Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukaemia cells

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Childhood acute lymphoblastic leukaemia (ALL) is treated by combination chemotherapy with a number of drugs, always including the enzyme L-asparaginase (ASNase). Although the initial remission rate is quite high, relapse and associated drug resistance are a significant problem. *In vitro* studies have demonstrated increased asparagine synthetase (AS) expression in ASNase-resistant cells, which has led to the hypothesis that elevated AS activity permits drug-resistant survival. The data presented show that not only is elevated AS expression a property of ASNase-resistant MOLT-4 human leukaemia cells, but that short-term (12 h) treatment of the cells with ASNase causes a relatively rapid induction of AS expression. The results also document that the elevated expression of AS in ASNase-resistant cells is not fully reversible, even 6 weeks after ASNase removal from the culture medium. Furthermore, ASNase resistance, assessed as both drug-insensitive cell growth rates and decreased drug-induced apoptosis, parallels this irreversible AS expression. Mimicking the elevated AS activity in ASNase-resistant cells by overexpression of the human AS protein by stable retroviral transformation of parental MOLT4 cells is sufficient to induce the ASNase-resistance phenotype. These data document that ASNase resistance in ALL cells is a consequence of elevated AS expression and that although other drug-induced metabolic changes occur, they are secondary to the increased asparagine biosynthetic rate.

Key words: acute lymphoblastic leukaemia, apoptosis, glutamine.

INTRODUCTION

The enzyme L-asparaginase (ASNase) is a standard component of the chemotherapy for childhood acute lymphoblastic leukaemia (ALL). ASNase, when used alone, causes complete remission in 40–60 % of pediatric ALL cases [1–3]. Although the initial remission rate for childhood ALL is quite high, relapse and associated drug resistance remain a significant problem with regard to disease-free survival rates [4]. A number of laboratories have investigated *in vitro* drug sensitivity in patient-derived ALL cells using short-term *in vitro* drug treatments. Using this approach, Pieters and co-workers [5–7], as well as others [8], have documented that decreased drug sensitivity *in vitro*, including that to ASNase, is correlated with drug resistance and poor prognosis *in vivo*. There is a need to obtain a better understanding of the biochemical basis of ASNase resistance in ALL, and this need is underscored further by studies that have concluded that ASNase treatment may also be helpful in some adult ALL patients [9,10], in certain subgroups of childhood acute myelogenous leukaemia [11,12] and in refractory AIDS-related non-Hodgkin's lymphoma [13].

Based largely on *in vitro* observations demonstrating increased asparagine synthetase (AS) expression in ASNase-resistant cells, it has been hypothesized that elevated AS activity permits ASNase-resistant human leukaemia cells to survive *in vivo*. A number of non-leukaemic and/or non-human cell lines exhibit increased AS expression *in vitro* following selection for ASNase resistance [14–19]. Despite this proposed cause and effect, there is only a single study that directly correlates AS expression and ASNase resistance in primary human leukaemia cells. In a 1969 study, Haskell and Canellos [20] showed higher AS enzymic activity in five ASNase-resistant leukaemia patients compared with four drug-sensitive patients. Using the MOLT-4 human ALL cell line, Hutson et al. [18] isolated ASNase-resistant clones by maintenance in the presence of increasing concentrations of ASNase. Consistent with previous *in vitro* studies, the ASNase-resistant MOLT-4 cells contained elevated levels of AS mRNA, protein and enzymic activity [18].

The present research was designed to address two specific questions. First, is there a rapid activation of AS expression in parental (ASNase-sensitive) MOLT-4 cells, or is the known transcriptional control of the AS gene by amino acid starvation defective in these cells? Secondly, can the ASNase-resistant phenotype be induced by overexpression of AS alone (i.e. without drug selection), or does ASNase resistance depend on drug-induced changes in cell function that are independent of AS activity? The results demonstrate that there are two forms of adaptation, short-term and long-term, that occur in response to ASNase exposure. The first of these mechanisms is illustrated by the rapid induction of AS expression in response to acute ASNase treatment. In contrast, culture of resistant cells incubated in the absence of ASNase for 6 weeks (resistant/6wk − ASNase cells) demonstrated that both ASNase-dependent cytotoxicity and the elevated expression of AS are not fully reversible. Furthermore, the stable overexpression of AS in parental MOLT-4 cells induces the ASNase-resistant phenotype without any exposure to the drug. These results document the biochemical basis of ASNase resistance and suggest possible mechanisms for therapeutic intervention.

Abbreviations used: ALL, acute lymphoblastic leukaemia; AS, asparagine synthetase; ASNase, L-asparaginase; FBS, fetal bovine serum; GFP, green fluorescent protein; IRES, internal ribosome entry sequence; PE, phycoerythrin; resistant/6wk − ASNase cells, resistant cells incubated in the absence of ASNase for 6 weeks; RT, reverse transcriptase; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulphonate.

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EXPERIMENTAL

Cell culture

The human ALL cell line MOLT-4 (A.T.C.C.; CRL 1582) was propagated in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS) and 10 ml/l antibiotic/antimyotic solution ('ABAM'; 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; Gibco BRL, Gaithersburg, MD, U.S.A.). All suspension cultures were maintained at 37 °C in a 5 % CO_2 incubator. Cells were collected by centrifugation for 5 min at 228 ***g***, washed twice in PBS, and resuspended at a density of approximately 5×10^5 cells/ml in fresh medium 24 h before all experiments. To establish a model of ASNase resistance, a MOLT-4 subline was selected by incubation of parental MOLT-4 cells in increasing concentrations of ASNase (Merck, West Point, PA, U.S.A.) from 1×10^{-5}–1 unit/ml [18]. A drug-resistant cell line was subcloned by limiting dilution and was continuously maintained in RPMI 1640 medium supplemented with 1 unit/ml ASNase. To examine the reversibility of ASNase effects, resistant MOLT-4 cells were transferred and maintained in RPMI 1640 medium lacking ASNase for a period of at least 6 weeks (i.e. resistant/6wk − ASNase cells).

ASNase challenge protocol, and analysis of cell growth and apoptosis

The effect of ASNase on the growth rate of MOLT-4 cells was determined by a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulphonate (WST-1) cell proliferation assay (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Briefly, MOLT-4 cells were collected by centrifugation, washed twice with PBS, and resuspended at a concentration of 4×10^5 cells/ml in RPMI 1640 medium. The cells were treated as described in the Figure legends, then seeded in 96-well plates at a final volume of 100 μl/well. After incubation for 48 h in ASNase-containing medium at 37 °C and 5 % CO_2, 10 μl of WST-1 reagent was added per well, and the cells were incubated for an additional 2 h. The absorbance of the WST-1-containing cell supernatant, linearly related to viable cell number, was determined at 450 nm with a reference wavelength of 690 nm, as described by the manufacturer. The data are expressed as the percentage of the control [(mean absorbance of the treated wells/mean absorbance of the untreated wells) × 100]. The results are presented as means ± S.D. of assays performed in quadruplicate.

The apoptotic profile of MOLT-4 cells was determined by flow cytometry using either an annexin V-FITC or annexin V-phycoerythrin (PE) apoptosis detection kit (R&D Systems, Minneapolis, MN, U.S.A.). For each experiment, MOLT-4 cells were collected by centrifugation, washed twice with PBS, and resuspended at a concentration of 1×10^6 cells/100 μl in binding buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$]. To each 100 μl sample of cells, 10 μl of 50 μg/ml propidium iodide and 1 μl of 25 μg/ml FITC- or PE-conjugated annexin V was added, and the cells were allowed to incubate in the dark for 20 min. Following incubation, the samples were subjected to flow cytometry analysis on a Becton Dickinson FACScan instrument, University of Florida Flow Cytometry Laboratory.

Northern-blot analysis for AS mRNA

Total cellular RNA was isolated with a Qiagen RNeasy kit (Qiagen, Valencia, CA, U.S.A.) from MOLT-4 cells (4×10^5 cells/ml) that had been incubated in RPMI-1640 medium with or without ASNase (1 unit/ml) for 12 h at 37 °C. For Northern-blot analysis, 15 μg of total RNA/lane was size-fractionated in a 1 % (w/v) agarose/6.6 % (v/v) formaldehyde gel and then blotted by downward capillary transfer on to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL, U.S.A.) in 10 × standard sodium citrate ('SSC'; 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). The membranes were cross-linked by ultraviolet exposure in a Gene Linker UV chamber (Bio-Rad, Hercules, CA, U.S.A.) and hybridized with a ^{32}P-radiolabelled AS cDNA probe generated with a Strip-EZ DNA random primed StripAble synthesis kit (Ambion, Austin, TX, U.S.A.). Unincorporated nucleotides were removed by centrifugation through a ProbeQuant G-50 micro-spin column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Hybridization was performed at 61 °C in a buffer solution [0.5 M sodium phosphate (pH 7.2), 7 % (w/v) SDS, 1 mM EDTA (pH 8.0) and 1 % (w/v) BSA; pH values correspond to the pH values of stock solutions]. After overnight incubation, all membranes were washed four times for 10 min with a high-stringency wash solution [0.04 M sodium phosphate (pH 7.2), 1 mM EDTA (pH 8.0) and 1 % (w/w) SDS; pH values correspond to the pH values of stock solutions] at 65 °C. The membranes were exposed to BioMax MR film (Kodak, Rochester, NY, U.S.A.), and the exposed films were quantified using an UN-SCAN-IT software package (Silk Scientific, Orem, UT, U.S.A.). Each experiment was repeated using different batches of cells and medium to ensure reproducibility. The human AS cDNA probe was obtained by reverse transcriptase (RT)-PCR using specific primers (5′-TTGTCGACATCACCCTGACCTGCTTACGCC-3′ and 5′-TTGTCGACGTTCCCCTATCTACCCACAGTCC-3′), based on the published rat AS cDNA sequence [21]. This RT-PCR reaction yielded the expected 1842 bp coding region of the human AS sequence, which was subsequently inserted into the pCR2.1 vector using a TA cloning system (Invitrogen, Carlsbad, CA, U.S.A.).

Immunoblotting analysis for AS protein content

MOLT-4 cells were collected and subjected to the same incubation conditions as described in the previous section. Following incubation, the cells were washed twice by centrifugation in PBS and then resuspended in sample dilution buffer [0.125 M Tris (pH 6.8), 1 % (w/v) SDS, 20 % (v/v) glycerol, 30 mg/ml Bromophenol Blue and 715 mM 2-mercaptoethanol]. The protein homogenates were incubated overnight at 4 °C with end-over-end rotation, sonicated by three 5 s bursts (Model 60 Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA, U.S.A.), and then centrifuged at 20800 ***g*** for 30 min at 4 °C to pellet insoluble debris. Protein concentration was determined on a 20 μl sample of each supernatant by adding 1.5 ml of ice-cold 10 % (w/v) trichloroacetic acid and incubating overnight at 4 °C. After centrifugation at 20800 ***g*** for 30 min at 4 °C, the supernatant was carefully removed, and the protein pellet was quantified using a modification of the Lowry assay [22]. A 40 μg sample of each extract was diluted 1:1 (v/v) with sample dilution buffer, heated at 65 °C for 10 min, and then subjected to immunoblotting using a monoclonal antibody specific for the AS protein [18].

Overexpression of AS cDNA by viral delivery

The human AS cDNA was cloned into the retroviral vector pBMN-IRES-GFP [23]. This viral vector contains a polylinker for cloning the cDNA of interest upstream from an internal ribosome entry sequence (IRES) which, in turn, is upstream of a

green fluorescent protein (GFP) sequence, and the entire transcription is driven by the viral long terminal repeat sequence. When transduced into target cells, the cDNA of interest is simultaneously expressed along with GFP, allowing the AS-producing cells to be sorted using FACS. To create pAS-IRES-GFP, the AS cDNA was subcloned into pBMN-IRES-GFP by digesting the vector with *Bam*HI and *Not*I, which was then gel-purified and combined with a purified AS insert having compatible ends. A positive clone with the correct AS orientation was grown for large-scale plasmid preparation and used to generate retrovirus. To overexpress AS in parental (ASNase-sensitive) MOLT-4 cells, a Moloney leukaemia virus retroviral infection system was used [23]. To generate retrovirus, the 293T-derived 'phoenix' cell line (2×10^6 cells/60 mm dish), stably transfected with the *gag-pol* and *env* viral proteins and having an ecotropic host range, was incubated in Dulbecco's modified Eagle's medium for 24 h and then transiently transfected with the pAS-IRES-GFP or the pBMN-IRES-GFP vector alone. The transfection mixture consisted of 4 μg of plasmid DNA complexed to 12 μl of FuGENE transfection reagent (Boehringer Mannheim). Following 48 h of culture, the medium was replaced with fresh RPMI 1640 medium containing 10 % (v/v) FBS, and the cells were cultured for an additional 24 h at 32 °C prior to collecting the virus-containing supernatant. To increase transduction efficiencies, the parental MOLT-4 cells were stably transduced with a retrovirus that expresses the ecotropic receptor and, therefore, enhances viral binding [24]. Transduction of these parental MOLT-4 cells was performed as follows. Cells (5×10^5) were pelleted and resuspended in 1 ml of the appropriate viral supernatant (AS-IRES-GFP or BMN-IRES-GFP) containing 1 μl of 5 mg/ml polybrene. They were then centrifuged for 90 min at 500 ***g*** to enhance the infectivity of the viral preparation. Following incubation at 32 °C for 24 h, the viral supernatant was removed, and the cells were resuspended in fresh medium and cultured at 37 °C. The expression of the transgenes was monitored by fluorescence microscopy to detect GFP expression.

RESULTS AND DISCUSSION

AS expression following ASNase treatment of MOLT-4 cells

Selection of the ASNase-resistant MOLT-4 human leukaemia cells used in the present study and the resulting increase in AS expression have been described previously [18]. Although it is known that incubation of cells in medium lacking an essential amino acid induces AS gene transcription [25,26], there are no reported investigations of the changes in AS content that occur immediately after ASNase treatment of human leukaemia cells. Therefore experiments were designed to test for both short-term and long-term adaptation of AS expression in response to ASNase. As reported previously [18], the ASNase-resistant MOLT-4 cells, continually maintained in the drug, exhibited a much higher level of basal AS mRNA than parental cells (Figure 1A, lanes 1 and 2). The AS mRNA content in parental MOLT-4 cells was increased significantly following a 12 h incubation in the presence of ASNase (Figure 1B, lanes 1 and 2). Consistent with previous data showing that protein and enzymic activity parallel mRNA levels, the level of AS protein was increased as well (results not shown). These data are consistent with the belief that ALL cells have relatively low expression of AS prior to ASNase therapy, but the results argue against the possibility that ALL cells are selectively sensitive to ASNase, because their AS activity is not adaptively up-regulated in response to the drug. Clearly, acute ASNase exposure causes induction of AS expression within hours. To determine if the increased AS expression in the ASNase-resistant cells was reversible, the cells were cultured in the absence of ASNase for 12 h (Figure 1B, lane 3) or 6 weeks (Figure 1A, lane 3). The elevated AS mRNA content present in the resistant cells declined during the 12 h incubation in the absence of the drug (Figure 1B, compare lanes 3 and 4), but even the resistant/6wk − ASNase cells contained an AS mRNA content greater than that in the parental cells (compare lanes 1 and 3 of Figure 1A). Interestingly, despite the elevated AS mRNA content of the resistant/6wk − ASNase cells,

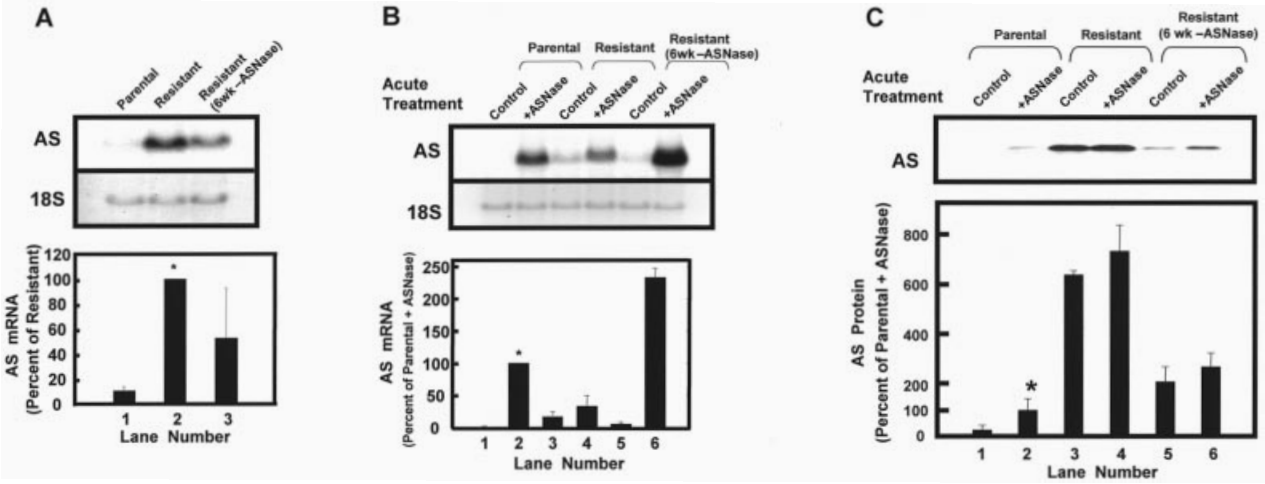

Figure 1 Expression of AS mRNA and protein following ASNase treatment of MOLT-4 Cells

Parental, resistant and resistant/6wk — ASNase MOLT-4 cells (1×10^4 cells/ml) were subjected to Northern-blot analysis for AS mRNA. The reversibility of the AS mRNA induction in drug-selected resistant cells was determined using the resistant/6wk — ASNase cells. The blot (15 μg/lane) was probed with a ^{32}P-radiolabelled AS cDNA and the ethidium bromide stain of the 18 S ribosomal RNA served as a measure of lane loading. The quantified data from three independent experiments were normalized to parental cells [the treatment condition is indicated with an asterisk above the bar (lane 2 in panels **A**–**C**)] and plotted as a bar graph. (**A**) Extended exposure of a Northern blot containing the mRNA samples from parental cells, resistant cells and resistant/6wk — ASNase cells (lanes 1, 3 and 5 from **B**) is presented to show the relative levels of AS expression in these cell lines. (**B**) The effect on all three cell populations of acute ASNase (1 unit/ml) treatment or withdrawal for 12 h. (**C**) Illustrates the immunoblot data from cells incubated under identical conditions to those described above for the Northern-blot analysis.

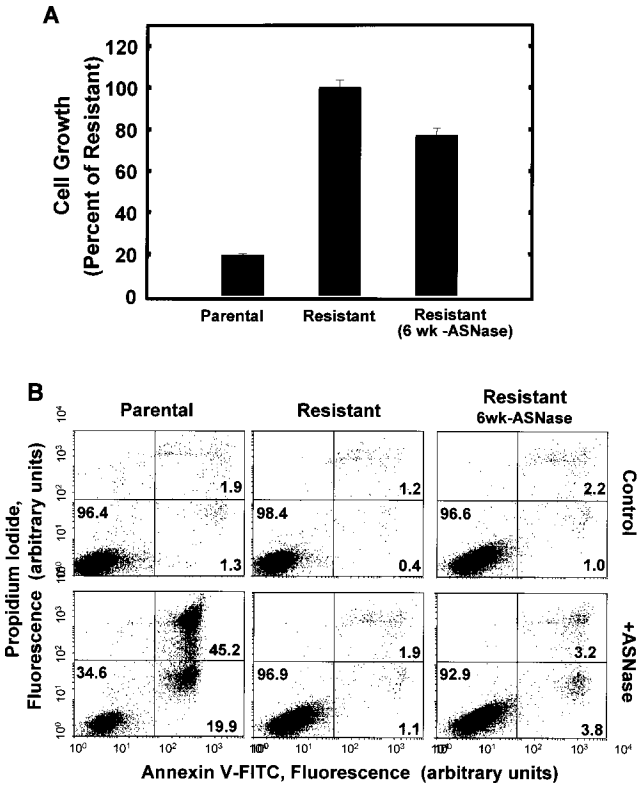

Figure 2 Effect of ASNase on cell growth and viability of MOLT-4 cells

Parental and resistant MOLT-4 cells (1×10^4 cells/ml) were incubated for 48 h in the absence (Control) or presence (+ASNase) of 1 unit/ml ASNase. Both a WST-1 cell proliferation assay (**A**) and an annexin V-FITC apoptosis assay (**B**) were performed, as described in the text. The reversibility of the ASNase-resistant phenotype was determined using the resistant/6wk−ASNase cells. For the WST-1 cell growth assay, the absorbance (i.e. cell number) of each cell population is plotted as a percentage (±S.D., $n = 4$) of the ASNase-resistant value. For the apoptosis analysis, the numbers in each quadrant indicate the percentage of cells from a total of 10000 cells. Viable cells appear in the lower left-hand quadrant (propidium iodide and annexin V negative), whereas cells in the right-hand quadrants denote apoptotic (lower quadrant) and apoptotic/necrotic (upper quadrant) cells. The apoptosis data presented are representative of three independent experiments.

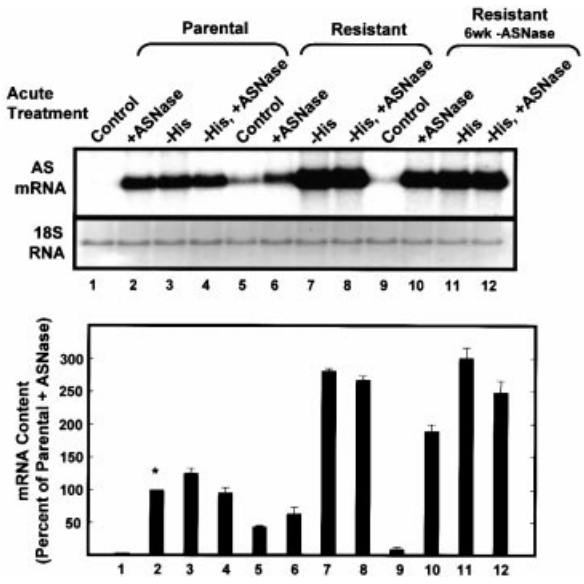

Figure 3 Expression of AS mRNA following histidine deprivation of MOLT-4 cells

To compare the regulation of AS mRNA following deprivation of an essential amino acid or ASNase treatment, parental, resistant and resistant/6wk−ASNase MOLT-4 cells (1×10^4 cells/ml) were subjected to an acute (12 h) incubation in medium containing 1 unit/ml ASNase (+ASNase) or lacking histidine (−His), or the combination of the two conditions (−His, +ASNase). The RNA blot (15 μg/lane) was probed with a ^{32}P-radiolabelled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18 S ribosomal RNA serving as a measure of lane loading. The quantified data from three independent experiments were normalized to the value for parental cells+ASNase (*) and plotted as a bar graph.

AS expression could be induced even further by an acute treatment with ASNase (compare lanes 5 and 6 of Figure 1B).

To monitor the AS protein content in response to acute and long-term ASNase treatment, each of the three cell populations was incubated for 12 h in the absence or presence of 1 unit/ml ASNase prior to protein extraction and immunoblotting. The amount of AS protein in the untreated parental MOLT-4 cells was below detection, but a 12 h exposure of parental MOLT-4 cells to ASNase caused a moderate, but detectable increase (compare lanes 1 and 2 in Figure 1C). In contrast, the drug-resistant cells continuously exposed to ASNase (lane 4, Figure 1C) contained a much higher AS protein content than parental cells either before (lane 1) or after (lane 2) the acute ASNase treatment. This elevated AS expression in the ASNase-resistant cells was not reversed during a 12 h incubation in the absence of the drug (compare lanes 3 and 4 in Figure 1C). Whereas the absolute amount of AS protein was decreased following 6 weeks of culture in the absence of ASNase (lane 5), these cells still contained 10–20-fold more AS protein than the parental control cells (compare lanes 1 and 5 in Figure 1C) showing that the ASNase-induced elevation in AS expression is not fully reversible.

Effect of ASNase on cell growth and apoptosis

Given that the ASNase-induced expression of AS was not completely reversible, it was of interest to determine whether drug resistance, as measured by cell growth and viability following an ASNase challenge, is a reversible or an irreversible phenotype. Parental, ASNase-resistant and resistant/6wk−ASNase cells were subjected to a 1 unit/ml ASNase challenge for 48 h, and then subjected to the WST-1 assay, to monitor cell growth, or the annexin V binding assay, to assess apoptosis/necrosis. Cell growth of parental cells declined by 85% during the 48 h ASNase challenge (Figure 2A). Consistent with the AS expression data (Figure 1), ASNase challenge of the resistant/6wk−ASNase cells resulted in a cell growth pattern that more closely resembled the resistant cells continuously maintained in the presence of ASNase, indicating that the drug-resistant phenotype is not reversible. When apoptosis/necrosis of the cells was assayed by annexin V and propidium iodide binding, the 48 h ASNase treatment of parental leukaemia cells caused a 65% decline in cell viability, whereas, as expected, continued incubation of the ASNase-resistant cells in the presence of the drug did not significantly affect cell viability (Figure 2B). Remarkably, when ASNase-resistant cells were transferred to ASNase-free medium for 6 weeks and then subjected to the 48 h ASNase challenge, the percentage of viable cells declined by less than 5% (Figure 2B). Thus even after 6 weeks of culture in the absence of ASNase, the drug-selected cells maintained a high degree of drug resistance. Once again, the data clearly show that the ASNase-resistant phenotype of the leukaemia cells is irreversible. These *in vitro* results parallel the clinical observation

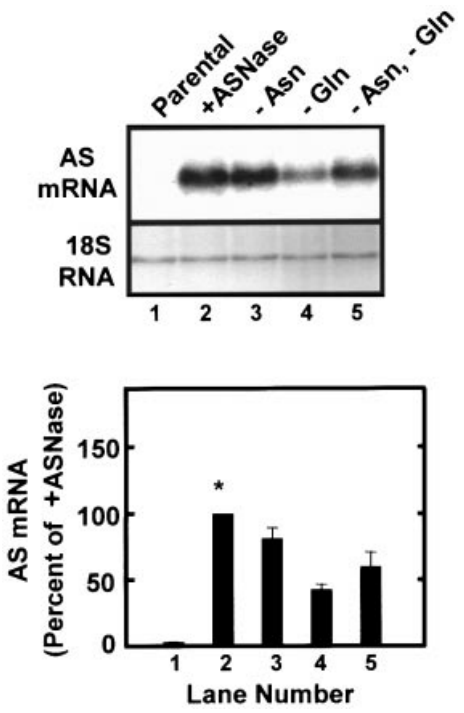

Figure 4 Comparison of AS mRNA induction following ASNase treatment or glutamine and/or asparagine deprivation of MOLT-4 cells

To assess the impact of deprivation of asparagine and/or glutamine, and compare the effect with acute (12 h) ASNase treatment, AS mRNA content was analysed in parental MOLT-4 cells (1×10^4 cells/ml) incubated in complete minimal essential medium (MEM; control), MEM lacking asparagine (—Asn), glutamine (—Gln), or both (—Asn, —Gln), as well as in cells treated with 1 unit/ml ASNase. The RNA blot (15 μg/lane) was probed with a ^{32}P-radiolabelled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18 S ribosomal RNA serving as a measure of lane loading. The quantified data from three independent experiments were normalized to the value for parental cells treated with ASNase (*) and plotted as a bar graph.

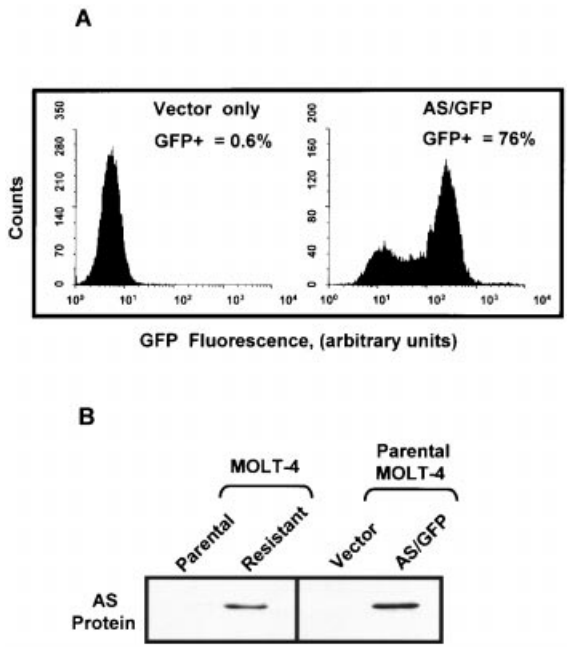

Figure 5 AS protein content in parental MOLT-4 cells transduced with a retrovirus expressing AS and GFP

To determine the percentage of MOLT-4 cells expressing AS following viral transduction with the AS-IRES-GFP construct, FACS analysis for GFP expression was performed comparing control and AS-IRES-GFP transduced cells (**A**). To compare levels of AS expression in the non-transduced MOLT-4 parental and ASNase-resistant cells with AS-IRES-GFP transduced cells, total cell protein extracts (40 μg/lane) were subjected to immunoblot analysis using a monoclonal antibody specific for AS (**B**).

that relapse of ALL patients is often associated with increased AS activity [20] and drug resistance [5,6].

AS expression following histidine deprivation

To test whether AS was induced to a maximal level during ASNase treatment, parental, resistant and resistant/6wk—ASNase MOLT-4 cells were incubated with ASNase, histidine-free medium or both for 12 h prior to Northern-blot analysis for AS mRNA content. Histidine deprivation has been documented to elicit maximum AS expression in Fao hepatoma cells [21]. For parental MOLT-4 cells, there was no difference in the degree of AS mRNA induction between ASNase-treated or histidine-depleted cells, and ASNase-treated/histidine-depleted cells did not enhance the increased expression (Figure 3). Conversely, although the basal level of AS is higher in resistant cells, the induction in response to ASNase was only a fraction of that following histidine depletion (Figure 3). These data show that resistant cells do not express AS at maximal levels during continuous culture in ASNase-containing medium, indicating that the AS activity required for survival in the presence of ASNase is within the dynamic range of the cell. Interestingly, the resistant/6wk—ASNase cells responded to the ASNase treatment and histidine deprivation in a manner similar to the parental MOLT-4 cells (Figure 3). Collectively, the results indicate that an acute ASNase treatment (12 h) of human leukaemia cells causes an AS expression response that is at, or near, the maximum of the cellular capability. In contrast, cells continuously exposed to ASNase have adapted to express a higher basal level of AS, but transfer to fresh ASNase-containing medium does not elicit a maximal response.

Comparison of ASNase treatment with deprivation of asparagine and glutamine

The ASNase preparations used therapeutically are known to exhibit an endogenous glutaminase activity that is 1–3% of that for asparagine [27]. To determine if both asparagine and glutamine deprivation caused induction of AS, parental MOLT-4 cells were incubated for 12 h in medium lacking one or both of these amino acids (Figure 4). Depriving the cells of asparagine was nearly as effective as ASNase treatment. Although glutamine removal from the medium caused induction of AS mRNA content, it was only about half as effective as ASNase. Starving cells of both amino acids yielded a result intermediate between that for asparagine or glutamine alone (Figure 4). These results suggest that ASNase-dependent depletion of glutamine may contribute to the activation of AS expression, but that asparagine deprivation alone is sufficient to cause the changes in AS mRNA content observed in parental leukaemia cells.

Overexpression of AS and ASNase resistance

An important question is whether or not elevated AS alone is sufficient to cause ASNase resistance. To determine if the

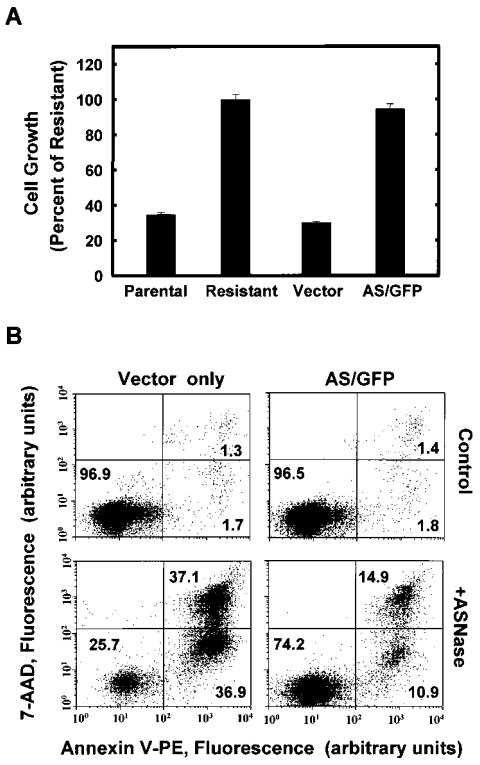

Figure 6 Effect of ASNase on cell growth and viability in virally-transduced MOLT-4 cells overexpressing AS

To determine the effect of an ASNase challenge on cell growth and viability in parental MOLT-4 cells transduced with either AS-IRES-GFP or vector only, the cells were incubated for 48 h (1×10^4 cells/ml) in the absence (Control) or presence (+ ASNase) of 1 unit/ml ASNase. Cell growth rates were assessed by the WST-1 assay (**A**), and the amount of cell death by apoptosis/necrosis was measured by annexin V binding (**B**). For the WST-1 cell growth assay, the absorbance of the treated cells is given as a percentage ($\pm$ S.D., $n = 4$) of drug-selected ASNase-resistant cells. For the apoptosis analysis, the numbers in each quadrant indicate the percentage of cells from a total of 10000 cells. Viable cells appear in the lower left-hand quadrant (propidium iodide and annexin V negative), whereas cells in the right-hand quadrants denote apoptotic (lower quadrant) and apoptotic/necrotic (upper quadrant) cells. Given the green fluorescence of GFP, 7-amino-actinomycin D (7-AAD) was used instead of propidium iodide for necrosis and annexin V-PE was used instead of annexin V-FITC for apoptosis. The apoptosis data presented are representative of two independent experiments.

enhanced expression of AS in drug-selected cells is solely responsible for the ASNase-resistant phenotype, AS was overexpressed in parental MOLT-4 cells at a level similar to that in ASNase-resistant cells. These cells were subjected to an ASNase challenge, and then the cytostatic and cytoxic effects were monitored. To overexpress AS, a Moloney mouse retrovirus system was utilized and the transduction efficiency was enhanced by prior stable transduction of the parental MOLT-4 cells with a virus that expresses the murine retroviral ecotropic receptor [24]. These cells were subsequently transduced with either a control or an AS-expressing virus. It was determined that stable expression of the viral ecotropic receptor did not alter the response to ASNase cytotoxicity (results not shown). To monitor the efficiency of viral infection, a viral vector was utilized that had an IRES and a GFP coding region downstream of the AS cDNA (pAS-IRES-GFP). To enrich for AS-overexpressing cells, FACS was used to separate the GFP-containing cells. Figure 5(A) shows the expression of GFP in the AS/GFP coexpressing cells after infection and two rounds of FACS sorting, as compared with parental MOLT-4 cells infected with control virus. As shown, 76% of the cells were GFP positive. To determine the amount of AS expression in the virally-transduced MOLT-4 cells versus ASNase parental or resistant MOLT-4 cells, AS protein expression was monitored by immunoblot analysis (Figure 5B). The level of AS protein the cells receiving the AS cDNA via viral infection was comparable with that present in the ASNase-selected drug-resistant cell population. Therefore a comparison of the ASNase sensitivity between the AS-expressing cells and the non-expressing controls would represent a valid test of the contribution of this activity to ASNase resistance.

An ASNase challenge was performed by incubating the AS-expressing and vector-only transduced cells in the absence or presence of 1 unit/ml ASNase for 48 h, followed by a WST-1 assay for cell growth (Figure 6A), or an annexin-V apoptosis assay (Figure 6B). The cells transduced with vector-only virus were as sensitive to ASNase as the parental MOLT-4 cells, resulting in a greater than 70% decrease in cell growth (Figure 6A). Conversely, the 48 h growth rate of the parental MOLT-4 cells overexpressing AS was comparable with that for drug-selected resistant MOLT-4 cells. Furthermore, the overexpression of AS in the parental MOLT-4 cells also resulted in a reduction of ASNase-induced cytotoxicity, as monitored by apoptosis/necrosis, from 74% to 26% (Figure 6B). These results demonstrate that increased AS activity alone can convert parental MOLT-4 cells into the ASNase-resistant phenotype with regard to both cell growth and cytotoxicity. Although some ASNase-induced cell death (26%) was observed in the AS-overexpressing cells, the sorting analysis in Figure 5(A) indicates that approximately 25% of GFP/AS transduced cells do not express a detectable level of GFP. Therefore this subpopulation of the cells may also not express AS at a sufficient level to induce the ASNase-resistant phenotype. If that is the case, then virtually all of the AS-overexpressing cells exhibit ASNase resistance.

To establish that AS overexpression would cause the ASNase-resistant phenotype in other leukaemia cells, Jurkat leukaemia cells were tested. Jurkat cells are known to be ASNase-sensitive and have been shown to develop ASNase resistance in response to drug selection [28]. Analysis of cell viability after a 48 h ASNase treatment was repeated in parental or AS-overexpressing Jurkat leukaemia cells with the following results: control = 96% viable, control + AS = 95% viable, ASNase treated = 46% viable, ASNase treated + AS = 75% viable. It was established that only 89% of the cells transduced with the AS vector expressed a detectable level of the GFP marker, so the actual ASNase resistance of the AS-expressing cells is likely to be somewhat higher than measured.

Conclusion

The results document that there is both short-term and long-term adaptation of asparagine AS expression in human MOLT-4 leukaemia cells exposed to ASNase, a drug used in the treatment of childhood ALL. With regard to the short-term response following a 12 h ASNase treatment, the results are consistent with the regulatory properties of the AS gene by amino acid availability. A rapid transcriptional induction of the AS gene occurs following amino acid deprivation of any single essential amino acid [18,25]. Although asparagine is typically considered to be non-essential with regard to the nutritional status of an intact animal, given the low basal activity of AS in leukaemia cells, ASNase-dependent depletion of the intracellular asparagine pool is probably sufficient to activate AS expression. Indeed, the results show that incubation of cells in medium lacking asparagine elicited a response similar in magnitude to ASNase treatment. It is known that *Escherichia coli* ASNase, used in most therapeutic

protocols and the enzyme used in the present studies, has an inherent glutaminase activity that represents 1–3% of the ASNase activity [27]. Therefore one could propose that cellular glutamine content may also play a regulatory function similar to that of asparagine. In support of this hypothesis, following induction of the rat AS gene by complete amino acid deprivation of Fao hepatoma cells, strong repression was observed by refeeding the cells with as little as 50 μM extracellular glutamine [21]. However, for the human MOLT-4 leukaemia cells, the increase in AS expression following glutamine deprivation was not as great as that observed after asparagine starvation or ASNase treatment. The data clearly demonstrate that ASNase treatment, through depletion of asparagine, glutamine or both, causes a relatively rapid induction of AS expression, but this response is insufficient to prevent the cytotoxic effects on human ALL cells.

Unlike amino acid deprivation/refeeding, the long-term activation of AS expression observed in ASNase-selected drug-resistant cells was only partially reversible. The present results for MOLT-4 cells are similar to the observations of Kiriyama et al. [15], who studied ASNase resistance in U937 lymphoma cells. It is possible that this irreversibility arises from an adaptation of the entire cell population or from selection for a subpopulation of leukaemia cells that contain elevated AS expression prior to ASNase treatment. With regard to mechanism, it has been documented that gene amplification is not the cause of the ASNase-induced AS expression in resistant cells [18,29]. However, the expression of AS has been shown to be inversely proportional to the degree of cytosine methylation in the region surrounding the transcriptional start site [16,30]. Indeed, Worton et al. [16] obtained data documenting an ASNase-dependent 25% reduction in global DNA methylation. Whereas the relationship between gene expression and cytosine methylation remains controversial, hypomethylation often correlates with increased expression of certain genes. Therefore these observations on methylation and AS transcription may underscore the irreversible changes that occur in response to ASNase treatment.

Despite an adaptive increase in AS expression within 12 h of initial ASNase exposure, 65% of the MOLT-4 cells underwent apoptosis/necrosis within 48 h. These data extend reports by others in non-ALL cells indicating that ASNase can induce cell death via apoptosis [31,32]. One explanation for the cytotoxicity of ASNase may be that the apoptosis process is triggered before the adaptive mechanisms have increased AS activity sufficiently. The data in Figure 1(C) illustrate that the AS protein content is moderately increased 12 h after ASNase treatment of parental cells, but the absolute level is still quite low relative to that present in drug-resistant cells and may be too low to prevent progression on to cell death. An alternative explanation may be provided by Broome [33], who showed that ASNase-sensitive lymphoma cells preferentially use extracellular asparagine for protein synthesis, rather than intracellularly generated asparagine. Interestingly, this preference was not as strong in ASNase-resistant cells, an observation that led Broome to hypothesize that the requirement for extracellular asparagine was why the parental cells were killed, despite having a cytoplasmic asparagine concentration equal to that in the drug-resistant cells.

An important question that has remained unanswered in previous studies of ASNase resistance is whether or not an elevated level of AS alone is sufficient to generate the ASNase-resistant phenotype. Parental MOLT-4 cells, transduced with AS-expressing virus to achieve an AS level similar to that observed in drug-selected cells, became resistant to ASNase-induced apoptosis. These results demonstrate, for the first time, that an elevated AS activity alone can cause ASNase resistance. While there is little doubt that other metabolic perturbations exist in ASNase-resistant cells, these changes are secondary to the alteration of AS expression.

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REFERENCES

1 Jaffe, N., Traggis, D., Das, L., Moloney, W. C., Hann, H. W., Kim, B. S. and Nair, R. (1971) L-asparaginase in treatment of neoplastic diseases in children. Cancer Res. **31**, 934–949
2 Sutow, W. W., Garacia, F., Starling, K. A., Williams, T. E., Lane, D. M. and Gehan, E. A. (1971) L-asparaginase therapy in children with advanced leukemia. Cancer **28**, 819–824
3 Tallal, L., Tan, C., Oettgen, H., Wollner, N., McCarthy, M., Helson, L., Burchenal, J., Karnofsky, D. and Murphy, M. L. (1970) *E. coli* L-asparaginase in the treatment of leukemia and solid tumors in 131 children. Cancer **25**, 306–320
4 Cortes, J. E. and Kantargian, H. M. (1995) Acute lymphoblastic leukemia. Cancer **76**, 2393–2417
5 Pieters, R., Klumper, E., Kaspers, G. J. L. and Veerman, A. J. P. (1997) Everything you always wanted to know about cellular drug resistance in childhood acute lymphoblastic leukemia. Crit. Rev. Oncol. Hematol. **25**, 11–26
6 Kaspers, G. J. L., Veerman, A. J. P., Pieters, R., Van Zantwijk, C. H., Smets, L. A., Van Wering, E. R. and Van Der Does-Van Den Berg, A. (1997) In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood **90**, 2723–2729
7 Kaspers, G. J. L., Pieters, R., Van Zantwijk, C. H., Van Wering, E. R., Van Der Does-Van Den Berg, A. and Veerman, A. J. P. (1998) Prednisone resistance in childhood acute lymphoblastic leukemia: *vitro-vivo* correlations and cross-resistance to other drugs. Blood **92**, 259–266
8 Asselin, B. L., Ryan, D., Frantz, C. N., Bernal, S. D., Leavitt, P., Sallan, S. E. and Cohen, H. J. (1989) *In vitro* and *in vivo* killing of acute lymphoblastic leukemia cells by L-asparaginase. Cancer Res. **49**, 4363–4368
9 Preti, A. and Kantarjian, H. M. (1994) Management of adult acute lymphocytic leukemia: present issues and key challenges. J. Clin. Oncol. **12**, 1312–1322
10 Larson, R. A., Dodge, R. K., Burns, C. P., Lee, E. J., Stone, R. M., Schulman, P., Duggan, D., Davey, F. R., Sobol, R. E., Frankel, S. R. et al. (1995) A five-drug remission induction regimen with intensive consolidation for adults with acute lymphoblastic leukemia: cancer and leukemia group B study 8811. Blood **85** 2025–2037
11 Codegoni, A. M., Biondi, A., Conter, V., Masera, G., Rambaldi, A. and D'Incalci, M. (1994) Human monocytic leukemia expresses low levels of asparagine synthase and is potentially sensitive to L-asparaginase. Leukemia **9**, 360–361
12 Hudson, M. M., Pui, C.-H. and Kalwinsky, D. K. (1990) Methotrexate plus L-asparaginase. An active combination for children with acute nonlymphocytic leukemia. Cancer **65**, 2615–2618
13 Tulpule, A., Espina, B., Palmer, M., Schiflett, J., Harvey-Buchanan, L. and Levine, A. M. (1998) Preliminary results of PEG-L-asparaginase (ONCASPAR) in the treatment of relapsed/refractory AIDS-related lymphomas. Blood **92**, 240B–241B
14 Prager, M. D. and Bachynsky, N. (1968) Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem. Biophys. Res. Commun. **31**, 43–47
15 Kiriyama, Y., Kubota, M., Takimoto, T., Kitoh, T., Tanizawa, A., Akiyama, Y. and Mikawa, H. (1989) Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia **3**, 294–297
16 Worton, K. S., Kerbel, R. S. and Andrulis, I. L. (1991) Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer Res. **51**, 985–989
17 Martin, J. K., Sun, W., Maraga, D., Schuster, S. M. and Wylie, D. E. (1993) An investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells. Amino Acids **5**, 51–69
18 Hutson, R. G., Kitoh, T., Amador, D. A. M., Cosic, S., Schuster, S. M. and Kilberg, M. S. (1997) Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. Am. J. Physiol. **272**, C1691–C1699
19 Waye, M. M. and Stanners, C. P. (1981) Role of asparaginase synthetase and asparagyl-transfer RNA synthetase in the cell-killing activity of asparaginase in Chinese hamster ovary cell mutants. Cancer Res. **41**, 3104–3106
20 Haskell, C. M. and Canellos, G. P. (1969) L-asparaginase resistance in human leukemia-asparagine synthetase. Biochem. Pharmacol. **18**, 2578–2580
21 Hutson, R. G. and Kilberg, M. S. (1994) Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content. Biochem. J. **303**, 745–750

22 Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**, 206–210
23 Hitoshi, Y., Lorens, J., Kitada, S. I., Fisher, J., LaBarge, M., Ring, H. Z., Francke, U., Reed, J. C., Kinoshita, S. and Nolan, G. P. (1998) Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. Immunity **8**, 461–471
24 Baker, B. W., Boettiger, D., Spooncer, E. and Norton, J. D. (1992) Efficient retroviral-mediated gene transfer into human B lymphoblastoid cells expressing mouse ecotropic viral receptor. Nucleic Acids Res. **20**, 5234
25 Gong, S. S., Guerrini, L. and Basilico, C. (1991) Regulation of asparagine synthetase gene expression by amino acid starvation. Mol. Cell. Biol. **11**, 6059–6066
26 Guerrini, L., Gong, S. S., Mangasarian, K. and Basilico, C. (1993) *Cis*- and *trans*-acting elements involved in amino acid regulation of asparagine synthetase gene expression. Mol. Cell. Biol. **13**, 3202–3212
27 Chakrabarti, R. and Schuster, S. M. (1996) L-Asparaginase: Perspectives on the mechanisms of action and resistance. International J. Pediatric Hemat./Oncol. **4**, 597–611
28 Iiboshi, Y., Papst, P. J., Hunger, S. P. and Terada, N. (1999) L-Asparaginase inhibits the rapamycin-targeted signaling pathway. Biochem. Biophys. Res. Commun. **260**, 534–539
29 Andrulis, I. L., Argonza, R. and Cairney, A. E. L. (1990) Molecular and genetic characterization of human cell lines resistant to L-asparaginase and albizziin. Somatic Cell Mol. Gen. **16**, 59–65
30 Andrulis, I. L. and Barrett, M. T. (1989) DNA methylation patterns associated with asparagine synthetase expression in asparagine-overproducing and -auxotrophic cells. Mol. Cell. Biol. **9**, 2922–2927
31 Bussolati, O., Belletti, S., Uggeri, J., Gatti, R., Orlandini, G., Dall'Asta, V. and Gazzola, G. C. (1995) Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. Exp. Cell Res. **220**, 283–291
32 Story, M. D., Voehringer, D. W., Stephens, L. C. and Meyn, R. E. (1993) L-Asparaginase kills lymphoma cells by apoptosis. Cancer Chemother. Pharmacol. **32**, 129–133
33 Broome, J. D. (1968) Studies on the mechanism of tumor inhibition by L-asparaginase. J. Exp. Med. **127**, 1055–1072

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