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Article type : Original Article - Other

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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Conflict of interest statement: All authors declare that they do not have any conflict of interest

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 κB (NFκB) expression was evaluated with western blot because it was reported that the overexpression of NFκB 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 NFκB 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 NFκB 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; NFκB.

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^2 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 κB (NFκB) has been shown to contribute to tumor cell survival, proliferation, and migration (20), the effect of asparaginase on NFκB 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_{50} 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_{50} 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 inductor 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, NFκB was decreased in SCC-9 cells treated with asparaginase. The NFκB 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). NFκB not only promotes tumor cell proliferation, suppresses apoptosis, and induces angiogenesis, but also induces epithelial-mesenchymal transition, which facilitates distant metastasis (28). I was demonstrated that turmeric inhibited NFκB activation and down-regulated the products of NFκB-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 -20ºC 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_2 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 5×10^3 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_{50}) 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_{50} 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 4ºC. After incubation, the membrane was washed and incubated with the secondary antibody (Abcam, Cambridge, UK) for 1 hour at 4ºC. 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_{50} 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 µL of CellEvent Caspase-3/7 Green Detection Reagent was added to samples containing 1 mL of cell suspension at $1x10^5$ cells/mL and incubated for 25 minutes at 37 °C. Then, 1 µL 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_{50} 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 $p < 0.05$.

Acknowledgements

The authors are grateful to FAPDF (Fundação de Apoio à Pesquisa do Distrito Federal - grant 193.000.972/2013, and DPG/UnB No 05/2018 - Postdoctoral Fellowship Program Abroad/FAPDF), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - grant 407851/2013-5), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

Conflict of Interest

None declared.

REFERENTES

1. Pelucchi C, Bosetti C, Galeone C, La Vecchia C. Dietary acrylamide and cancer risk: an updated meta-analysis. Drugs 2015;136(12):2912-2922.
2. Xu F, Oruna-Concha MJ, Elmore JS. *T*he use of asparaginase to reduce acrylamide levels in cooked food. Food Chem. 2016;210:163-171.
3. WHO. Model List of Essencial Medicine. 2015; 19th Edition:http://www.who.int/selection_medicines/committees/expert/20/EML_2015_FINAL_amended_AUG2015.pdf?ua=1. Accessed February 2019.
4. Ali U, Naveed M, Ullah A, Ali K, Shah SA, Fahad S, Mumtaz AS. L-asparaginase as a critical component to combat Acute Lymphoblastic Leukaemia (ALL): A novel approach to target ALL. Eur J Pharmacol.2016;771:199-210.
5. Zheng W, Ren H, Ke X, Xue M, Zhang Y, Xie Y, Lin N, Tu M, Liu W, Ping L, Ying Z, Zhang C, Deng L, Wang X, Song Y, Zhu J. PEG-asparaginase in BFM-90 regimen improves outcomes in adults with newly diagnosed lymphoblastic lymphoma. Chin J Cancer Res. 2017;29(1):66-74.
6. Bu S, Yuan F, Wei X, Yin Q, Li Y, Mi R, Yang H, Li H, Ge S, Liu Y, Song Y. L-asparaginase-based regimen as a first-line treatment for newly diagnosed nasal type extranodal natural killer cell/T-cell lymphoma. Exp Ther Med. 2016;11(6):2437-2445.
7. Lorenzi PL, Llamas J, Gunsior M, Ozbun L, Reinhold WC, Varma S, Ji H, Kim H, Hutchinson AA, Kohn EC, Goldsmith PK, Birrer MJ, Weinstein JN. Asparagine synthetase is a predictive biomarker of L-asparaginase activity in ovarian cancer cell lines. Mol Cancer Ther. 2008;7(10):3123-3128.
8. Lorenzi PL, Reinhold WC, Rudelius M, Gunsior M, Shankavaram U, Bussey KJ, Scherf U, Eichler GS, Martin SE, Chin K, Gray JW, Kohn EC, Horak ID, Von Hoff DD, Raffeld M, Goldsmith PK, Caplen NJ, Weinstein JN. Asparagine synthetase as a causal, predictive biomarker for L-asparaginase activity in ovarian cancer cells. Mol Cancer Ther. 2006;5(11):2613-2623.
9. Yu M, Henning R, Walker A, Kim G, Perroy A, Alessandro R, Virador V, Kohn EC. L-asparaginase inhibits invasive and angiogenic activity and induces autophagy in ovarian cancer. J Cell Mol Med. 2012;16(10):2369-2378.
10. Abakumova OY, Podobed OV, Karalkin PA, Kondakova LI, Sokolov NN. Antitumor activity of L-asparaginase from Erwinia carotovora against different human and animal leukemic and solid tumor cell lines. Biochem (Mosc) Suppl Ser B Biomed Chem. 2012;6(4):307-316.
11. Taylor CW, Dorr RT, Fanta P, Hersh EM, Salmon SE. A phase I and pharmacodynamic evaluation of polyethylene glycol-conjugated L-asparaginase in patients with advanced solid tumors. Cancer Chemother Pharmacol. 2001;47(1):83-88.

12. Kim K, Jeong JH, Lim D, Hong Y, Lim HJ, Kim GJ, Shin SR, Lee JJ, Yun M, Harris RA, Min JJ, Choy HE. L-Asparaginase delivered by Salmonella typhimurium suppresses solid tumors. Mol Ther Oncolytics 2015;2:15007.
13. Kyriakidis DA, Tsirka SA, Tsavdaridis IK, Iliadis SN, Kortsaris AH. Antiproliferative activity of L-asparaginase of Tetrahymena pyriformis on human breast cancer cell lines. Mol Cell Biochem. 1990;96(2):137-142.
14. Knott SRV, Wagenblast E, Khan S, Kim SY, Soto M, Wagner M, Turgeon MO, Fish L, Erard N, Gable AL, Maceli AR, Dickopf S, Papachristou EK, D'Santos CS, Carey LA, Wilkinson JE, Harrell JC, Perou CM, Goodarzi H, Poulogiannis G, Hannon GJ. Asparagine bioavailability governs metastasis in a model of breast cancer. Nature 2018;554(7692):378-381.
15. Tardito S, Chiu M, Uggeri J, Zerbini A, Da Ros F, Dall'Asta V, Missale G, Bussolati O. L-Asparaginase and inhibitors of glutamine synthetase disclose glutamine addiction of beta-catenin-mutated human hepatocellular carcinoma cells. Curr Cancer Drug Targets.2011;11(8):929-943.
16. Zhang B, Dong LW, Tan YX, Zhang J, Pan YF, Yang C, Li MH, Ding ZW, Liu LJ, Jiang TY, Yang JH, Wang HY. Asparagine synthetase is an independent predictor of surgical survival and a potential therapeutic target in hepatocellular carcinoma. Br J Cancer. 2013;109(1):14-23.
17. Avramis VI. Asparaginases: biochemical pharmacology and modes of drug resistance. Anticancer Res. 2012;32(7):2423-2437.
18. Lanvers-Kaminsky C. Asparaginase pharmacology: challenges still to be faced. Cancer Chemother Pharmacol. 2017;79(3):439-450.
19. Ji Y, Li L, Tao Q, Zhang X, Luan J, Zhao S, Liu H, Ju D. Deprivation of asparagine triggers cytoprotective autophagy in laryngeal squamous cell carcinoma. Cell Death Differ. 2017;101(12):4951-4961.
20. Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, Yeh NT, Mukaida N, and Van Waes. IL (interleukin)-1alpha promotes nuclear factor-kappaB and AP-1 – induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clin Cancer Res. 2001; 7:1812–1820.
21. Kalavrezos N, Scully C. Mouth cancer for clinicians. Part 2: Epidemiology. Dent Update 2015;42(4):354-356, 358-359.
22. Moreira J, Tobias A, O'Brien MP, Agulnik M. Targeted Therapy in Head and Neck Cancer: An Update on Current Clinical Developments in Epidermal Growth Factor Receptor-Targeted Therapy and Immunotherapies. Drugs 2017;77(8):843-857.
23. Shrivastava A, Khan AA, Khurshid M, Kalam MA, Jain SK, Singhal PK. Recent developments in L-asparaginase discovery and its potential as anticancer agent. Crit Rev Oncol Hematol. 2016;100:1-10.
24. Fung MKL, Chan GC. Drug-induced amino acid deprivation as strategy for cancer therapy. J Hematol Oncol. 2017;10(1):144.
25. Zhang B, Fan J, Zhang X, Shen W, Cao Z, Yang P, Xu Z, Ju D. Targeting asparagine and autophagy for pulmonary adenocarcinoma therapy. Appl Microbiol Biotechnol. 2016;100(21):9145-9161.
26. Xia Y, Shen S, Verma IM. NF-kappaB, an active player in human cancers. Cancer Immunol Res. 2014;2(9):823-830.
27. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. Signal Transduct Target Ther. 2017;2:e17023.
28. Kim JH, Gupta SC, Park B, Yadav VR, Aggarwal BB. Turmeric (Curcuma longa) inhibits inflammatory nuclear factor (NF)-kappaB and NF-kappaB-regulated

gene products and induces death receptors leading to suppressed proliferation, induced chemosensitization, and suppressed osteoclastogenesis. Mol Nutr Food Res. 2012;56(3):454-465.
29. Squarize CH, Castilho RM, Sriuranpong V, Pinto Jr DS, Gutking JS. Molecular crosstalk between the NFkB and STAT3 signaling pathways in head and neck squamous cell carcinoma. Neoplasia 2006;8(9):733-746.
30. Igelmann S, Neubauer HA, Ferbeyre G. STAT3 and STAT5 activation in solid cancers. Cancers (Basel) 2019;11(10)1428;doi.org/10.3390/cancers11101428.
31. Zhang W, Liu Y, Li YF, Que Y, Yang X, Peng L. Targeting of survivin pathways by YM155 inhibitis cell death and invasion in oral squamous cell carcinoma cells. Cell Physiol Biochem 2016;38:2426-2437.
32. Duarte VM, Han E, Veena MS, Salvado A, Sua JD, Liang LJ, Fauli KF, Srivatsan ES, Wang MB. Curcumin enhances the effect of cisplatin in supression of head and neck squamous cell carcinoma via inhibition of IKKβ protein of the nuclear factor kB pathway. Mol Cancer Ther. 2010:9(10):2665-2675.
33. Panosyan EH, Wang Y, Xia P, Lee WN, Pak Y, Laks DR, Lin HJ, Moore TB, Cloughesy TF, Kornblum HI, Lasky JL, 3rd. Asparagine depletion potentiates the cytotoxic effect of chemotherapy against brain tumors. Mol Cancer Res. 2014;12(5):694-702.
34. Tardito S, Uggeri J, Bozzetti C, Bianchi MG, Rotoli BM, Franchi-Gazzola R, Gazzola GC, Gatti R, Bussolati O. The inhibition of glutamine synthetase sensitizes human sarcoma cells to L-asparaginase. Cancer Chemother Pharmacol. 2007;60(5):751-758.

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 ± 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 ± 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 NFκB. A) Treatment with asparaginase at IC_{50} for 48h reduced the levels of NFκB. 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 NFκB 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
Abakumora et al. 2012 (10)	*In vitro*	LnCap (human prostate carcinoma), MCF7 (human breast carcinoma), SKOV-3, CaOV (human ovarian carcinoma), HepG2 (human hepatocelular carcinoma), HT-1080 (human fibrosarcoma, GGNC-1 (rat Gasser's ganglion neurinoma cells), and EPNT-5 (murine glioblastoma) cell lines	L-asparaginase (derived from *Erwinia carotovora* - ECAR LANS); L-asparaginase (from *Escherichia coli* - 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.
Ji et al. 2017 (19)	*In vitro*	Tu212 and Tu686 (human laryngeal squamous cell carcinoma) cell lines	Asparaginase (from *E. chrysanthemi*) - 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.
Kim et al. 2015 (12)	*In vitro* and *in vivo*	*In vitro:* MC38 (murine colon adenocarcinoma), 4T1 (murine mammary carcinoma) and AsPC1 (human pancreas carcinoma) cell lines; *In vivo:* 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 *Salmonella typhimurium* engineered to express the enzyme; Leunase - 5 IU/mL	*In vitro* cytotoxicity induced by both *S. typhimurium*-derived L-asparaginase (L-asparaginase-containing bacterial culture supernatant) and Leunase (asparaginase used clinically). *In vivo* tumor size regression induced by L-asparaginase expressed and released by *S. typhimurium* within the tumor tissue.
Knott et al. 2018 (14)	*In vivo*	*In vivo:* mice with tumors induced by inoculation of 4T1 (murine mammary carcinoma) or MDA-MB-231 (human breast carcinoma) cells	*In vivo:* 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.
Kyriakidis et al. 1990 (13)	*In vitro*	T47D, BT20, MCF-7 (human breast carcinoma), Walker 256 (rat breast carcinoma) and HeLa (human cervical carcinoma) cell lines	L-asparaginase (from *Tetrahymena pyriformis* or *E. coli*) - 0.01 IU	Asparaginase derived from both *T. pyriformis* and *E. coli* 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.
Lorenzi et al. 2006 (8)	*In vitro*	OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, IGROV1, and SKOV-3 (human ovarian cancer) cell lines	L-asparaginase (from *E. coli*) 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.
Lorenzi et al. 2008 (7)	*In vitro*	A total of 19 ovarian cancer cell lines	L-asparaginase (from *E. coli*) 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.
Panosyan et al. 2014 (33)	*In vitro* and *in vivo*	*In vitro:* DAOY, GBM-ES, U87 (human brain tumor) and GL-261 (murine glioma) cell lines; *In vivo*: mice with tumors induced by inoculation of DAOY cells	Asparaginase (from *E. coli)* *In vitro:* 0.0625-2 IU/mL *In vivo*: 2 IU/g of body weight	Asparaginase *in vitro* resulted in dose-dependent and variable growth inhibition and in reduction of neurosphere formation. Co-treatment with gemcitabine or etoposide resulted significantly better. *In vivo* results showed sustained deamination of asparagine after treatment with asparaginase. Asparaginase combined with temozolomide resulted in significant growth suppression.
Tardito et al. 2007 (34)	*In vitro*	HT1080 (human fibrosarcoma), RD (human rhabdomyosarcoma), SW872 (human liposarcoma), HOS, SAOS-2, and U2OS (human osteosarcomas) cell lines	Asparaginase (from *E. chrisanthemy*) - 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.

Tardito et al. 2011(15)	*In vitro*	HepG2, Hep3B, Huh-7, PLC-PRF-5, and Huh-6 (human hepatocellular carcinoma) cell lines	Asparaginase (from *E. chrisanthemy*) - 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_2/M cell cycle arrest.
Taylor et al. 2001(11)	*In vitro* and phase I clinical trial	*In vitro*: human tumor clonogenic assay with fresh breast, colon, lung and ovary tumors, melanoma, and non-Hodggkin's lymphoma samples Clinical trial: malignant melanoma (11), non-small cell lung cancer (6), sarcoma (2), colon cancer (2), bladder cancer (1), Cholangio-carcinoma (1), Multiple myeloma (1), Renal cell carcinoma (1), Salivary gland tumor (1), Small cell lung cancer (1)	*In vitro*: 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^2	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^2 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^2. 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 *in vitro* sensitivity.
Yu et al. 2012 (9)	*In vitro*	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.
Zhang et al. 2013 (16)	*In vitro* and *in vivo*	*In vitro:* MHCCLM3, MHCC97H, SMMCCLM3, and PLC (hepatocellular carcinoma) cell lines; *In vivo*: mice with tumors induced by inoculation of MHCCLM3 cells	L-asparaginase *In vitro*: 0.5 and 1 μml^{-1} *In vivo*: 20 μg^{-1} administered intraperitoneally every other day	Cells with low asparagine synthetase expression were more sensitive to L-asparaginase then cells with high expression. Asparagine synthetase-knockdown cells, both *in vitro* and *in vivo* (in xenograft tumors), had respective proliferation and tumor size significantly decreased and reduced after treatment with L-asparaginase.
Zhang et al. 2016 (25)	*In vitro*	A549 and H1975 (human pulmonary carcinoma) cell lines	Asparaginase (derived from *Erwinia*) - 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 (± Range) (%)**		***p*-value**
	48h	**72h**	
0.04	99 (±93.9-110.8)	86.1 (±77.1-97.4)	*
0.16	82.5 (±76.8-94.8)	67.1 (±51.7-80.3)	***
0.64	63.5 (±51.9-77.3)	40.3 (±35.5-58.1)	***
1	55.5 (±49.7-64.4)	37 (±33-45.2)	***
1.5	56.1 (±48.6-64.35)	37 (±31.9-45.4)	***
2.5	59 (±44.1-74.3)	34.2 (±30.6-43.7)	***
5	53.5 (±42.7-71.74)	34.2 (±31.5-42.6)	***

HaCaT			
Concentration IU/mL	**Median Cell Viability (± Range) (%)**		***p*-value**
	48h	**72h**	
0.04	95.7 (±88-98.3)	92.1 (±76.4-99.4)	0.5972
0.16	95.6 (±85.8-99.3)	88.6 (±68.5-98)	0.0706
0.64	86 (±75.9-97)	73.8 (±62-87.3)	**
1	75.7 (±65.1-97.1)	69.2 (±54.5-81.5)	*
1.5	69.6 (±61.3-77)	63.8 (±51.3-76)	0.0656
2.5	64.1 (±57-82.8)	62.2 (±53.2-68.4)	0.2486
5	60.7 (±30.5-69.1)	56.9 (±49.5-67.8)	0.9316

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