shown to affect the N-terminus, and two cleavage sites generating p18 and either a 28 or a 33 amino acid peptide have been suggested (Cao et al., 2003; Toyota et al., 2003; Wood et al., 1998). More important for understanding the putative role of Bax in arsenic trioxide-induced neuroblastoma cell death is the observation in other cell systems that truncation of Bax results in a more pro-apoptotic form and that this p18 Bax seems to have its effects in mitochondria (Cao et al., 2003; Cartron et al., 2004; Choi et al., 2001; Gao and Dou, 2000; Toyota et al., 2003; Wood and Newcomb, 2000). The precise site for p18 Bax action in neuroblastoma cells can be discussed as we have preliminary experimental evidence to claim that p18 Bax is not located to mitochondria (Karlsson J., Pietras A., Beckman S., Pettersson H.M., Larsson C., and Pählman S. To be published).

Proteolytic activation of Bax in neuroblastoma cells is not uniquely associated with arsenic trioxide-induced death. In drug-sensitive human neuroblastoma cell lines, such as IMR-32 and SH-SY5Y, all four cytotoxic drugs used during induction treatment of neuroblastoma patients, etoposide, doxorubicin, vincristine and carboplatin, triggers the cleavage of p21 Bax to its p18 form (Karlsson et al., 2004). Thus, proteolytic activation of Bax seems to be a common death route in cells dying from cytotoxic stress and our preliminary data regarding localization of p18 Bax suggest that this route does not primarily involve the mitochondrion as opposed to Bax or Bad activation caused by conformational changes reported in other cell systems (Panaretakis et al., 2002). Our findings that pan-caspase inhibition does not block arsenic trioxide-induced cell death or Bax cleavage and that the fraction of cytoplasmic vs mitochondrial cytochrome c in arsenic trioxide-treated neuroblastoma cells is small, further support a non-mitochondrial p18 Bax dependent death

route (Karlsson et al., 2004). We also show that in conformity with arsenic trioxide treatment in normoxia, proteolytic activation of the proapoptotic protein Bax takes place when hypoxic neuroblastoma cells are treated with arsenic trioxide (Karlsson et al., 2005). Bax cleavage is not as pronounced, however, and it is possible that other cell death mechanisms are activated as a complement under hypoxic conditions. In conclusion, the death route by which p18 Bax works in neuroblastoma cells is at present not fully clarified and needs further attention.

A further elucidation of arsenic trioxide-evoked death routes in neuroblastoma might turn out to be rewarding as some of the effects seen so far suggest that they could be novel and lead to conceptually new routes by which xenobiotics trigger death in mammalian cells. To mechanistically understand why arsenic trioxide as opposed to many other cytotoxic drugs can kill multidrug-resistant neuroblastoma cells is another challenge that also might unravel new death executors and pathways. The comparatively slow death process induced by arsenic trioxide and a putative triggering phase, open up for transcriptional events to be important. Preliminary data from a global gene expression analysis of arsenic trioxide-treated neuroblastoma cells do indeed demonstrate changes in mRNA levels of several genes (Pettersson H.M., Dahlman A., Fredlund E., Karlsson J., and Pålman S., unpublished data). This observation suggests that altered transcription of a set of specific genes could be directly involved in the death process and their involvement is currently investigated.

There is no clinical evidence that arsenic trioxide is effective as a single drug against neuroblastoma but molecular studies suggest that there is an additive and sometimes synergistic effect with conventional cytotoxic drugs. Combination treatment with arsenic trioxide is already under investigation in adult oncology as well as in children with high risk neuroblastoma (Table 1) and we look forward with great interest to the results from these clinical trials.

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Table 1. Ongoing clinical trials using arsenic trioxide as single agent or in combination therapy, listed at the U.S. National Institutes of Health (<http://clinicaltrials.gov>) in October 2006.

Condition(s)	Phase	Title
Myelodysplastic Syndrome	Phase II	Research Study of Arsenic Trioxide (Trisenox) in Patients With Myelodysplastic Syndrome (MDS)
Acute Myelogenous Leukemia	Phase II	Trial of Arsenic Trioxide With Ascorbic Acid in the Treatment of Adult Non-APL Acute Myelogenous Leukemia
Brain Tumor	Phase I	Trial of Arsenic Trioxide and Stereotactic Radiotherapy for Recurrent Malignant Glioma
Untreated Childhood Brain Stem Glioma; High-Grade Childhood Cerebral Astrocytoma	Phase I	Arsenic Trioxide and Radiation Therapy in Treating Young Patients With Newly Diagnosed Gliomas
Stage II Multiple Myeloma; Stage III Multiple Myeloma; Refractory Plasma Cell Neoplasm; Stage I Multiple Myeloma	Phase I	Arsenic Trioxide and Ascorbic Acid Combined With Bortezomib, Thalidomide, and Dexamethasone in Treating Patients With Relapsed or Refractory Multiple Myeloma or Plasma Cell Leukemia
Metastatic Pheochromocytoma; Recurrent Pheochromocytoma; Regional Pheochromocytoma; Recurrent Neuroblastoma	Phase II	Iodine I 131 Metaiodobenzylguanidine and Arsenic Trioxide in Treating Patients With Recurrent, Progressive, or Refractory Neuroblastoma or Malignant Pheochromocytoma or Paraganglioma
Atypical Chronic Myeloid Leukemia; Chronic Idiopathic Myelofibrosis; Chronic Myelomonocytic Leukemia; Myelodysplastic and Myeloproliferative Disease; ...	Phase II	Arsenic Trioxide, Ascorbic Acid, Dexamethasone, and Thalidomide in Treating Patients With Chronic Idiopathic Myelofibrosis or Myelodysplastic or Myeloproliferative Disorders
Adult Primary Hepatocellular Carcinoma; Advanced Adult Primary Liver Cancer; Recurrent Adult Primary Liver Cancer	Phase II	Arsenic Trioxide in Treating Patients With Metastatic Liver Cancer That Cannot Be Removed by Surgery
Chronic Myelomonocytic Leukemia; Myelodysplastic Syndromes; Secondary Acute Myeloid Leukemia	Phase II	Arsenic Trioxide and Gemtuzumab Ozogamicin in Treating Patients With Advanced Myelodysplastic Syndromes
Adult Acute Erythroid Leukemia; Adult Acute Monoblastic and Acute Monocytic Leukemia; Adult Acute Myeloid Leukemia; Secondary Acute Myeloid Leukemia	Phase I	Arsenic Trioxide, Cytarabine, and Idarubicin in Treating Patients With Acute Myeloid Leukemia
Chronic Myelomonocytic Leukemia; Myelodysplastic Syndromes Childhood Cancer	Phase I/II Phase II	Azacitidine and Arsenic Trioxide in Treating Patients With Myelodysplastic Syndromes Arsenic Trioxide in Treating Patients With Advanced Neuroblastoma or Other Childhood Solid Tumors
Acute Myelogenous Leukemia; Myelodysplastic Syndrome	Phase I/II	Arsenic Trioxide in Combination With Cytarabine in Patients With High-Risk MDS and Poor-Prognosis AML
Promyelocytic Acute Leukemia Relapsed or Refractory Acute Promyelocytic Leukemia	Phase III Phase IV	Acute Promyelocytic Leukemia 2006 (APL) Treatment Protocol for Relapsed APL With Arsenic

Figure 1.

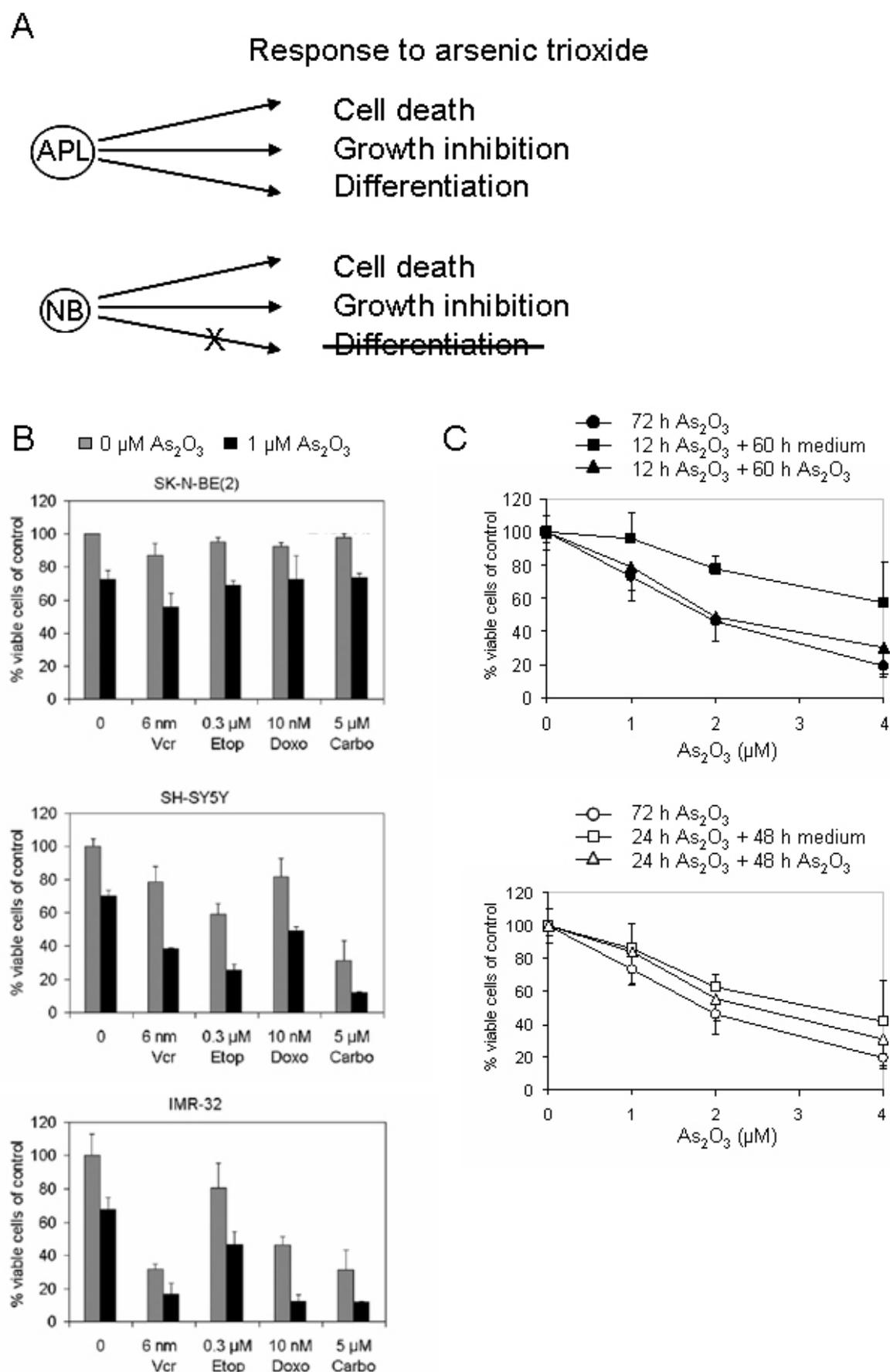


Figure 1. A, schematic picture of arsenic trioxide-induced responses in APL and neuroblastoma (NB) cells, respectively. B, additive and synergistic cytotoxic effects of arsenic trioxide and cytostatics. Neuroblastoma cells were treated with the indicated drugs for 72 h, after which cell viability was measured using the MTT assay. Data (mean \pm SD; n = 9) are presented as percentage of viable cells as compared to untreated controls. C, Neuroblastoma cells need a triggering phase of arsenic trioxide exposure before the drug confers its toxic effect. SK-N-BE(2)c cells were treated with arsenic trioxide for indicated periods of time and grown for a total of 72 h. Some of the samples were washed after 12 or 24 h of treatment, thereafter new medium or arsenic trioxide were added and the cells were grown for an additional 60 and 48 h, respectively. The amount of viable cells was evaluated using the MTT assay. Data (mean \pm SD; n = 9) are presented as percentage of viable cells as compared to untreated controls.