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***Original Contribution***

# THE EFFECT OF AZASERINE ON CYSTEINE TRANSPORT IN ERYTHROCYTES

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**ABSTRACT**

PURPOSE: The objective of the present study was to investigate the effects of azaserine on cysteine influx and efflux in erythrocytes. Azaserine is a potent carcinogen and induces tumors especially in the pancreas. Azaserine structurally resembles glutamine and thus interferes with a variety biological processes including enzymatic activities and transmembrane transport of solutes. Glutamine and cysteine have been shown to share similar transport mechanisms. In this respect azaserine, structurally resembling glutamine, may effect the cysteine transport across the cell membrane or conversely cysteine availability may effect the uptake or release of azaserine.

METHODS: In the present study, erythrocytes were treated with different concentrations of azaserine and cysteine and then cysteine influx and efflux processes in erythrocytes were measured through detection of nonprotein, soluble free –SH levels. The effect of glutamine on cysteine transport was also measured in a similar way.

RESULTS: Our results indicate that azaserine has an effect on bi-directional transport of cysteine through the erythrocyte membranes. An induction of cysteine influx was observed in the presence of 2 mM of azaserine (2.4 ± 0.07 μmol/ml erythrocyte) compared to the absence of azaserine (1.9 ± 0.14 μmol/ml erythrocyte). However, the effect of azaserine on cysteine efflux from erythrocytes was more pronounced and started at 0.5 mM of azaserine and then increased in a concentration dependent manner. In the presence of 2 mM of azaserine, the cysteine efflux reached to 0.71± 0.05 μmol/ml erythrocyte and in the absence of azaserine the efflux reached to 0.51± 0.01 μmol/ml erythrocyte. Glutamine at 2 mM concentration also significantly increased the cysteine efflux from erythrocytes.

CONCLUSION: Our results suggest that carcinogenic compound azaserine affects the flux of cysteine through erythrocyte membranes. This result suggest that azaserine may enter the erythrocytes by an azaserine/cysteine exchange mechanism. Glutamine which shares structural similarity with azaserine did not show any effect on cysteine influx. We suggest that in addition to its effect on induction of DNA damage and enzymatic activities, the described effects of azaserine in this study may contribute to its carcinogenity. Azaserine may limit the cysteine availability as it gains entry into the cells in exchange with cysteine. This process may represent a mechanism by which azaserine is accumulated in tissues in which it leads to carcinogenesis.

**Key words:** Azaserine, Toxicity, Cysteine transport

## INTRODUCTION

Cysteine is a nonessential amino acid intracellularly synthesized from methionine and serine amino acids (1). The other source of intracellular cysteine is the uptake of cystine from extracellular space and intracellular reduction to two molecules of cysteine (2). Cysteine thus obtained by the cells is used as a precursor for the biosynthesis of proteins which are needed by the cells. Cells also utilize cysteine for the synthesis of an important tripeptide glutathione (GSH) (3). GSH functions as an antioxidant and protects the cells and tissues from the deleterious effects of several free radicals (4). GSH is also involved in cellular detoxification of several xenobiotics. This activity mainly occur through conjugation reactions catalyzed by glutathione S-transferase (5). In these reactions cysteine

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provides the reactive sulfhydryl group and acts as the functional group of GSH. Except being incorporated into GSH, cysteine itself and when used as N-Acetyl-L-cysteine (NAC) also functions as an antioxidant against free radicals (6). In this respect, cysteine mainly functions in maintaining a proper intracellular and extracellular redox status. In addition to these effects, cysteine has also been shown to play a role in carcinogenesis. It was demonstrated that plasma concentrations of cysteine is inversely related to the occurrence of breast cancer (7). Thus cysteine availability may play a role in carcinogenesis.

Azaserine (O-diazoacetyl-L-serine) is a toxic and carcinogenic compound which was first isolated from the cultures of Streptomyces (8). Azaserine was initially used as antibiotic and antineoplastic compound (9,10). However, it was later discovered that azaserine was a potent tumor inducing agent. Azaserine has been shown to induce tumors in rat pancreas in 12 to 18 moths following weekly injections for several weeks (11). Azaserine is a glutamine analog and it interferes with the activity of several enzymes that use this amino acid as a substrate. Glutamine is especially utilized excessively in *de nova* purine synthesis. In this metabolic pathway, azaserine reacts with the peptide -SH groups in the active site of the amidotransferase enzymes that transfer ammonia from glutamine to an acceptor and causes irreversible inhibition (12). In addition to its effect on enzymatic activities, azaserine has also been shown to induce DNA damages (13). In human cells, azaserine has been demonstrated to induce two different lethal DNA damages which are carboxymethylated bases and $O^6$ - methylguanine (14).

These described effects of azaserine are readily accepted as significant contributors to azaserine induced carcinogenesis. However, there should be some other factors that play a role and should be present and functioning during azaserine induced carcinogenic transformation because azaserine does not induce tumors in all tissues. Azaserine induced tumors rather predominates more in the exocrine part of the pancreatic tissue. Azaserine has been shown to interfere with the transmembrane transport of some amino acids. It was shown that azaserine inhibits the uptake of glutamate and alanine in γ-glutamyl transpeptidase implanted erythrocytes (15). It has also been shown that azaserine strongly inhibits glutamine uptake (16). Thus we suggest that the effect of azaserine on solute transport may contribute to its carcinogenity. In fact there are some solid reasons to suggest that azaserine may effect the solute transport through the membrane. One is that, as mentioned for purine synthesis, it is able to irreversibly inactivate enzymes through reaction with their -SH groups. Sulfhydryl reactive compound NEM (N-ethylmalemide) has been shown to react with the -SH groups of the membrane and affect the transport characteristics of cells (17-19). If azaserine covalently attaches to critical proteins in the membrane, the transport characteristic of the cell membrane may change. The second is that azaserine itself structurally resembles the amino acid glutamine and thus may share similar transport pathways with other amino acids or inhibit the uptake or release of certain amino acids. Thus azaserine may limit the availability of certain amino acids. In this respect we investigated the effect of azaserine on cysteine transport by using human erythrocytes as a model system. Our results presented suggest that azserine significantly interferes with the transmembrane transport of cysteine. We suggest that these effects of azaserine may contribute to its carcinogenity by at least two different mechanisms. One, azaserine may limit the cysteine availability by stimulating its efflux and the second, azaserine may gain entry into the cells through exchange with cysteine, thus accumulate in the cells, then cause cellular damage and lead to carcinogenesis .

## MATERIALS AND METHODS

### Materials:

L-cysteine, glutamine, and azaserine were obtained form Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). 5,5'-Dithiobis(-nitrobenzoate) (DTNB) was obtained from Fluka BioChemica, (Switzerland). Blood was obtained from the blood bank of SSK Hospital, (Antakya/Turkey) as 300 ml units derived from people with no prerecorded medical conditions.

### Preparation of erythrocytes:

Plasma was separated by centrifugation at 2000 g for 5 min. The plasma and the buffy coat were then removed and discarded. The resulting erythrocyte pellet was washed three times with two volumes of phosphate buffered saline (PBS) (9 parts of 0.15 M NaCl and 1 part of 0, 1 M potassium phosphate buffer, pH 7, 4). PBS-glucose contained 8 mM of glucose in the PBS (20).

**L-cysteine influx studies:**

0, 25 ml of washed erythrocytes were suspended in 0.5 ml of Krebs Ringer phosphate buffer (135 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 5 mM Glucose, 10 mM NaH2PO4, pH 7.4 ) containing 1, 2.5, and 5 mM concentrations of L-cysteine in the presence and absence of azaserine and incubated for 60 and 120 min at 37 $^{o}$C in a water bath. At the end of incubation erythrocytes were removed, centrifuged and the supernatants were discarded. The free -SH concentrations in erythrocytes were then determined as described by Sedlak (21). Briefly 100 µl of erythrocytes were lysed in 100 µl of 10 % TCA prepared in sodium phosphate-EDTA buffer (0,01 M sodium phosphate/0, 005 M EDTA). The erythrocyte lysates were then centrifuged at 12, 000 g for 5 minutes. At the end of centrifugation 100 µl of the supernatant was mixed with 1, 9 ml of Tris-EDTA buffer containing 0, 6 µM/ml DTNB (400 mM Tris base, 20 mM EDTA, pH 8, 9). Samples were allowed to stand for 5 minutes to develop color. The absorbances of the samples were then measured at 412 nm and the concentrations of free -SH were calculated by using the mM extinction coefficient of 13, 1.

**L-cysteine efflux studies:**

0, 25 ml of washed erythrocytes were resuspended in 0.5 mL of Krebs Ringer phosphate bufer in the presence of different concentrations of L-cysteine. Erythrocytes were incubated at 37 $^{o}$C in a water bath for 1 hour to allow the uptake process. At the end of incubation erythrocytes were centrifuged and the supernatants were discarded. The erythrocytes were then resuspended in 0.5 ml of fresh Krebs Ringer buffer in the presence or absence of indicated compounds and incubated at 37 $^{o}$C for indicated times to allow the efflux process. At the end of incubation erythrocytes were centrifuged and the supernatants were transferred to fresh tubes. The free -SH concentrations in the supernatant was then measured as described above.

**Statistical analysis**

One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison tests were applied to process the data statistically. All tests were performed on triplicate samples. Results were expressed as mean ± S.D. $p < 0.05$ values were considered to be significant.

**RESULTS**

Results of cysteine influx by erythrocytes has been shown in **Figure 1**. As seen, erytrocytes influx cysteine in a concentration dependent manner. The higher the amount of cysteine concentration outside in the buffer the higher the amount of cysteine that the erythrocytes accumulated. In order to show that erythrocytes may efflux cysteine back into the media we first incubated the erythrocytes in the presence of cysteine and then transferred them to fresh buffer and allowed them to efflux the intracellular cysteine to the environment. As seen in **Figure 2** the erythrocytes effluxed cysteine to the environment when initially cysteine is absent from the environment probably to maintain a balanced intracellular and extracellular cysteine concentration. Both influx and efflux processed were stabilized at the end of 1 hour of incubation. Additional incubation for another hour did not result in a significant increase in cysteine influx or efflux.

Following the construction of cysteine influx and efflux processes we tried to determine if azaserine effects these processes. Thus we incubated the erythrocytes both in the presence of both cysteine and different concentrations of azaserine. As shown in **Figure 3**, only 2 mM of azaserine significantly increased the cysteine influx into the erythrocytes. The other lower concentrations of azaserine did not show any effect on cysteine influx. We then determined if azaserine has an effect on cysteine efflux. Thus, we first loaded the erythrocytes with cysteine and then measured the efflux process in the presence of different concentrations of azaserine. As shown in **Figure 4**, the presence of azaserine in the environment significantly increased the amount of cysteine that is effluxed from the erythrocytes. In contrast to its effect on influx process, the effect of azaserine on cysteine efflux process started at 0.5 mM of azaserine and then increased in a concentration dependent manner. Thus it is obvious that azaserine more efficiently affects the efflux of cysteine compared to cysteine influx. These results may point to a cysteine/azaserine exchange mechanism in erythrocytes.

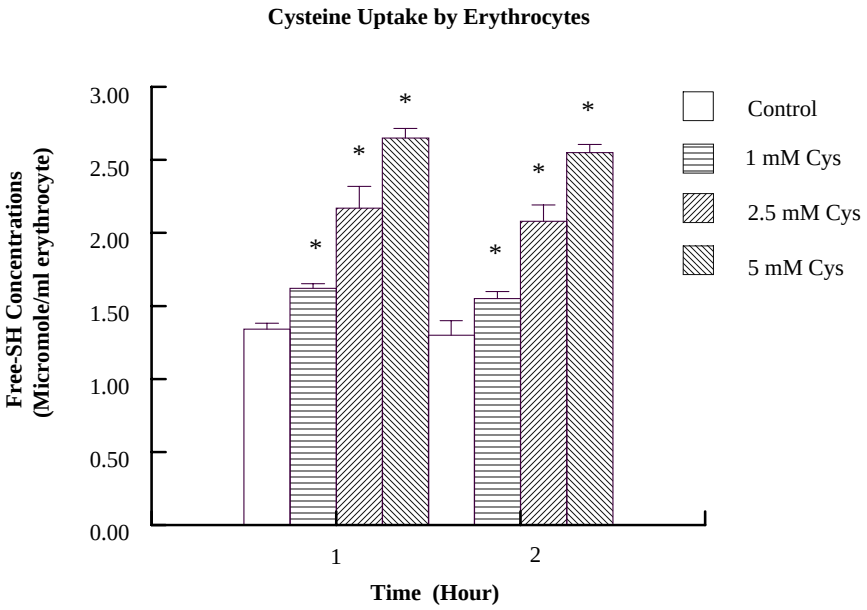


Figure 1 Cysteine uptake by erythrocytes: Erythrocytes were incubated in the presence of different concentrations of cysteine for 1 and 2 hours. Following incubation erythrocytes were washed and free-SH levels were measured in erythrocytes. Results are the mean, and S.D. of three seperate experiments. $p < 0.05$
*Significantly different from the control

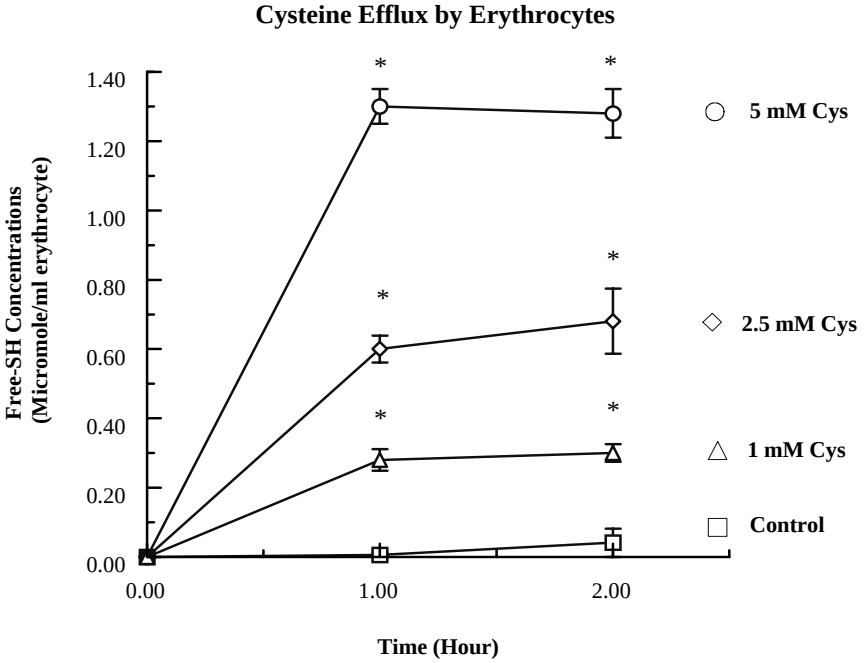


Figure 2 Cysteine efflux by erythrocytes: Eryhtrocytes were preincubated with different concentrations of cysteine for 1 hour. Erythrocytes were then washed and transferred to clean buffer and incubated further for 1 and 2 hours. At the end of incubation erythrcoytes were removed by centrifugation and free-SH levels in the supernatant were measured. Results are the mean and S.D. of three seperate experiments. $p < 0.05$
* Significantly different from the control.

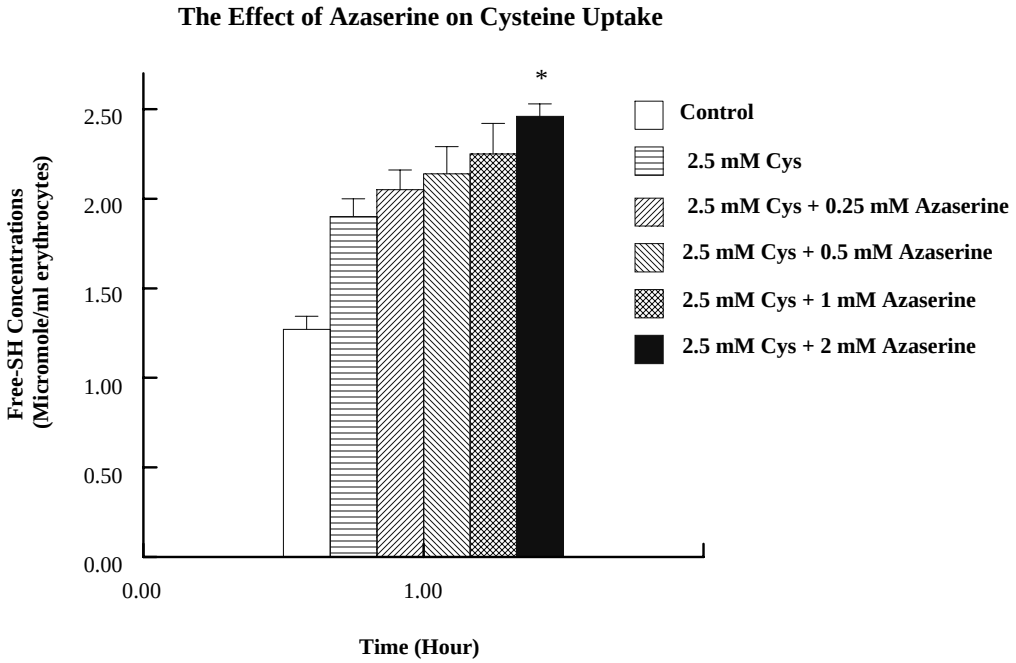


Figure 3. The effect of azaserine on cysteine uptake: Erytrocytes were incubated in the presence of 2.5 mM of cysteine and different concentrations of azaserine for 1 hour. At the end of incubation erythrocytes were washed and then the free-SH concentrations in erythrocytes were measured. Results are the mean and S.D. of three separate experiments. $p < 0.05$
*Significantly different from the 2.5 mM Cys group.

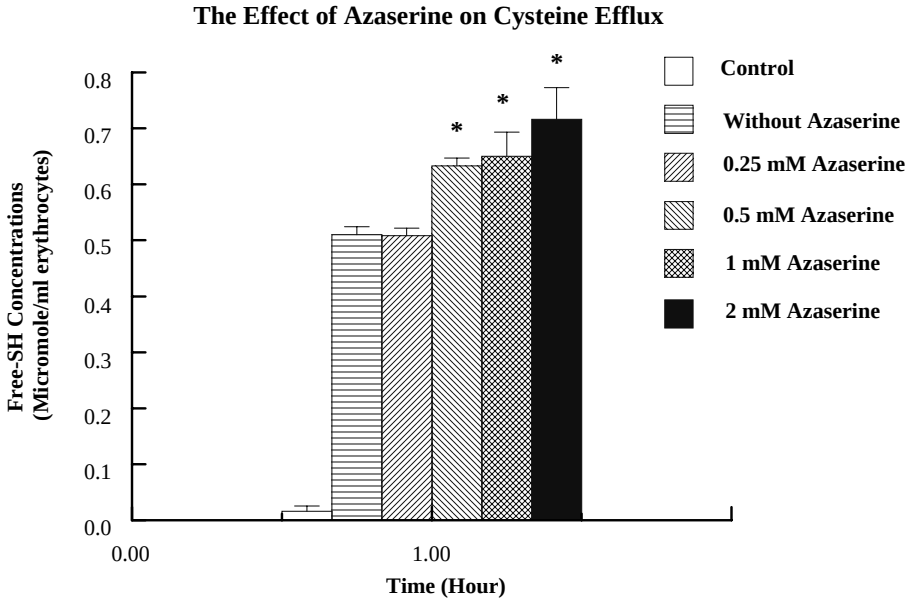


Figure 4 The effect of azaserine on cysteine efflux: All groups except the control were preincubated in the presence of 2.5 mM cysteine for 1 hour. At the end of 1 hour erythrocytes were removed and washed. The washed erythrocytes were transferred to fresh buffer containing the indicated concentrations of azaserine and further incubated for 1 hour. At the end of incubation erythrocytes were removed and free-SH concentrations were measured in the supernatant. Results are the mean and S.D. of three seperate experiments. $p < 0.05$
* Significantly different from the Without Azaserine group.

In order to show if azaserine induces any irreversible effect we exposed the erythrocytes to azaserine for a prolonged time and then measured its effect on cysteine uptake. As shown in **Figure 5**, azaserine did not induce any irreversible effect in cysteine influx. In the next step we tried to show if glutamine displays similar effects because of its structural similarity to azaserine. Thus we investigated whether glutamine effects the cysteine influx by erythrocytes. **Figure 6** shows that glutamine does not induce cysteine uptake. However, as shown in **Figure 7** glutamine significantly increases cysteine efflux from eryhtrocytes.

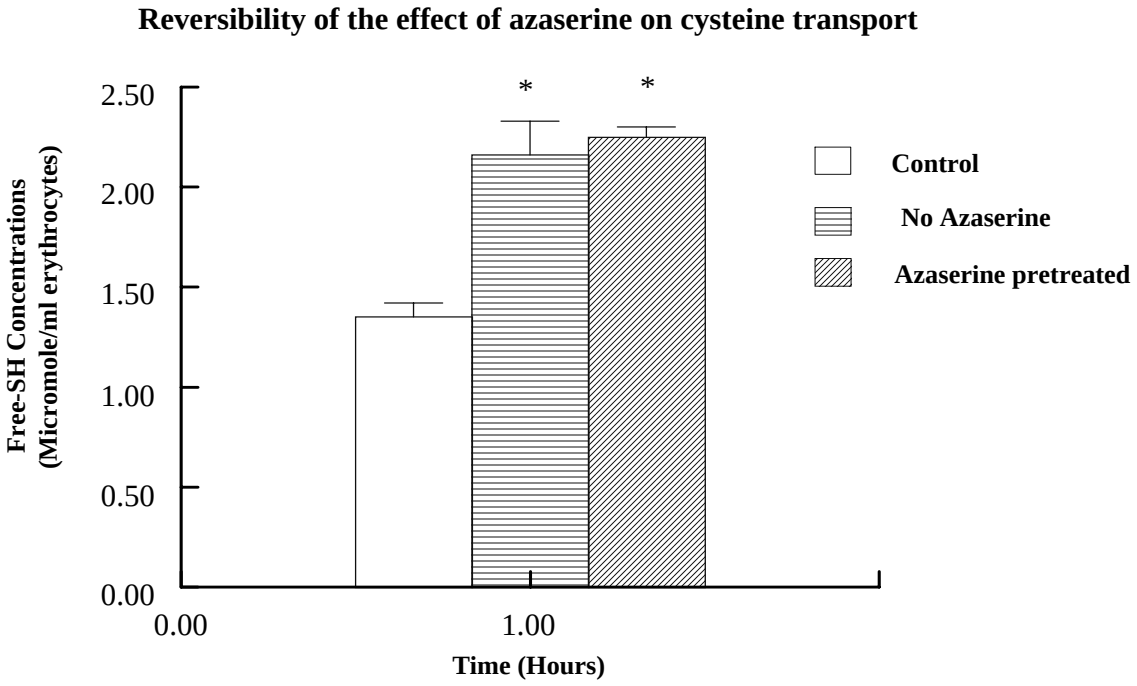


Figure 5 Reversibility of the effect of azaserine on cysteine transport: Azaserine preated group was incubated in the presence of 2 mM of azaserine for 5 hours. The other two groups were incubated in the buffer only. At the end of incubatin the erythrocytes were removed and washed. The washed erythrocytes, except the control group, were transferred to buffer containing 2.5 mM of cysteine and further incubated for 1 hour. At the end of incubation erythrocytes were removed, washed and free-SH levels were measured in erythrocytes. Results are the mean and S.D. of three seperate experiments. $p < 0.05$
* Significantly different from the control group

## DISCUSSION

Our results demonstrate that erythrocytes take up cysteine from the extracellular space and then release it back to the media when cysteine is absent outside of the cells. This behavior of erythrocytes may be related to a regulation of redox status of the plasma as was disscussed previously (22). In the present study, the effects of azaserine on these described cysteine influx and efflux process in erythrocytes were investigated. The first question that we tried to answer was whether azaserine caused any irreversible change in the activity of certain transporter proteins in the membranes as it causes irreversible enzyme inhibition. Our results propose that azaserine, in our experimental conditions, is not able to induce any irreversible effect in cysteine transporters. Thus we do not suggest a role for irreversible change in membrane transport characteristics in azaserine induced carcinogenesis.

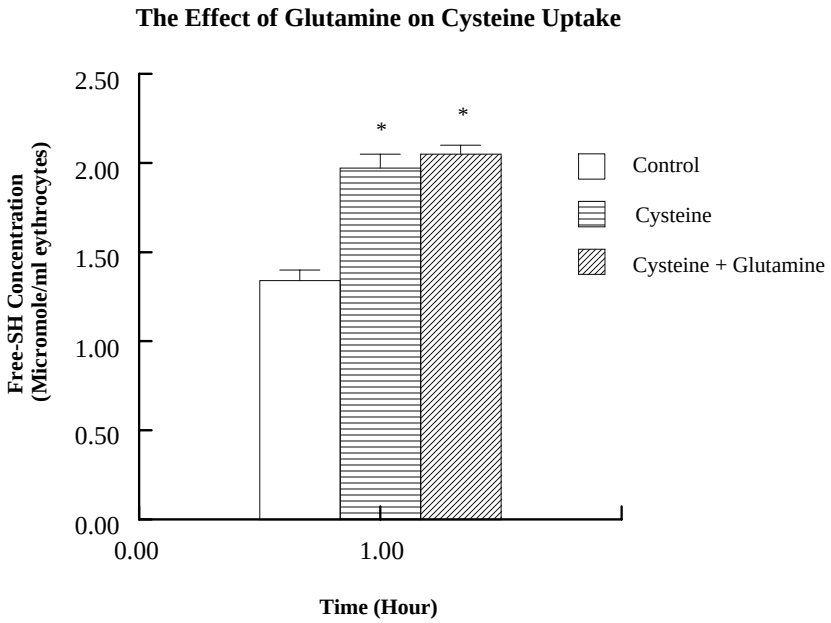


Figure 6 The effect of glutamine on cysteine uptake: Erythrocytes were incubated in Krebs Ringer containing 2. 5 mM of cysteine in the presence and absence of 2 mM of glutamine for 1 hour. At the end of incubation erythrocytes were removed, washed and then free-SH levels in erythrocytes were measured. Results are the mean and S.D. of three seperate experiments. $p < 0.05$
* Significantly different from the control group

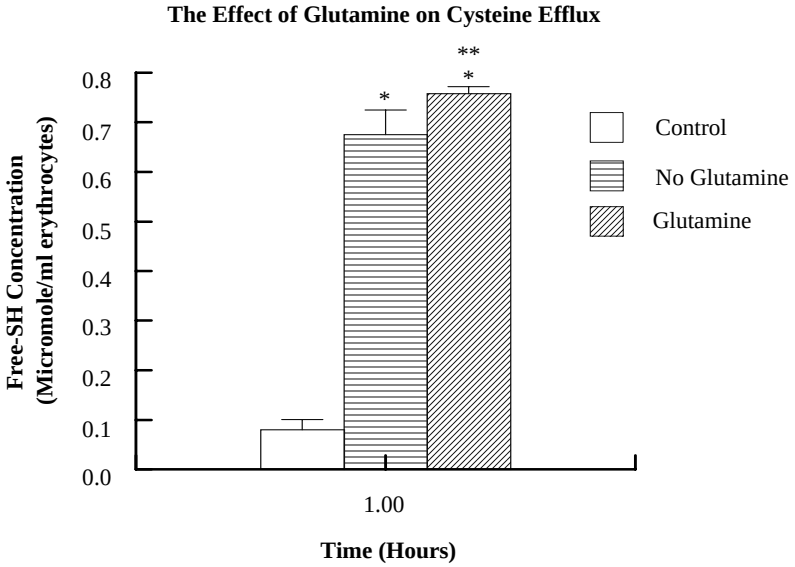


Figure 7 The effect of glutamine on cysteine efflux: Erythrocytes were incubated in the presence of 2.5 mM of cysteine for 1 hour. Then the erythrocytes were removed and washed. The washed erythrocytes were further incubated for 1 hour in the presence and absence of 2 mM of glutamine. At the end of incubation free-SH levels in the supernatants were measured. Results are the mean and S.D. of three seperate experiments. $p < 0.05$
* Significantly different from the control
** Significantly different from No Glutamine group

Azaserine is a toxic compound and induces tumor formation. We propose that in order to exert these already demonstrated effects, azaserine has to be accumulated into the cells or has to reach to a critical concentration. There are several evidences that support this hypothesis. In prokaryotes, azaserine is taken up by the aromatic amino acid transporters (23). Another structural analog of azaserine, 6-diazo-5-oxo-L-norleucine is also taken up by the aromatic amino acid transporters in E.coli (24). Mutations or defects in these transporters have been to show to limit intracellular azaserine availability and thus generate resistance to azaserine treatment (25). Studies using the D- and L- isomers of azaserine in pancreas again suggest that azaserine availability is critical for its effects to be observed (26). Only the L-isomer of the azaserine induced significant DNA damage and atypical acinar cell nodules (AACN) in rat pancreas. D-azaserine, however did not induce either DNA damage or AACN in rat pancreas. We conclude that azaserine especially induces tumors in pancreas because pancreatic cells have a mechanism that serve to maintain a higher azaserine concentration compared to other tissues. In this sense, we suggest a role for cysteine in azaserine uptake and accumulation by the pancreatic cells. Azaserine is an amino acid anolog and thus shares similar transport mechanism with other amino acids. These transport mechanisms usually involve either a co transport of two different amino acids into the cells or a counter transport of amino acids in which inside transport of one amino acid results in subsequent release of another different amino acid in reverse direction. These transport mechanisms normally serve to maintain an optimal intracellular concentartion of all amino acids required growth and propogation. An important example that is related to azaserine movement is the activity of the bacterial integral membrane protein YfiK (27). Overproduction of this protein leads to a parallel secretion of cysteine and O-acetylserine. O-acetylserine is an azaserine analog and expression of this protein also leads to azaserine resistance possibly by concomitant excretion of cysteine and azaserine. Our results presented in this study suggest the movement of azaserine and cysteine in reverse directions rather than a cotransport. A slight increase was observed when the erythrocytes were incubated together with azaserine and cysteine. However, efflux rate of cysteine was more efficient when azaserine was present initially at the trans side of erythocytes as observed in Figure 4. In addition, this result was observed at lower azaserine concentrations. An evidence which may support the hypothesis that azaserine and cysteine is concomitantly transported in reverse directions comes from an earlier study. In this study, it was demonstrated that azaserine analog glutamine is effluxed from the cells when cysteine is present in the extracellular space (28).

Azaserine induces tumors in pancreatic tissue especially. This action is mostly attributed to DNA damage and enzyme inhibitions. However, there are no explanations for why azaserine induces tumors especially in pancreatic tissue. Depending on our results we propose that in addition to DNA damaging and enzymatic activity inhibiting actions, azaserine induced carcinogenesis involves differential accumulation of azaserine in different tissues. Tissues that do not immediately respond to azaserine by induction of tumor formation may posses cysteine coupled coefflux of azaserine from the cells, an activity similar to bacterial YifK protein. Tissues that respond to azaserine by induction of tumor formation, in contrast, may not posses this type of a function. They may rather accumulate azaserine by an azaserine/cysteine exchange mechanism and absence of an azaserine removal system may accelerate or induce carcinogenesis. If such a system works this would also limit the cysteine availability for the cells in addition to azaserine accumulation. The whole proposal is also supported by azaserine resistance study in microorganisms (25). If presence of certain amino acids limit the azaserine availability and thus prevent azaserine toxicity. It could be suggested that the presence of certain amino acids that share similar transport pathways with azaserine may prevent azaserine induced carcinogenesis. One candidate amino acid that may interfere with azaserine induced carcinogenesis is cysteine. However, identification and demonstration of such mechanisms functioning in azaserine mediated carcinogenesis requires further investigations including in vivo studies.

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