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**Asparaginase induces selective dose- and time-dependent cytotoxicity, apoptosis, and reduction of NFκB expression in oral cancer cells**

**Short Title:** Asparaginase affects oral cancer cells

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## Abstract

Asparaginase is fundamental to the treatment of hematological malignancies. However, little has been studied on the effects that asparaginase could exert on solid tumors. Thus, this study aimed to evaluate the effects of asparaginase on an oral carcinoma cell line. The cytotoxicity of asparaginase in SCC-9 (tongue squamous cell carcinoma) and HaCaT (human keratinocyte) cell lines was evaluated with MTT cell viability assay. The cells were treated with asparaginase at 0.04, 0.16, 0.63, 1.0, 1.5, 2.5, and 5.0 IU/mL. Dose-response curves and  $IC_{50}$  values were obtained and the Tumor Selectivity Index (TSI) was calculated. The effect of asparaginase on procaspase-3 and nuclear factor  $\kappa B$  (NF $\kappa B$ ) expression was evaluated with western blot because it was reported that the overexpression of NF $\kappa B$  has been shown to contribute to tumor cell survival, proliferation, and migration. Caspase 3/7 staining was performed to identify cell death using flow cytometry. Effective asparaginase concentrations were lower for SCC-9 cells when compared to HaCaT cells. The cytotoxicity results at 48h and 72h were significantly different for SCC-9 cells. The TSI indicated that asparaginase was selective for the tumor cells. A decrease in procaspase-3 and NF $\kappa B$  protein levels was observed in SCC-9 cells. Furthermore, asparaginase resulted in significant apoptosis after 48h and 72h. Based on these results, asparaginase was cytotoxic in a dose- and time-dependent manner, induces apoptosis, and reduces NF $\kappa B$  expression in oral cancer cells. These results encourage further studies on the effectiveness of this enzyme as a treatment for solid tumors, especially head and neck cancer.

**Keywords:** Apoptosis; Oral cancer cells; Asparaginase; NF $\kappa B$ .

## INTRODUCTION

Asparaginase is an enzyme that is commercially employed by the food industry to decrease the concentration of acrylamide, a potentially carcinogenic substance (1) that results from the reaction between the asparagine amino acid and reducing sugars during the thermal processing of food (2).

- Asparaginase is also used as coadjuvant in the treatment of hematological malignancies such as Acute Lymphoblastic Leukemia (ALL), therefore it is included in the list of essential medicines of the World Health Organization (3). Even though it is primarily used to treat ALL (4), clinical trials have been conducted to evaluate its effects on tumors of the lymphatic and hematopoietic system, such as lymphoblastic lymphoma (5) and extranodal lymphoma of NK / T cells (6).

Yet, studies focusing on the effect of asparaginase on solid tumors are scarce. The *in vitro* studies on the antitumor activity of asparaginase against breast (7-11), ovarian (10-14) and hepatocellular cancer (10,15,16) cell lines, among others, are reported in Table 1. Furthermore, one clinical trial demonstrated that polyethylene glycol-conjugated L-asparaginase is generally well tolerated in patients with advanced solid tumors, and that a dosage of 2,000 IU/m<sup>2</sup> by intramuscular injection every 2 weeks resulted in a significant depletion of serum L-asparagine (11).

The enzyme acts by reducing plasma levels of asparagine amino acid, catalyzing the conversion of asparagine to aspartic acid (17). Unlike normal cells, tumor cells are unable to guarantee their supply of asparagine, which makes them dependent on extracellular asparagine (17). Thus, when asparaginase is administered, asparagine is depleted, which leads to cell cycle arrest and cell death (17).

Although the exact mechanism by which asparaginase exerts its anticancer effects is not fully understood (18), distinct processes have been proposed. A recent *in vitro* study

on laryngeal carcinoma cells found that treatment with asparaginase lead to an increase in cleaved caspase-3, which in turn resulted in cell death by apoptosis in a dose- and time-dependent manner (19). Even though the overexpression of nuclear factor  $\kappa$ B (NF $\kappa$ B) has been shown to contribute to tumor cell survival, proliferation, and migration (20), the effect of asparaginase on NF $\kappa$ B in head and neck carcinoma cell lines has not yet been reported.

Treatment of head and neck squamous cell carcinoma (HNSCC) involves a combination of surgery, radiotherapy, and chemotherapy, depending on the stage of cancer (1). Despite the different forms of treatment, the mortality rate is considered high with an overall 5-year survival rate of approximately 50% to 60% (21). Therefore, it is necessary to develop new therapies that are more effective and less invasive (22).

In a literature search (Table 1), no studies were found reporting on the effects of asparaginase on oral carcinoma. Therefore, this study aimed to investigate the antineoplastic effects of asparaginase on an oral carcinoma cell line.

## RESULTS

### **Asparaginase reduces oral cancer cell viability in a dose- and time-dependent manner**

The dose-response curves, established for both the SCC-9 and HaCaT cell lines after treatment with asparaginase for 48 or 72 hours, are shown in Figure 1. At concentrations higher than 1 IU/mL, the cell viability tended to stabilize and form a plateau with SCC-9 cells (Figures 1A and 1B). This was not the case with the keratinocyte cell line (HaCaT), in which the viability decreased proportionally to the dose of asparaginase (Figures 1C and 1D). Additionally, it is important to note that, with SCC-9 cells, the difference in viability between cells treated with the vehicle and those treated

with asparaginase was statistically significant at concentrations equal to or greater than 1 IU/mL after 48h and 0.64 IU/mL after 72h of treatment (Figure 1E and 1F). With HaCaT cells, this difference was only significant at concentrations equal to or greater than 1.5 IU/mL after 48h and 1 IU/mL after 72h (Figure 1G and 1H). These results suggest that asparaginase was more effective against the HNSCC cell line (SCC-9) than the keratinocyte cell line (HaCaT).

The median cell viability after treatment of both cell lines with different concentrations of asparaginase for 48h or 72h is presented in Table 2. There was a statistically significant difference between the 48h and 72h results for all concentrations with SCC-9 cells, while for HaCaT cells this difference was significant only for 0.64 IU/mL and 1 IU/mL. In view of these results, it is considered that asparaginase affects SCC-9 cells in a time-dependent manner.

The dose-response curve results also enabled the calculation of  $IC_{50}$  values (Figure 1A-D), which were 4.7 and 0.6 IU/mL for SCC-9 at 48h and 72h, respectively. For HaCaT cells, the  $IC_{50}$  values were 6.6 and 7.8 IU/mL for 48h and 72h, respectively.

### **TSI indicates that asparaginase is selective for oral cancer cells**

The TSI was calculated for each experimental treatment period, according to the formula previously described. The TSI for 48h and 72h were respectively 1.4 and 13. Considered that a TSI higher than 1 indicates that the treatment is selective for the tumor cell line, asparaginase is markedly selective for the SCC-9 cell line, especially after a 72-hour treatment.

### **Asparaginase reduces expression of Procaspase-3 and NFκB in oral cancer cells**

A western blot assay to evaluate protein expression in SCC-9 cells treated with asparaginase at IC<sub>50</sub> showed reduced procaspase-3 after 48 and 72h (Figure 2 and Supplementary Figure 1). The blot quantification indicates that asparaginase treatment causes reduction of the procaspase-3 levels to a median of 50.4% in 48h and 41.6% in 72h when compared to the vehicle-treated cells. The decrease in procaspase-3 levels indicated that it was cleaved into caspase-3, which is its active form. Given that this protein is responsible for the induction of apoptosis, these results suggest that asparaginase may induce apoptosis in SCC-9 cells, which was then confirmed by flow cytometry assay.

As shown in Figure 3, treatment with asparaginase at IC<sub>50</sub> for 48 hours reduced the expression of the NFκB transcriptional factor in SCC-9 cells (Supplementary Figure 2). The blot quantification suggests that asparaginase caused the reduction of NFκB (to 47.1%) when compared to vehicle-treated cells.

### **Asparaginase induces apoptosis in oral cancer cells**

Aligned with the findings from western blotting, we also showed that asparaginase induced apoptosis in SCC-9 through increased levels of Caspase3/7 under 48h and 72h treatment with asparaginase (Fig 4 A and B, \*\*\*p < 0.0001). Therefore, it is clear the apoptotic effect of asparaginase upon oral cancer cells.

## **DISCUSSION**

To our knowledge, this is the first study to report on the effects of asparaginase on the oral cancer cell line. In fact, only a few studies have been published on the potential

of the enzyme on solid tumors. Ji *et al.* (19) described a significant reduction in the cell viability of Tu212 and Tu686 laryngeal squamous cell carcinoma cell lines treated for 48h with asparaginase at concentrations ranging from 0.063 to 2 IU/mL, with 2 IU/mL resulting in less than 50% viability in both cell lines. Tardito *et al.* (15) proposed that a much lower dose of asparaginase is effective against the HepG2 hepatocellular carcinoma cell line, with an  $IC_{50}$  value of 0.1 IU/mL after a 48-hour treatment. Our results indicate that a higher concentration of asparaginase is necessary for the induction of comparable effects in SCC-9 ( $IC_{50}$  4.7 IU/mL – 48h). However, a comparable  $IC_{50}$  value was obtained for the SCC-9 cell line after 72h (0.6 IU/mL). This difference between the  $IC_{50}$  values after 48h and 72h, along with the analyses of cell viability displayed in Table 2, highlights the importance of time on the treatment of SCC-9 cells with asparaginase. Therefore, it is reasonable to hypothesize that the prolonged activity of asparaginase on SCC-9 cells results in continuous asparagine depletion, which reduces protein synthesis and ultimately inhibits proliferation, induces apoptosis (12,23,24) and reflects on cell viability (4).

The  $IC_{50}$  values for the HaCaT cells after both 48- and 72-hour treatment (6.6 IU/mL and 7.8 IU/mL, respectively) were higher than 5 IU/mL, the highest concentration used in the dose-response curve. As seen in Figure 1, no tested concentration of asparaginase could reduce cell viability to less than 40% in the keratinocyte cell line, therefore these  $IC_{50}$  values are approximated, calculated by the non-linear regression model. In contrast, the  $IC_{50}$  measures for the SCC-9 cell line are within the concentration range used in the dose-response curve. These observations demonstrate that asparaginase was less effective against HaCaT cells in comparison to SCC-9 cells.

Additionally, asparaginase was considered selective for the tumor cells, with a TSI of 1.4 for 48h and 13 for 72h. The especially high TSI after a 72-hour treatment might be due to the time-dependent effect as previously described. Considering the low or



absent expression of asparagine synthetase in most cancers, there is a higher demand for asparagine and glutamine (17). Yet, we know that asparaginase reduces the bioavailability of both amino acids thereby oral cancer cells ought to be more susceptible to asparaginase than the keratinocyte cells (23, 24).

A decrease in procaspase-3 was observed upon asparaginase treatment for both 48 and 72 hours in a time-dependent manner (Figure 2). Ji *et al.* (19) report a dose and time-dependent increase in the number of annexin-V marked cells that suggest apoptosis, and in the levels of cleaved caspase-3 and poly ADP-Ribose polymerase (PARP), both proapoptotic proteins, after a 24- or 48-hour treatment of Tu212 and Tu686 laryngeal squamous cell carcinoma cells with asparaginase. Zhang *et al.* (25) also identified asparaginase as an inducer of apoptosis in A549 and H1975 pulmonary adenoma cell lines, describing a significant increase in annexin-V-marked cells after 24-hour of treatment. The reduced bioavailability of asparagine and glutamine is associated with apoptosis once that the cells are unable to properly synthesize proteins due to cell death (12,23,24).

In our study, NF $\kappa$ B was decreased in SCC-9 cells treated with asparaginase. The NF $\kappa$ B family, also called p65, is comprised of five transcription factors that form distinct protein complexes that bind to DNA sequences in the promoter regions of responsive genes. It regulates cellular processes that are relating to immune and inflammatory responses and cancer progression (26, 27). NF $\kappa$ B not only promotes tumor cell proliferation, suppresses apoptosis, and induces angiogenesis, but also induces epithelial-mesenchymal transition, which facilitates distant metastasis (28). It was demonstrated that turmeric inhibited NF $\kappa$ B activation and down-regulated the products of NF $\kappa$ B-controlled genes linked to cancer survival (Bcl-2, cFLIP, XIAP, and cIAP1), proliferation (cyclin D1 and c-Myc), and metastasis (CXCR4) (28). Squarize *et al.* (29) showed that in

HNSCC cells (HN12, HN13, HN30) the activity of interleukin-6 (IL-6) is increased and depended on NFκB. NFκB inhibition resulted in the downregulation of the IL-6 promoter and inhibition of the signal transducer and activator of transcription-3 (STAT3) activity in HNSCC cells (29). STAT3 promotes tumor progression by regulating the expression of the cell cycle, survival, and pro-inflammatory genes, in addition to controlling mitochondrial function, metabolism, and stemness (30). Also, NFκB downregulation in SCC-9 cells results in growth inhibition, increased apoptosis, and reduced cell migration and invasion (31). The inhibition growth caused by NFκB decreased activity was found in another HNSCC cell line (CAL-27) treated with curcumin (32). Thus, asparaginase treatment could be linked to caspase-3 activation or the regulation of proliferation mechanisms mediated by reduced NFκB levels.

Based on these results, we concluded that asparaginase is cytotoxic to the oral cancer cell line in a dose- and time-dependent manner; and it induces apoptosis, which was confirmed by flow cytometry assays. Moreover, asparaginase down-regulates NFκB, an important molecule that modulates cell growth and migration. Altogether, these results encourage further studies on the effectiveness of this enzyme as a treatment for solid tumors, including head and neck cancer.

## **MATERIAL AND METHODS**

### **Asparaginase**

The lyophilized asparaginase enzyme, derived from *Escherichia coli*, was purchased from Sigma-Aldrich (A3809) (St. Louis, MO, USA). 100 IU of asparaginase was diluted in 1 mL deionized water and kept at -20°C until used.

### **Cell lines and culture conditions**

A tongue squamous cell carcinoma cell line (SCC-9) and a human keratinocyte cell line (HaCaT) were used for the experiments. They were purchased from ATCC (Rockville, MD, USA), considered that all experiments were conducted on commercially available cell lines, our study was not submitted to an ethics committee. The SCC-9 cell line was cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM) / F12 with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin). The HaCaT cell line was cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were incubated in 5% CO<sub>2</sub> at 37°C. Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

#### **Cell viability assay – dose-response curve**

SCC-9 and HaCaT cells were seeded into 96-well plates at a density of 5×10<sup>3</sup> cells/well and incubated overnight. Cells were treated with 0.04, 0.16, 0.64, 1.0, 1.5, 2.5 or 5.0 IU/mL asparaginase for 48 and 72 hours. Deionized water was used as a vehicle. After the treatment, 10 μL of MTT solution (3,[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/mL was added to each well, followed by incubation for 4h at 37°C. The medium was then aspirated and 100 μL of acidified isopropanol was added to dissolve formazan crystals. Absorbance was measured at 570 nm in a DTX 800 reader (Beckman Coulter, CA, USA). The assays were performed in triplicate.

The concentration capable of inducing 50% cell cytotoxicity (IC<sub>50</sub>) in each cell line was estimated from the cell viability assay results through a non-linear regression, and the Tumor Selectivity Index (TSI) was calculated from those values, according to the formula:  $TSI = \frac{IC_{50}Control\ cell(HaCaT)}{IC_{50}Tumor\ cell(SCC-9)}$ . A TSI higher than 1 suggests that asparaginase is selective to the tumor cell line (SCC-9), in comparison to the control cell line (HaCaT).

## **Western Blot analysis**

SCC-9 cells were treated with asparaginase at the IC<sub>50</sub> concentration for 48 or 72 hours, with deionized water as a vehicle. They were then washed, lysed in a buffer and centrifuged. The protein fraction was collected and quantified by the Lowry method. Samples (30 µg denatured proteins in 30µL buffer) were loaded in a 10% acrylamide gel and electrophoresed at 100V. The proteins were then transferred to a PVDF membrane, which was blocked with a 5% blocking solution (milk albumin) for one hour and incubated overnight with the Caspase-3 (SC7148), NFκB p65 (D14E12) (Cell Signaling Technology, Danvers, MA, USA) or GAPDH (SC47724) (Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies at 4°C. After incubation, the membrane was washed and incubated with the secondary antibody (Abcam, Cambridge, UK) for 1 hour at 4°C. After washing again, the membrane was covered with ECLPRIME (GE Health Care, Little Chalfont, UK) and images were acquired with a chemiluminescence imager (Amersham Imager 600, GE Health Care, Little Fort, UK). Image J software (National Institute of Health, Bethesda, MD, EUA) was used to measure the grey mean value (GMV) of each blot and its surrounding region (background). The measurements were inverted and the background was deducted from the blots GMVs. The protein bands were relatively quantified as a ratio of the net procaspase-3 or NFκB value over its relative net GAPDH value.

## **Apoptosis assay**

In order to confirm the results from the western blot, flow cytometry was used to analyze cell death events. SCC-9 cells were treated with asparaginase at the IC<sub>50</sub> concentration for 48 or 72 hours, with deionized water as a vehicle. The CellEvent

Caspase-3/7 Green Flow Cytometry Assay Kit (Life Technologies, Carlsbad, California, USA) was used according to the kit's instructions. Briefly, 1  $\mu$ L of CellEvent Caspase-3/7 Green Detection Reagent was added to samples containing 1 mL of cell suspension at  $1 \times 10^5$  cells/mL and incubated for 25 minutes at 37 °C. Then, 1  $\mu$ L of 1 mM SYTOX AADvanced dead cell stain solution was added to samples and incubated for 5 minutes at 37 °C. The analysis of the samples was performed on the BD Accuri™ C6 Plus flow cytometer (BD Life Sciences, San Jose, CA, USA). A minimal of eight readings were performed.

### **Statistical analyses**

The statistical analysis for the cell viability assay was performed on the results from nine wells at each concentration. The Kruskal-Wallis test and Dunn's posttest were applied to compare the results of multiple groups (different concentrations) for each cell line in each different treatment period. To assess time-dependency the Mann-Whitney test was used to compare the results of each concentration at 48h and 72h. The IC<sub>50</sub> values were calculated through a non-linear regression model. The Student's T-test was applied to the flow cytometry data. All statistical tests were performed with the GraphPad 5.0 program for Windows. The significance level was  $p < 0.05$ .

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## Conflict of Interest

None declared.

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## FIGURE LEGENDS

**Figure 1.** Dose-response curves,  $IC_{50}$  values and cell viability for each cell line treated with asparaginase for 48h and 72h in different concentrations. A-D: Dose-response curves (normalized cell viability vs asparaginase concentration in the log) and non-linear regression (red line). A plateau formed when SCC-9 cells were treated with higher concentrations of asparaginase (A and B), which was not observed with HaCaT cells (C and D). Under the experimental conditions described in this study, asparaginase was not able to reduce cell viability by 50% or more. Representation of medians  $\pm$  range. E-H: Cell viability results. Difference between cell viability in vehicle-treated cells (0 IU/mL) and cells treated with asparaginase becomes significant at lower concentrations with SCC-9 cells (E and F) in comparison to HaCaT cells (G and H). Representation of medians  $\pm$  range. Kruskal-Wallis test and Dunn's posttest were used to compare all groups (different concentrations) in the same experimental condition. \* =  $p < 0.05$ .

**Figure 2.** Expression of procaspase-3. A) Treatment with asparaginase at  $IC_{50}$  reduced procaspase-3, which suggests its cleavage into caspase-3 and is indicative of apoptosis. B) Relative quantification of the grey means values of the western blots for 48h and 72h. Veh = Vehicle; Asp = Asparaginase.

**Figure 3.** Expression of NF $\kappa$ B. A) Treatment with asparaginase at  $IC_{50}$  for 48h reduced the levels of NF $\kappa$ B. B) Relative quantification of the grey means values of the western blots for 48h. Veh = Vehicle; Asp = Asparaginase.

**Figure 4.** Asparaginase induced apoptosis. A) Asparaginase increased the number of cells undergoing apoptosis in 48h. B) Asparaginase resulted in a higher number of apoptotic cells in 72h of treatment. \*\*\* =  $p < 0.0001$ .

**Supplementary Figure 1:** Blots from western blot assay to evaluate protein expression in SCC-9 cells treated with asparaginase at IC50 showed reduced procaspase-3 after 48 and 72h.

**Supplementary Figure 2:** Blots from western blot assay to evaluate protein expression in SCC-9 cells treated with asparaginase at IC50 for 48 hours showing the reduction of the expression of the NF $\kappa$ B transcriptional factor.

**Table 1** – Summary of descriptive characteristics of the studies that assessed the effects of asparaginase on solid tumors.

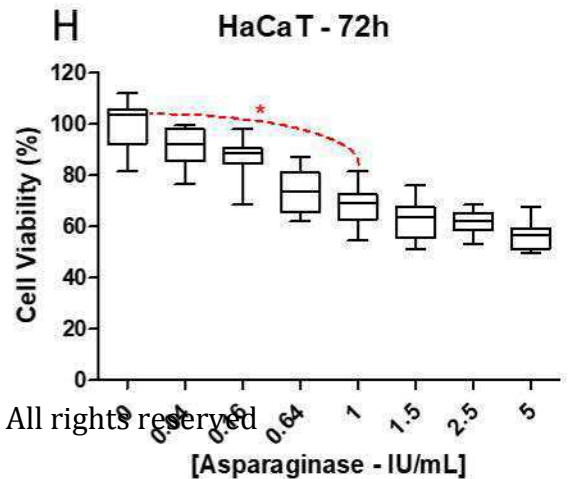
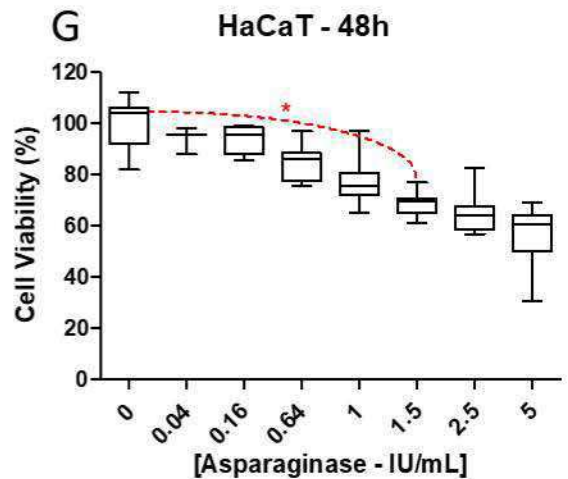
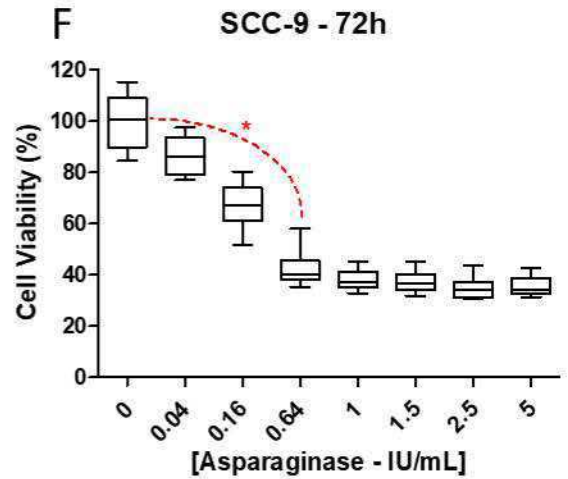
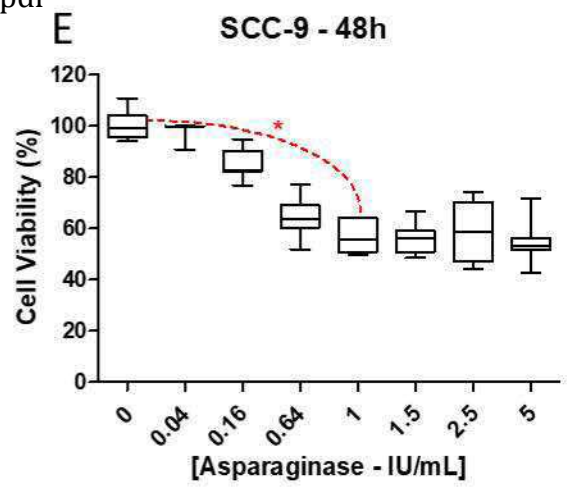
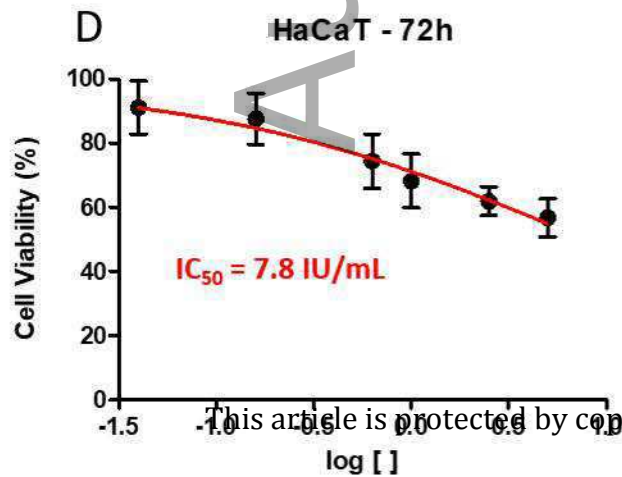
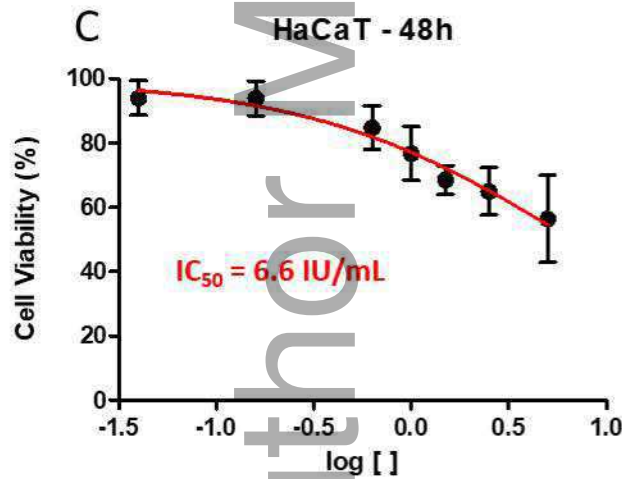
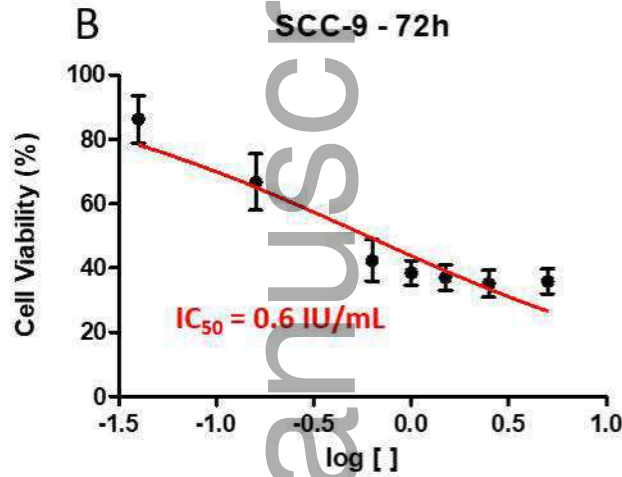
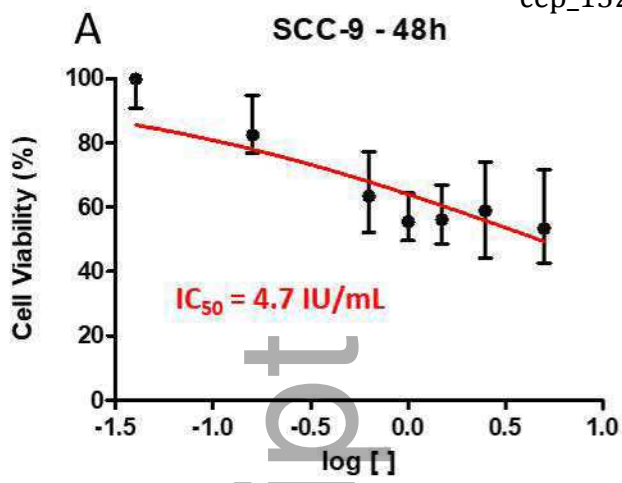
	Study Design	Cell type/ Tumor	Asparaginase	Main results
<b>Abakumora et al. 2012 (10)</b>	<i>In vitro</i>	LnCap (human prostate carcinoma), MCF7 (human breast carcinoma), SKOV-3, CaOV (human ovarian carcinoma), HepG2 (human hepatocellular carcinoma), HT-1080 (human fibrosarcoma, GGNC-1 (rat Gasser's ganglion neurinoma cells), and EPNT-5 (murine glioblastoma) cell lines	L-asparaginase (derived from <i>Erwinia carotovora</i> - ECAR LANS); L-asparaginase (from <i>Escherichia coli</i> - Medac) - 0.2-10 IU/mL	L-asparaginases resulted in a significant dose-dependent decrease in viable cells. SKOV-3, HepG2, GGNC-1 and EPNT-5 exhibited the highest sensitivity to the cytostatic effect of L-asparaginases. Medac asparaginase was effective in increasing the number of LnCap apoptotic cells. L-asparaginase combined with doxorubicin (0.5 µg/mL) increased the number of MCF7 apoptotic cell.
<b>Ji et al. 2017 (19)</b>	<i>In vitro</i>	Tu212 and Tu686 (human laryngeal squamous cell carcinoma) cell lines	Asparaginase (from <i>E. chrysanthemi</i> ) - 0-2 IU/mL	Asparaginase induced remarkable cytotoxicity, autophagy and caspase-dependent apoptosis in Tu212 and Tu686 cells. Autophagy was mediated by inactivation of Akt/mTOR and activation of the Erk signaling pathway.
<b>Kim et al. 2015 (12)</b>	<i>In vitro</i> and <i>in vivo</i>	<i>In vitro</i> : MC38 (murine colon adenocarcinoma), 4T1 (murine mammary carcinoma) and AsPC1 (human pancreas carcinoma) cell lines; <i>In vivo</i> : mice with tumors induced by inoculation of MC38, 4T1 or AsPC1 cells	L-asparaginase secreted (0.2 mg/mL culture supernatant) or delivered to tumor tissue by <i>Salmonella typhimurium</i> engineered to express the enzyme; Leunase - 5 IU/mL	<i>In vitro</i> cytotoxicity induced by both <i>S. typhimurium</i> -derived L-asparaginase (L-asparaginase-containing bacterial culture supernatant) and Leunase (asparaginase used clinically). <i>In vivo</i> tumor size regression induced by L-asparaginase expressed and released by <i>S. typhimurium</i> within the tumor tissue.
<b>Knott et al. 2018 (14)</b>	<i>In vivo</i>	<i>In vivo</i> : mice with tumors induced by inoculation of 4T1 (murine mammary carcinoma) or MDA-MB-231 (human breast carcinoma) cells	<i>In vivo</i> : 200 µL of 60 U L-asparaginase five times a week intraperitoneally	Treatment with L-asparaginase reduced serum asparagine to undetectable levels, not significantly altering primary tumors, but reducing metastasis. When asparagine synthetase-silenced cells were injected into mice treated with L-asparaginase, metastases were nearly undetectable, and a reduction of primary tumor volume was observed.
<b>Kyriakidis et al. 1990 (13)</b>	<i>In vitro</i>	T47D, BT20, MCF-7 (human breast carcinoma), Walker 256 (rat breast carcinoma) and HeLa (human cervical carcinoma) cell lines	L-asparaginase (from <i>Tetrahymena pyriformis</i> or <i>E. coli</i> ) - 0.01 IU	Asparaginase derived from both <i>T. pyriformis</i> and <i>E. coli</i> resulted in antiproliferative activity on the breast cancer cell lines (T47D, BT20 MCF-7 and Walker 256). No difference was observed in HeLa cells when compared to control.
<b>Lorenzi et al. 2006 (8)</b>	<i>In vitro</i>	OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, IGROV1, and SKOV-3 (human ovarian cancer) cell lines	L-asparaginase (from <i>E. coli</i> ) serially diluted for a dose-response curve	Asparagine synthetase silencing enhanced the cytotoxic effects of L-asparaginase, which suggests asparaginase synthetase as a biomarker for selection of patients who shall undergo treatment with L-asparaginase.
<b>Lorenzi et al. 2008 (7)</b>	<i>In vitro</i>	A total of 19 ovarian cancer cell lines	L-asparaginase (from <i>E. coli</i> ) serially diluted for a dose-response curve	The expression of asparagine synthetase, measured by immunoassay, is a strong predictor of L-asparaginase activity in ovarian cancer cell lines.
<b>Panosyan et al. 2014 (33)</b>	<i>In vitro</i> and <i>in vivo</i>	<i>In vitro</i> : DAOY, GBM-ES, U87 (human brain tumor) and GL-261 (murine glioma) cell lines; <i>In vivo</i> : mice with tumors induced by inoculation of DAOY cells	Asparaginase (from <i>E. coli</i> ) <i>In vitro</i> : 0.0625-2 IU/mL <i>In vivo</i> : 2 IU/g of body weight	Asparaginase <i>in vitro</i> resulted in dose-dependent and variable growth inhibition and in reduction of neurosphere formation. Co-treatment with gemcitabine or etoposide resulted significantly better. <i>In vivo</i> results showed sustained deamination of asparagine after treatment with asparaginase. Asparaginase combined with temozolomide resulted in significant growth suppression.
<b>Tardito et al. 2007 (34)</b>	<i>In vitro</i>	HT1080 (human fibrosarcoma), RD (human rhabdomyosarcoma), SW872 (human liposarcoma), HOS, SAOS-2, and U2OS (human osteosarcomas) cell lines	Asparaginase (from <i>E. chrysanthemi</i> ) - 0.001-10 IU/mL	Asparaginase produced a marked decrease of cell viability and significant activation of Caspase-3 in HT1080 and SW872 cells. In contrast, RD, SAOS-2, HOS, and U2OS cells exhibited only a partial growth suppression. Flow cytometry indicated apoptosis in all cells (except for RD) treated with asparaginase, and its association to a glutamine synthetase inhibitor (MSO) increased even further the number of apoptotic cells.

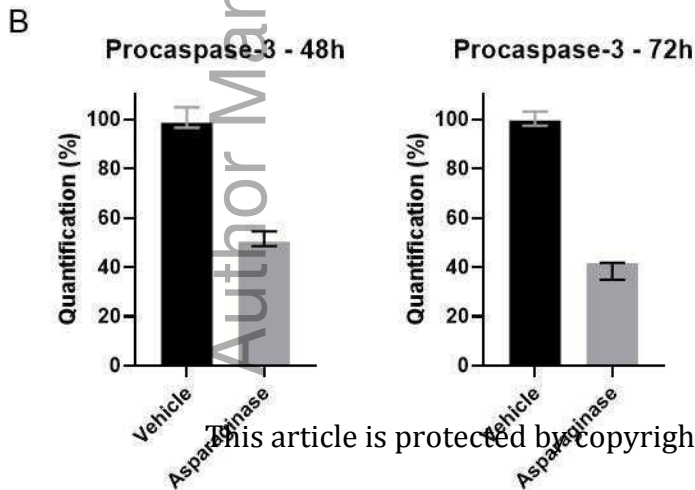
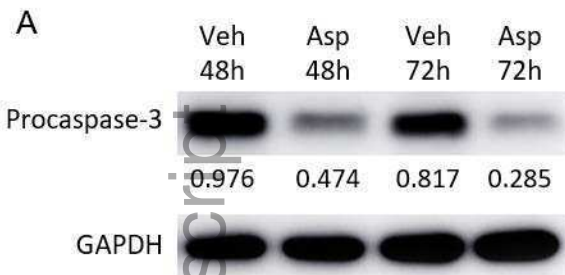
<b>Tardito et al. 2011(15)</b>	<i>In vitro</i>	HepG2, Hep3B, Huh-7, PLC-PRF-5, and Huh-6 (human hepatocellular carcinoma) cell lines	Asparaginase (from <i>E. chrisanthemy</i> ) - 1 IU/mL	Asparaginase increased glutamine synthetase expression and activity. Only HepG2 cells were sensitive to asparaginase alone, while its association to a glutamine synthetase inhibitor resulted in significantly decreased viability. Asparaginase caused apoptosis and increased caspase-3 in HepG2 cells and combined with MSO induced G <sub>2</sub> /M cell cycle arrest.
<b>Taylor et al. 2001(11)</b>	<i>In vitro</i> and phase I clinical trial	<i>In vitro</i> : human tumor clonogenic assay with fresh breast, colon, lung and ovary tumors, melanoma, and non-Hodgkin's lymphoma samples Clinical trial: malignant melanoma (11), non-small cell lung cancer (6), sarcoma (2), colon cancer (2), bladder cancer (1), Cholangiocarcinoma (1), Multiple myeloma (1), Renal cell carcinoma (1), Salivary gland tumor (1), Small cell lung cancer (1)	<i>In vitro</i> : polyethylene glycol-conjugated (PEG) L-asparaginase - 0.075 and 0.75 IU/mL Clinical trial: PEG-L-asparaginase administered by intramuscular injection every 2 weeks - 250-2000 IU/m <sup>2</sup>	Malignant melanoma was the most sensitive tumor type to PEG-L-asparaginase, especially at the highest concentration. PEG-L-asparaginase at a dosage of 2,000 IU/m <sup>2</sup> every 2 weeks results in consistent depletion of serum L-asparagine. Treatment with PEG-L-asparaginase was generally well tolerated, with grade 1 and grade 2 fatigue/weakness, nausea/vomiting, and anorexia/weight loss occurring more consistently at 2,000 IU/m <sup>2</sup> . Further studies are needed to determine the antitumor activity of PEG-L-asparaginase in patients with solid tumors, especially those with malignant melanoma, since this tumor demonstrated <i>in vitro</i> sensitivity.
<b>Yu et al. 2012 (9)</b>	<i>In vitro</i>	HEYA-8, CAOV-3, SKOV-3, and OVCAR-8 (human ovarian cancer) cell lines	L-asparaginase - 0.3-3 IU/mL	L-asparaginase significantly decreased invasiveness and adhesion of ovarian cancer cells, as did reduce quantity and organization of actin stress fibers. L-asparaginase also activated autophagy pathways.
<b>Zhang et al. 2013 (16)</b>	<i>In vitro</i> and <i>in vivo</i>	<i>In vitro</i> : MHCCLM3, MHCC97H, SMMCCLM3, and PLC (hepatocellular carcinoma) cell lines; <i>In vivo</i> : mice with tumors induced by inoculation of MHCCLM3 cells	L-asparaginase <i>In vitro</i> : 0.5 and 1 μm <sup>-1</sup> <i>In vivo</i> : 20 μg <sup>-1</sup> administered intraperitoneally every other day	Cells with low asparagine synthetase expression were more sensitive to L-asparaginase than cells with high expression. Asparagine synthetase-knockdown cells, both <i>in vitro</i> and <i>in vivo</i> (in xenograft tumors), had respective proliferation and tumor size significantly decreased and reduced after treatment with L-asparaginase.
<b>Zhang et al. 2016 (25)</b>	<i>In vitro</i>	A549 and H1975 (human pulmonary carcinoma) cell lines	Asparaginase (derived from <i>Erwinia</i> ) - 0.0625-2 IU/mL	Asparaginase induced remarkable cytotoxicity in a dose-dependent manner, apoptosis and autophagy in lung adenocarcinoma cells. By treating cancer cells with asparaginase and inhibiting autophagy, cytotoxicity was increased, as well as the activity of caspase 3.

**Table 2** – Median cell viability after treatment of SCC-9 and HaCaT with asparaginase in different concentrations and in different treatment periods.

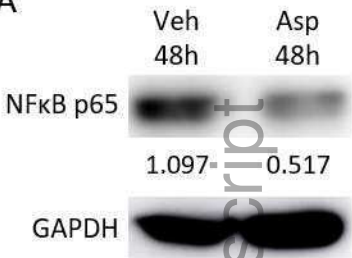
SCC-9			
Concentration IU/mL	Median Cell Viability ( $\pm$ Range) (%)		p-value
	48h	72h	
0.04	99 ( $\pm$ 93.9-110.8)	86.1 ( $\pm$ 77.1-97.4)	*
0.16	82.5 ( $\pm$ 76.8-94.8)	67.1 ( $\pm$ 51.7-80.3)	***
0.64	63.5 ( $\pm$ 51.9-77.3)	40.3 ( $\pm$ 35.5-58.1)	***
1	55.5 ( $\pm$ 49.7-64.4)	37 ( $\pm$ 33-45.2)	***
1.5	56.1 ( $\pm$ 48.6-64.35)	37 ( $\pm$ 31.9-45.4)	***
2.5	59 ( $\pm$ 44.1-74.3)	34.2 ( $\pm$ 30.6-43.7)	***
5	53.5 ( $\pm$ 42.7-71.74)	34.2 ( $\pm$ 31.5-42.6)	***
HaCaT			
Concentration IU/mL	Median Cell Viability ( $\pm$ Range) (%)		p-value
	48h	72h	
0.04	95.7 ( $\pm$ 88-98.3)	92.1 ( $\pm$ 76.4-99.4)	0.5972
0.16	95.6 ( $\pm$ 85.8-99.3)	88.6 ( $\pm$ 68.5-98)	0.0706
0.64	86 ( $\pm$ 75.9-97)	73.8 ( $\pm$ 62-87.3)	**
1	75.7 ( $\pm$ 65.1-97.1)	69.2 ( $\pm$ 54.5-81.5)	*
1.5	69.6 ( $\pm$ 61.3-77)	63.8 ( $\pm$ 51.3-76)	0.0656
2.5	64.1 ( $\pm$ 57-82.8)	62.2 ( $\pm$ 53.2-68.4)	0.2486
5	60.7 ( $\pm$ 30.5-69.1)	56.9 ( $\pm$ 49.5-67.8)	0.9316

Mann-Whitney test was used to compare 48h and 72h results. \* =  $p \leq 0.05$ ; \*\* =  $p \leq 0.005$ ; \*\*\* =  $p \leq 0.0002$

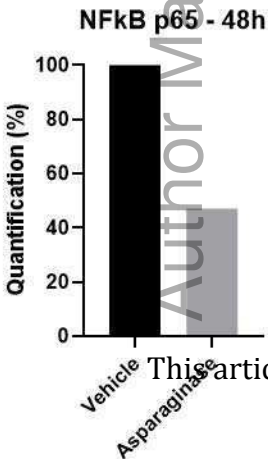




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