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**MASTER**

# Anti-inflammatory actions of a taurine analogue, ethane β-sultam, in phagocytic cells, in vivo and in vitro

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**Abstract**

The ability of a taurine prodrug, ethane β-sultam, to reduce cellular inflammation has been investigated, *in vitro*, in primary cultures of alveolar macrophages and an immortilised N9 microglial cell line and *in vivo* in an animal model of inflammation and control rats. Ethane β-sultam showed enhanced ability to reduce the inflammatory response in alveolar macrophages, as assayed by the lipopolysaccharide-stimulated –nitric oxide release, (LPS stimulated-NO), in comparison to taurine both *in vitro* (10 nM, 50 nM) and in vivo (0.15mmol/kg/day by gavage). In addition, ethane β-sultam, (50, 100 and 1000nM) significantly reduced LPS-stimulated glutamate release from N9 microglial cells to a greater extent than taurine. The anti-inflammatory response of taurine was shown to be mediated via stabilisation of IkBα. The use of a taurine prodrug as therapeutic agents, for the treatment of neurological conditions, such as Parkinson's and Alzheimer's disease and alcoholic brain damage, where activated phagocytic cells contribute to the pathogenesis, may be of great potential.

Key Words: taurine: anti-inflammatory action: ethane-β sultam: nitric oxide: glutamate

Abbreviations: NO-nitric oxide: TauT-taurine transporter: LPS-lipopolysaccharide: ROS- reactive oxygen species: IκB-IkappaB: IκBa-IkappaBalpha: NFκB-nuclear factor kappa B: TNFα-tumor necrosis factor alpha:

## 1. Introduction

The sulphonated amino acid taurine is widely distributed in mammalian tissues, and is present at high concentrations, ~ 50 mM, in leucocytes, microglia and macrophages, [1], where it plays an important anti-inflammatory role. The mechanisms underlying the cytoprotective actions of taurine appear to depend, to a large extent, on the cellular type. The anti-inflammatory actions of taurine are manifested in a variety of forms, including its reaction with hypochlorous acid, in the presence of myeloperoxidase, to form the more stable and less toxic, taurine chloramine (Tau-Cl) in activated neutrophils, as well as modulation of calcium ion homeostasis [2]. The mode of taurine's anti-inflammatory action remains undefined since most studies have investigated the effects of Tau-Cl in immortalised macrophage cell lines, such as RAW 264 and NR8383 (for example see [3]). Since activated phagocytic cells, namely microglia, play an important role in many neurodegenerative conditions, such as Parkinson's disease [4] and alcohol-induced brain damage [5], compounds with anti-inflammatory actions, which are able to traverse the blood brain barrier may have therapeutic actions in the retardation of the disease processes.

Although taurine can be synthesised intracellularly from cysteine and methionine, the diet is the main source. Taurine uptake from the plasma into cells is tightly controlled by the taurine transporter (TauT). TauT controls the influx of taurine within very narrow limits, such that exogenously administered taurine only transiently increases levels within a number of cellular types, including liver [6] and, to a lesser extent, brain [7] [8] [6]. TauT activity can be modified by a number of factors including inflammation, where it is decreased [9] [10] [11], calcium ions and nitric oxide [9], glucocorticoids [12], protein kinase C activation in rat astroglial cells

[13] and human glioma GL15 cells [14]. Therefore taurine analogues, which are able to traverse membranes independently of TauT, may enhance intracellular taurine levels thereby promoting anti-inflammatory pathways.

It remains unclear as to how modifications to the taurine molecule might enhance its uptake across the lipid membrane bi-layer into the cell or passage across the blood brain barrier, BBB, since it would be against a concentration gradient. The ability of the taurine analogue, acamprosate, calcium acetyl homotaurinate, to reduce alcohol craving in detoxified alcohol abusers, is attributed to the presence of the calcium moiety and the *N*-acetyl homotaurine, which is reputed to enhance its passage across the blood brain barrier to bind with the metabotrophic glutamate 5 receptors [15].

Macrophages play an integral role in the development of innate and adaptive immune responses against bacterial pathogens. NFkappaB, one of the most ubiquitous transcription factor, plays a pivotal role when macrophages are activated, partly via increased IL-6 and iNOS expression, [16] [17] [18]. Inducible nitric oxide synthase (iNOS) is responsible for generating high levels of nitric oxide (NO) in activated macrophages. Under conditions favouring the production of high concentrations of both NO and superoxide anion, the highly reactive peroxynitrite anion is also generated. Among the many inflammatory mediators produced by activated phagocytic cells, i.e. macrophages and microglia, NO production has been widely regarded as representative of inflammatory activation [19] [20]. Therefore inhibitors which, preferentially target molecules that are involved in NFkappaB activation may be of therapeutic value.

In the present studies, a new taurine prodrug has been synthesised, ethane β–sultam. Its mode of passage across the cellular membrane remains unknown.

Intracellularly ethane β-sultam will hydrolysed to taurine, **Figure 1.** It's anti-inflammatory action has been studied both *in vivo* and *in vitro,* in alveolar macrophages isolated from rats, supplemented or not with ethane β-sultam as well as *in vitro* in an immortalised microglial cell line, N9.

## 2. Material and Methods.

All tissue culture media and chemicals were purchased from Sigma (Belgium) unless stated otherwise.

### 2.1. *Synthesis of ethane β-sultam*

All chemicals were purchased from Aldrich and were used without further purification. Taurine sulfonyl chloride (30.4g, 169mmol) was added to finely ground sodium carbonate (35.9g, 339mmol) in ethyl acetate (950ml) and stirred at ambient temperature for 48 hours. The reaction mixture was filtered through celite and the solvent removed by reduced pressure rotary evaporation at 30°C, giving a fine white powder (15.9g, 89%). Melting points were determined on a Gallenkamp melting point apparatus, and were 50-51°C [lit. 51-52°C (Page, 2004)]. 400 MHz $^{1}H$ and 67 MHz $^{13}C$ NMR spectra were determined on a Bruker Advance 400MHz spectrometer, while for the 500Mhz $^{1}H$ and 100Mhz $^{13}C$ NMR spectra a Bruker AMX 500 spectrometer was utilized. The results were; $^{1}H$ NMR: δ ($CDCl_3$) 3.39 (2H, dt, J 4 and 7, $CH_2N$); 4.32 (2H, dt, J 2 and 7, $CH_2SO_2$); 5.53 (1H, bs, NH). $^{13}C$ NMR: δ ($CDCl_3$) 60.6, 26.8. Infra-red measurements were determined on a Gallenkamp melting point apparatus and were: $\nu_{max}$ ($cm^{-1}$): 3307, 3048, 3022, 2991, 2918, 1416, 1336, 1299, 1249, 1212, 1171, 1156, 1107, 966, 803, 760, 668, 615. GC-MS were determined on a Varian GC-MS with a Finnigan MAT ion trap detector. For mass spectrometry a Fisons Quatro VG quadrupole mass spectrometer was utilsed; m/z (GC-MS) ($M^{+}H$):108, 77, 54, 42 . The $pK_a$ of ethane β–sultam was determined by titration using the reversible chromophoric change at 230nm, while hydrolysis of ethane β–sultam to the β–amino acid taurine as a function of pH was followed at 300nm.

### 2.2.Cell culture studies

*Isolation and cultures of alveolar macrophages*

Alveolar macrophages were isolated from the rats by pulmonary lavage. In brief, the rats were anaesthetised with Nembutal, a catheter inserted into the trachea, and the lungs lavaged gently with phosphate buffered saline, pH 7.4, approximately 50 ml. The alveolar macrophages were recovered after centrifugation at 1,200rpm for 10 min, and cell viability measured by trypan blue uptake extrusion (>98%). The cells at densities of 1 $x10^5$ or 2 x $10^5$ cells, were plated in wells in Dubecco media supplemented with 10% foetal calf serum, containing penicillin (100 μg /ml) and streptomycin (100μg /ml). The cells were left to adhere overnight, washed, and re-suspended in culture media to which lipopolysaccharides, LPS, (1 μg/ml) or LPS (1μg/ml) + interferon-gamma, 100 U/ml were added; and the cells were left for a further 24 h. The supernatants were removed and stored at –20$^o$C prior to analyses.

*Preparation of immortalised N9 glial cells*

The N9 microglial cell line was donated by Dr Paola Ricciardi Castagnoli (CNR Cellular and Molecular Pharmacology Centre, Milan, Italy). The cell line was originally derived from embryonic day 13 mouse microglial cultures [21]. The responses from these cells are very similar to those from primary rat microglia [22]: [23]. N9 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 50 μM β-mercaptoethanol, 50 U/l penicillin and 50 μg/ml streptomycin at 37$^o$C in 5% $CO_2$. The immortilised glial cells, N9 were grown to confluence and cells recovered after centrifugation at 1,200 rpm for 10 minutes. Cell viability was measured by trypan blue uptake extrusion (>98%). The glial cells were then plated at densities of 1 $x10^5$ or 2 x $10^5$ cells, in Dubecco media supplemented with 10% foetal calf serum, containing penicillin (100 μg /ml) and streptomycin (100μg /ml).

**2.3.Cellular studies of the anti-inflammatory action of taurine and the pro-drug ethane β-sultam**

*In vitro administration of taurine and ethane β-sultam with alveolar macrophages*

Isolated alveolar macrophages were incubated with taurine or ethane β-sultam, 10 and 50 mM for 24h. The cells were then stimulated with LPS, 1 μg/ml, for a further 24 h. The cell supernatants were then removed and both nitrite and glutamate content assayed.

*In vitro studies of taurine and ethane β-sultam with immortalised N9 glial cells*

N9 glial cells were incubated with taurine or ethane β-sultam, for 24h. The cells were then incubated with LPS, (1μg/ml) for 24h. The supernatants were then removed and assayed for nitrite and glutamate content.

*Nitrite assay*

Aliquots, 100 μl, of the cell culture supernatant were mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthalene diamine dihydrochloride, and 2.5% phosphoric acid) to determine nitrite concentration. After incubation at room temperature for 10 minutes, the optical density was measured at 560 mm. Sodium nitrite standards, in the range of 1-50 μM, were prepared

*Glutamate assay*

Aliquots, 250 μl, of the cell culture supernatant, + 640 μl water, were mixed with Tris (0.1M)-EDTA (0.002M)-hydrazine (64%) to which β-nicotinamide adenine dinucleotide (30mM) and adenosine 5'-diphosphate (100mM) had been added. The absorbance was read at 340nm after which glutamic dehydrogenase, 24 U, was added. The absorbance was re-read after 40 minutes incubation. L-glutamate standards in the range 100-500mM were prepared.

*IL-6 quantification*

IL-6 ELISA kit (R&D Systems, Abingdon, UK) was used for the quantitative measurement of this cytokine in the supernatants.

**2.4. Animal experiments**

Male Wistar rats 200-250g, were housed in polypropylene cages (*Iffa Credo, Belgium*) and allowed *ad libitum* access to normal diet (*Credo, Belgium*) and water. All animal procedures were in strict accordance with the recommendations of EEC (86/609/CEF) and with the Belgian 'projet de loi' (Moniteur Belge 19.02.1992, p. 3437) on the care and use of laboratory animals.

*In vivo administration of taurine and ethane β-sultam to control rats*

Groups of rats, 200-220g, n=6 in each group, were administered, taurine or ethane β-sultam, 0.15 mmol/kg/day, by gavage, volume = 1.5ml, at 10.00 am each day for 7 days. After this time, the alveolar macrophages were isolated by pulmonary lavage from each group of rats as well as from control rats that had received water, 1.5 ml, by gavage. The cells were stimulated with LPS, 1 μg/ml, for a further 24 h. The cell supernatants were then removed and nitrite and glutamate content assayed.

*In vivo administration of ethane-β-sultam to a rat model of inflammation*

A rat model of neuroinflammation has recently been described where ethanol is administered 3x/day for 2 days which is followed by 5 days of abstinence, for a period of 3 weeks [24]. Rats, 100-150g, n=6 in each group were administered the 1g ethanol /kg regime, supplemented or not with one dose/day of ethane-β-sultam, 0.15 mmol/kg/day, which was given by gavage, 30 minutes before the first ethanol administration. Ethane-β-sultam was administered daily for 3 weeks. Alveolar macrophages were isolated, plated at densities of 1 $x10^5$, and then stimulated with LPS for 24h. The supernatants were then removed and both NO and IL-6 assayed.

**2.5.Mechanism of taurine's anti-inflammatory action**

*NFKappaB analysis in alveolar macrophages isolated from taurine supplemented rats*

Rats received taurine orally, 12.5g/l, in the drinking water for 7 days, daily intake = 500 mg/day. = 15mmol/kg/day. The alveolar macrophages were isolated by pulmonary lavage and stimulated with LPS 1 μg/ml and TNFα, 4 U/ml, for 15, 30 and 60 minutes. Nuclear proteins were then isolated from the alveolar macrophages. In brief, the cells were suspended in ice-cold buffer A (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.9) and left on ice for 20 min before being vortex-mixed and centrifuged at 15,000 x g for 30 s. The nuclear pellets were gently suspended in cold buffer B (50 mM HEPES/KOH, 50 mM KCl; 300 mM NaCl, 0.1 mM EDTA, 10% (by volume) glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9) and left on ice for 20min. After centrifugation at 15,000x g for 5 min at 4°C, aliquots of the supernatant containing the nuclear fraction were rapidly frozen in two aliquots in liquid nitrogen and stored at –80°C. The protein concentrations were assayed, by the BioRad method, in one of the aliquots prior to the electrophoretic mobility shift assay.

The nuclear fraction containing 20 μg protein was incubated for 30 min at room temperature with 0.2 ng $^{32}$P-labelled oligonucleotide probe,

5' GATCAGGGACTTTCCGCTGGGGACTTTCCAG-3', 1 mg BSA and 1.25 mg poly(dI-dC), poly(dI-dC) (Pharmacia Biotech Benelux) in buffer (20 mM HEPES/KOH, 75 mM NaCl, 1 mM EDTA, 5% (by volume) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT, pH 7.9) in a final volume 20 μl. DNA-protein complexes were resolved on a non-denaturing 6% (w/v) polyacrylamide gel, run for 4 h at 180 V in buffer (2.5 mM Tris, 2.5 mM $H_3BO_3$, 2 mM EDTA, pH 8.5). The gel was then dried and autoradiographed on Fuji X-ray film (General Electrics, Antwerp, Belgium). For

competition experiments, unlabelled probe, either wild type 5' GATCAGGGACTTTCCGCTGGGGACTTTCCAG-3' or mutated.

5'-GATCACTCACTTTCCGCTGCTCACTTTCCAG-3' was added in excess (50x) in buffer. In each experiment the band of the DNA-protein complex from the unstimulated and stimulated macrophages was verified by comparison to the band obtained after stimulation of U937 with LPS, 10 μg/ml. The intensity of each of the NFkappaB complex was quantitated.

*Ikbα studies in alveolar macrophages isolated from taurine supplemented rats*

Rats received taurine in their drinking water at three different concentrations, 6.25, 12.5 and 25 g/l for 8 days. Alveolar macrophages were isolated from each treatment group, pooled, and then activated with LPS, (1 μg/ml) and TNFα (4 U/ml) for 60 minutes. IκBα was detected by Western blot analysis using a specific anti-human full length IκBα polyclonal antibody (Euromedex, Souffel Weyersheim, France). Cytoplasmic extracts, each containing 10 μg protein, isolated from the activated macrophages, were mixed with the loading buffer (10mM Tris-HCl, pH6.8, 1% SDS, 25% glycerol, 0.1 mM 2-ME and 0.03% bromophemol blue), prior to their loading onto 10% poyacrylamide-SDS. After electrophoresis, the resultant gels were electro-transferred onto Immobiolon-P membranes (Millipore, Bedford, MA). Filters were incubated in a primary antibody for 120 minutes, (1/10000 dilution) and in peroxidase-conjugated rabbit anti-body IgG (1/1000 dilution:DAKP, Copenhagen, Denmark) for 60 minutes at room temperature and finally analysed by Amersham's enhanced chemiluminescence system (Amersham, Aylesbury, UK) with Fuji Xray film. Coomassie blue staining was used to confirm that equal amounts of protein had been applied to each lane. The density of each of the IkBα band was quantitated.

*Statistical analysis.*

All of the results are presented as mean ± standard deviation. Statistical analysis was by ANOVA 1. Significance was calculated by GB Stat programme and set at $P<0.05$ by Fischer test.

## 3. Results

*3.1.Hydrolysis of ethane-β-sultam to taurine.*

The hydrolysis of the ethane-1,2-β-sultam occurs with exclusive S-N fission and there is no NMR evidence of any reactions involving either C-S or C-N bond breaking, **Figure 2**. The pH rate profile for the unsubstituted β-sultam shows only reactions that are either first order in hydronium-ion or hydroxide-ion concentration, but in aqueous sodium hydroxide solutions the rate becomes pH independent.

The $pK_a$ of ethane β-sultam was found to be 12.1 by means of a reversible chromophoric change at 230nm. In aqueous sodium hydroxide (NaOH) solutions a slow exponential decay of the chromophore of ethane β-sultam was observed at 300 nm and 30$^{o}$C. The absorbance change had a very slow rate, which was found to be independent of hydroxide-ion concentration and gave a first order rate constant of $1.00 \times 10^{-5}$ $s^{-1}$. Above pH 7 ethane 1,2-β-sultam undergoes attack by hydroxide ion at the sulfonyl centre resulting in ring opening *via* cleavage of the S-N bond and formation of the β-amino sulfonic acid, taurine. As a result, in solutions of pH greater than the $pK_a$ of ethane β-sultam the rate of hydrolysis becomes pH independent as it depends on the concentration of the minor species, the neutral β-sultam, which decreases as the pH is increased. The second order rate constant, $k_{OH}$, for the alkaline hydrolysis of the ß-sultam is $5.16 \times 10^{-4}$ $M^{-1}$ $s^{-1}$ which is 20-fold lower than the second-order rate constants for the hydroxide ion hydrolysis, $k_{OH}$, of simple *N*-alkyl-β-sultams e.g. N-methyl ($1.41 \times 10^{-2}$ $M^{-1}$ $s^{-1}$). At pH 7 ethane-1,2-β-sultam is relatively stable and only very slowly hydrolysed to taurine. Below pH 7, ethane-1,2-β-sultam undergoes an acid catalysed hydrolysis to taurine.

*3.2. In vitro studies of the anti-inflammatory action of taurine or ethane β-sultam in alveolar macrophages isolated from control rat and immortalised N9 microglial cells.*

The release of NO and glutamate from isolated alveolar macrophages after LPS or LPS + IFNγ stimulation is shown in **Figure 3a.** A threefold increase in NO release was evident after LPS stimulation which increased to 4 fold with LPS + IFNγ stimulation. No changes in glutamate release were apparent after stimulation with either LPS or LPS + IFNγ, **Figure 3a**.. In these initial studies it was important to ascertain whether ethane-β-sultam showed superior anti-inflammatory effects to that of taurine. **Figure 3b** clearly showed that both taurine and ethane-β-sultam reduced nitrite release in LPS-stimulated alveolar macrophages, the latter to a greater extent than taurine. Levels of glutamate present in the supernatants from un-stimulated and stimulated alveolar macrophages did not alter after LPS stimulation (data not shown). **Figure 4a** shows the release of NO and glutamate from N9 cells after stimulation with LPS or LPS + IFNγ. As can be observed there was a dramatic increase in both of these mediators of inflammation, particularly after LPS + IFNγ. Supernatant glutamate levels in un-stimulated microglial cells were approximately 90 μM $\pm$ 9, and increased significantly after LPS stimulation, **Figure 4a**. Both ethane-β-and taurine showed an enhanced anti-inflammatory action, as assessed by nitrite release, in LPS-stimulated N9 glial cells, **Figure 4b**. The levels of glutamate released after LPS stimulation were reduced by all doses of ethane-β-sultam, 50 nM, 100 nM and 1000nM by comparison to stimulated controls, but only with the highest taurine dose, **Figure 4c.**

*3.3. In vivo studies of the anti-inflammatory action of taurine and ethane β-sultam in alveolar macrophages isolated from control rats.*

The alveolar macrophages were isolated from rats which had received daily doses of either ethane β-sultam or taurine, 0.15 mmol/kg/day, by gavage or water for 7 days. These cells were cultured and then stimulated with LPS for 24h. Nitrite release was significantly decreased by both taurine and ethane–β-sultam, the latter to a greater extent than taurine, **Figure 5**.

*3.4 In vivo studies of the anti-inflammatory action of ethane β-sultam in alveolar macrophages isolated from rats with alcohol-induced inflammation.*

Binge drinking over a 3 week period activates the innate immune system which is verified by activated macrophages in the periphery and neuro-inflammation in the brain (Ward et al., 2009). The administration of ethane-β-sultam during the binge drinking regime reduced the activation of the innate immune system which was confirmed by decreases in the release of both NO and IL-6, **Figure 6a and Figure 6b.**

**Investigation of taurine's anti-inflammatory action**

*3.5. NFkappaB activation in macrophages.*

**Figure 7a** shows the electrophoretic mobility-shift assay for the activation of NFκB by LPS and TNFα in alveolar macrophages isolated from either taurine supplemented, stimulated at 15, 30 and 60 minutes, or control rats, stimulated at 15, 30, 60 and 120 minutes. The density band values identified the higher NFκB activation in the controls at each time interval by comparison to the taurine supplemented rats, **Figure 7b.** It was noteworthy that NFkB activation was reduced at time 0 and at each of the subsequent time points assayed in the taurine supplemented alveolar macrophages by comparison to the controls.

*3.6. IkappaBα stabilisation*.

**Figure 8a** shows a Western blot for the macrophage cytosolic extracts, which had been immobilised on the membrane and then probed with IkBα protein. The levels of IkBα increased in the macrophage cytosolic fraction as the concentrations of taurine administered to the rats increased. The density band intensities showed that there was a significant increase with increasing taurine concentrations, **Figure 8b**.

## 4. Discussion

In these present studies we have shown that the pro-drug ethane β-sultam showed an enhanced ability to diminish the inflammatory response after stimulation with LPS in comparison to taurine. Variable results for the anti-inflammatory action of taurine *in vitro*, have previously been presented. For example, its protective effect in co-cultures was reported in intestinal Caco-2 cell monolayers from the damage by macrophage-like THP-1 cells,[25] while no effect was reported, in peritoneal neutrophils [26], RAW 264.7 cells [27],or peripheral blood mononuclear cells [28]. In contrast the anti-inflammatory actions of its chlorinated form, taurine chloramine, *in vitro*, inhibits pro-inflammatory mediators, such as nitric oxide and TNFα, in a variety of activated cell lines, including rodent macrophages [29] NR8383 cells, [3] and peripheral blood mononuclear cells [28]. The lack of an effect of taurine alone in such systems may be attributable to its limited ability to enter cells, due to TauT down-regulation, as well as to the specific cell line employed, [30].

The transcription of TauT is up-regulated by a number of compounds by virtue of the presence of various recognition sites on its promoter region. These sites include the TonE hypertonicity site (part of the TonE (tonicity-responsive element)/TonEBP (TonE- binding protein) system [31] the TPA responsive element (TGAGTCAG), which is responsible for gene regulation by the protein kinase C (PKC)-mediated signal transduction pathway[32] the glucose tumour suppressor gene, p53 [33], TNFα and an NFκB consensus-like sequence [34].

It was our intention in these studies to overcome such restrictions on its regulation by the use of taurine analogues and prodrugs that could circumnavigate the homeostatic control exerted by TauT. Previously other taurine pro-drugs have been investigated for their anti-inflammatory action, e.g. 5-aminosalicyltaurine, which

liberates taurine and 5-aminosalicylic acid, and was reported to ameliorate chemically induced colitis after rectal administration [35]. In our recent studies of chronically alcoholised rats, oral administration of Acamprosate (400 mg/kg/day), calcium acetyl homotaurinate, during the chronic ethanol intoxication procedure exhibited an anti-inflammatory effect by decreasing the formation of reactive oxygen radical species during the initial detoxification period, in comparison to rats chronically ethanol intoxicated alone [36]. The exact mode of action remains unknown, but the presence of the acetyl and calcium moiety, as well as homotaurine contributed to its protective properties, mediated by metabotrophic GluR5 receptors in various brain regions [15]. In the present studies, we clearly showed that in vitro and in vivo, ethane β-sultam exhibited greater anti-inflammatory actions than taurine in both alveolar macrophages and microglial cells after ex vivo LPS stimulation. Furthermore, it was shown that there was an increase in taurine levels in specific brain regions after three weeks of ethane-β-sultam (Della Corte, Dexter, Ward unpublished data) thereby confirming that the prodrug had indeed traversed cellular membranes. In contrast it is extremely difficult to increase brain taurine levels unless extremely high doses of taurine are administered.

Microglia are the resident immune cells of the central nervous system and play an important role in preserving the neurons. However when microglia are activated, by pro-inflammtory stimuli, substantial levels of glutamate are released [37]. This is due to the activation of NADPHoxidase which generate superoxide, leading to the formation of hydroxyl radical via Fenton chemistry, resulting in oxidative stress. Depletion of reduced glutathione will occur, which will be remedied by the synthesis of more of this antioxidant by the influx of cystine via the Xc exchange system which releases glutamate [37]. In these present studies increases in glutamate release were

apparent in microglia, but not macrophages, after bacterial stimulation. Expression of inducible nitric oxide synthase will also be enhanced after microglial activation as was evident in these present studies. Nitric oxide will deplete ATP production, and inhibit cytochrome oxidase in competition with oxygen, thereby inducing hypoxia and activating the hypoxia-inducible factors, which will result in the release of glutamate and excitoxicity [38]. However such glutamate release may also stimulate microglia to produce neurotrophic factors which will support neuronal survival and growth. This is mediated via increases in intracellular calcium mediated partly by the stimulation of NMDA receptors and group III metabotrophic glutamate receptors which induces intracellular calcium release from the endoplasmic reticulum, as well as stimulation of glutamate transporters to increase influxes of extracellular calcium [39]. Such increases in intracellular calcium will lead to activation of the protein kinase C pathway which induces microglial neurtrotrophic expression and production.

Beta sultams are sulfonyl analogues of β-lactams, and activated derivatives are able to inactivate serine enzymes, such as elastase, which is released in response to inflammatory stimuli and plays a major role in protein digestion following phagocytosis, and β-lactamase, (which is the main cause of bacterial resistance to antibiotics) by sulfonation of the active site serine [40]. The parent unsubstituted β-sultam does not inhibit serine enzymes but is slowly hydrolysed to taurine. The potential therapeutic use of ethane β–sultam as a prodrug has several advantages compared to taurine. These include an increased lipophilicity, which may facilitate its uptake by the cell prior to transformation to taurine [41]. However it should be emphasised that simple diffusion of taurine or its pro-drugs across a lipid bi-layer against a concentration gradient, (i.e. the high concentration of taurine within the cell), might indicate that such prodrugs have an alternative passage across the

membrane, possibly via a protein transporter. In the present studies ethane β–sultam showed superior anti-inflammatory properties, both *in vivo* and *in vitro* to those of taurine.

NFkappaB is an important transcription factor that regulates genes involved in immunity and inflammation [42]. Under normal conditions NFkappaB is present in the cytoplasm in an inactive state bound to the inhibitory protein IkappaB (IkB). Stimulation with pro-inflammatory cytokines such as TNFα initiates an intracellular signalling cascade, resulting in the phosphorylation and subsequent degradation of IkB by the 26S-proteasome. The degradation of IkBα releases NFkappaB allowing its translocation to the nucleus, resulting in the activation of cyclooxygensae-2 (COX-2) cytokines, chemokines, cell surface receptors and adhesion molecules that are pivotal mediators of the immune and inflammatory responses. Therefore, therapeutic intervention against NFkappaB activation might be advantageous in preventing the progression of inflammation-related diseases. Previous studies have focused primarily on the action of taurine chloramine on this transcription factor. Taurine chloramine was shown to depress the migration of NFkappaB to the nucleus of activated NR8383 cells, [3] and murine peritoneal macrophages and RAW 264.7 cells [43]. Attenuation of ERK½ activation was also reported in the latter study. Treatment with taurine chloramine will result in decreased phosphorylation [44] [3] and oxidation of IκBα [1] and a lower activity of IκB kinase, [3]. The present *in vivo* studies demonstrated a clear interaction between taurine and IkBα, with taurine showing a dose dependent stabilisation of this inhibitory protein. Although these studies were not undertaken with ethane-β-sultam, the corresponding changes in both NO and IL-6 after the incubation with this taurine pro-drug would confirm indirectly that these changes were occurring via changes in NFkappaB activation. Such changes

in IL-6 release are also important in decreasing the iron loading of the tissues, since decreases in IL-6 will diminish hepcidin secretion thereby preventing the internaliation of iron via the interaction of hepcidin with ferroportin [45].

It has been reported that elderly patients (61-81 years) have significantly lower blood taurine concentrations, compared with younger individuals (20-38 years) [46]. Such a decline could exacerbate the oxidative damage that occurs during the ageing process as well as the modulation of various neurotransmitter systems and immune responsiveness. Taurine supplementation might reverse these effects [47]. In this respect, the taurine prodrug, studied here, which may exert their beneficial effect independently of TauT, might be particularly valuable. With an ever-increasing older population, palliative measures to retard the effects of ageing and neurodegenerative processes could be of enormous financial and social advantages.

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**Figure Legends**

**Figure 1.** The hydrolysis of ethane-β-sultam to taurine .

**Figure 2**. Rate of hydrolysis of ethane-β-sultam to taurine from pH2- pH10. . The $pK_a$ of ethane β−sultam was determined by titration using the reversible chromophoric change at 230nm, while hydrolysis of ethane β−sultam to the β−amino acid taurine as a function of pH was followed at 300nm.

**Figure 3a.** Release of nitrite and glutamate from alveolar macrophages after stimulation with LPS, 1μg/ml[dark grey] or LPS + IFNγ 100 units/ml [light grey]. The data shown were obtained in one representative experiment of at least three independent experiments. Significance is represented by *, P <0.05, **, P<0.01.

**Figure 3b.** Release of nitrite from alveolar macrophages after ex vivo incubation with taurine, 10 mM and 50 mM, or ethane β-sultam, 10 mM and 50 mM for 24 h followed by stimulation with 1 μg/ml LPS for 24 h**.** The data shown were obtained in one representative experiment of at least three independent experiments. Significant reductions were calculated for each dose, **, P <0.01.

**Figure 4a.** Release of nitrite and glutamate from N9 microglial cells after stimulation with LPS, 1μg/ml or LPS + IFNγ 100 units/ml. The data shown were obtained in one representative experiment of at least three independent experiments. Significance is represented by *, P <0.05, **, P<0.01.

**Figure 4b.** Nitrite release into the culture media from N9 microglial cells after incubation with taurine or ethane-β-sultam, 1000 nM, 100 nM and 50 nM for 24h hr [open blocks] followed by LPS stimulation, 1 μg/ml, for a further 24 h [dark grey]. The results are representative of at least 3 separate incubation studies. Significance is represented by *, P <0.05, **, P<0.01.

**Figure 4c.** Glutamate release into the culture media from N9 microglial cells after incubation with taurine or ethane-β-sultam, 1000 nM, 100 nM and 50 nM for 24h hr [open blocks] followed by LPS stimulation, 1 μg/ml, for a further 24 h [dark grey].. The results are representative of 3 separate incubation studies. Significance is represented by **, P <0.01 by comparison to the non-stimulated controls; $ P <0.01 by comparison to stimulated controls.

**Figure 5.** Release of nitrite from alveolar macrophages isolated from rats, n=6 in each group, which had received a daily dose of taurine or ethane-β-sultam 0.15mmol/kg/l /day by gavage for 7 days. The alveolar macrophages were left for 24h (open blocks non-stimulated] before being stimulated ex vivo and with LPS, 1μg/ml, (black blocks] for 24h**.** Significance is represented by **, P <0.01; * P<0.05 by comparison to the stimulated controls; $ P <0.05 by comparison to un-stimulated controls.

**Figure 6.** Changes in the release of NO, **Figure 6a** and IL-6, **Figure 6b**, from alveolar macrophages isolated from rats, n=6 in each group, which had received intermittent ethanol administration 3x/day , +/- one daily dose of 0.15 mmol/kg/l of ethane-β-sultam by gavage. The alveolar macrophages were left for 24h [open blocks]

before being stimulated ex vivo with LPS, 1μg/ml, [black blocks] for 24h. Significance is represented by **, P <0.01; * P<0.05 by comparison to the corresponding stimulated and non-stimulated cells.

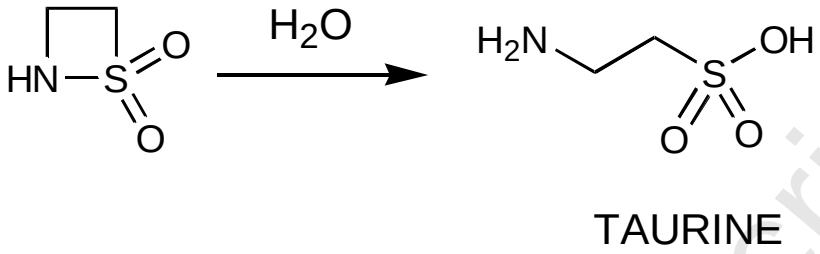
**Figure 7a**. Electromobility shift showing NFkappaB activation in alveolar macrophages isolated from control rats and rats which had received taurine supplementation in their drinking water, (12.5 g/l), after their stimulation with LPS, 1 μg/ml, and TNFα, 4U/ml, at different time points. Nuclear pellets were prepared and incubated with $P^{32}$ labelled primers.

**Figure 7b**. Density intensities for each NFkappaB band

**Figure 8a**. Western blot showing stabilisation of IkappaBα in alveolar macrophages isolated from rats which had received oral administration of taurine, 6.25, 12.5 or 25 g/l, for 7 days and then stimulated with LPS, 1 μg/ml, and TNFα, 4U/ml.

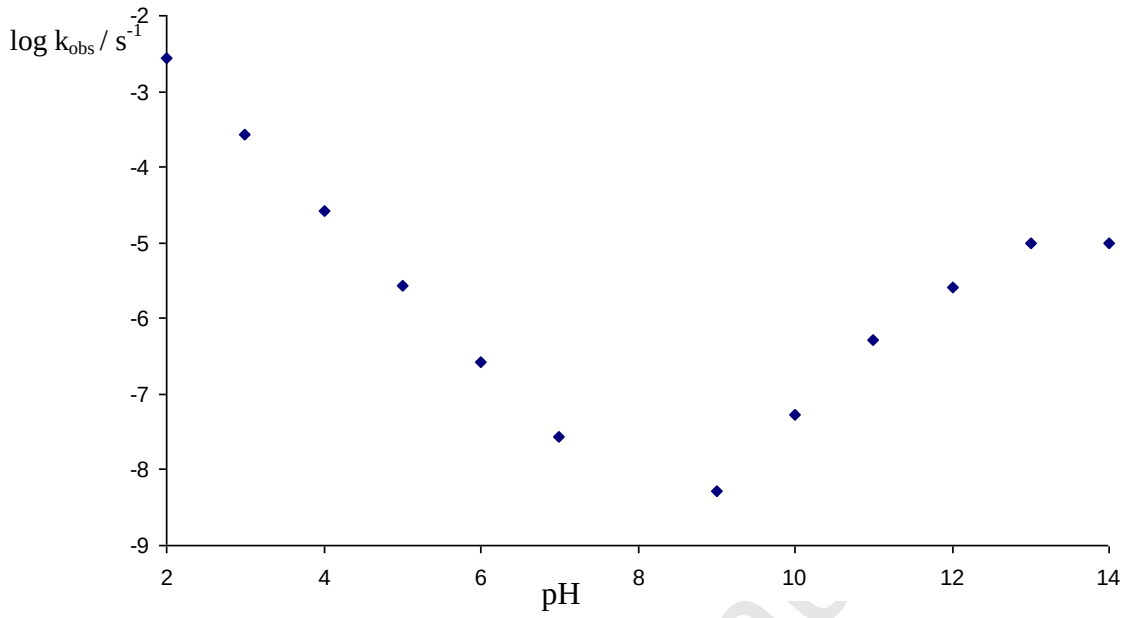
**Figure 8b**. Density intensities for Ikappabα, three individual bands from each concentration were scanned. The mean and standard deviation for each concentration is represented in the Figure.

**Figure 1.**




TAURINE

**Figure 2**



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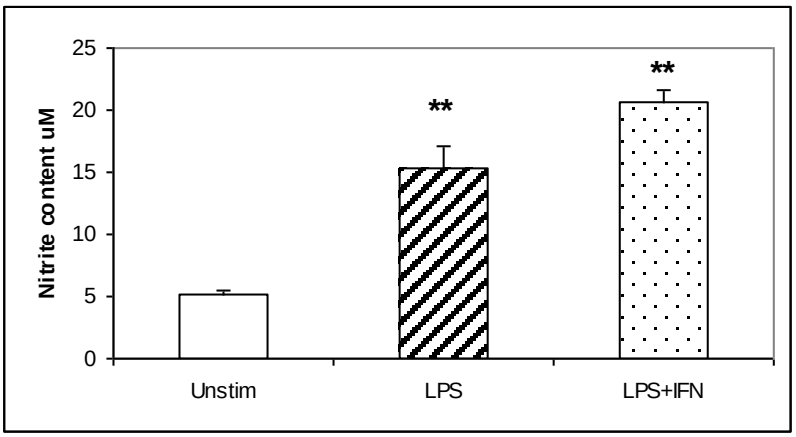


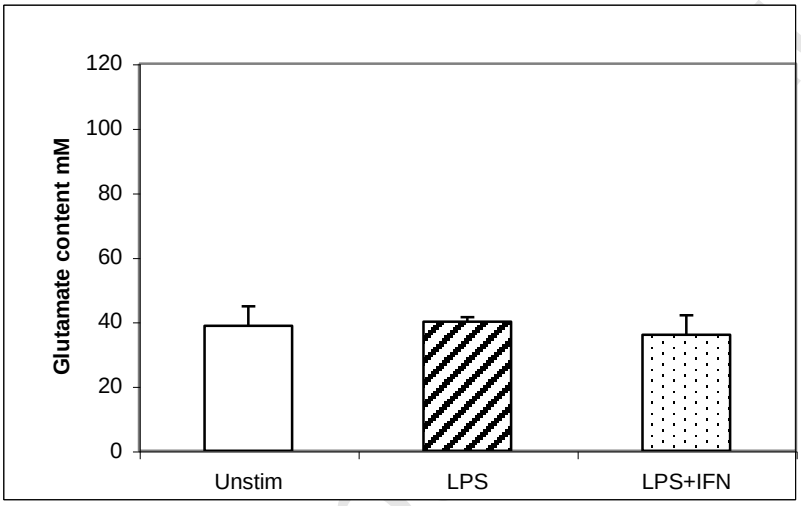

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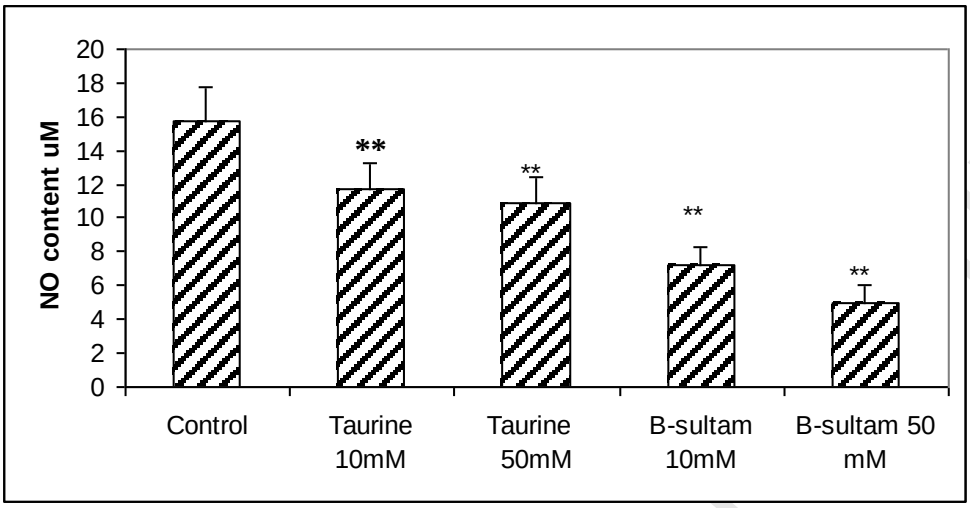
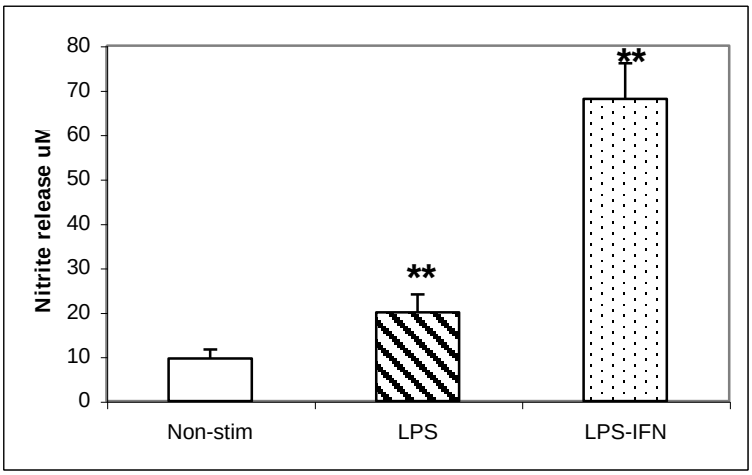
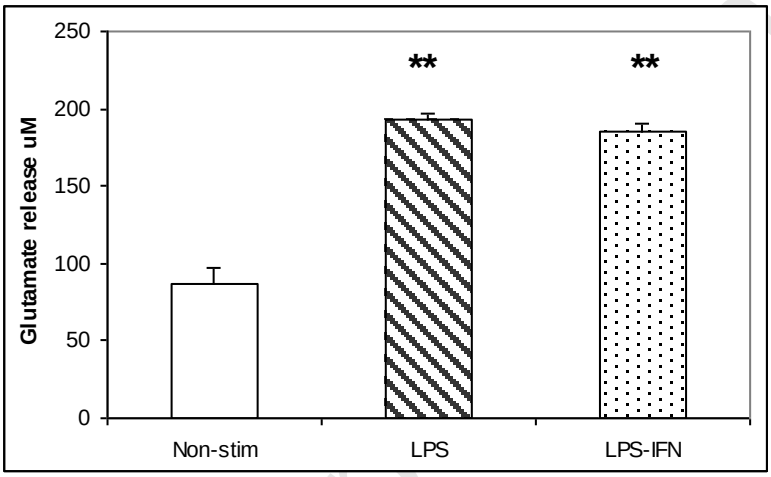


**Figure 4a**



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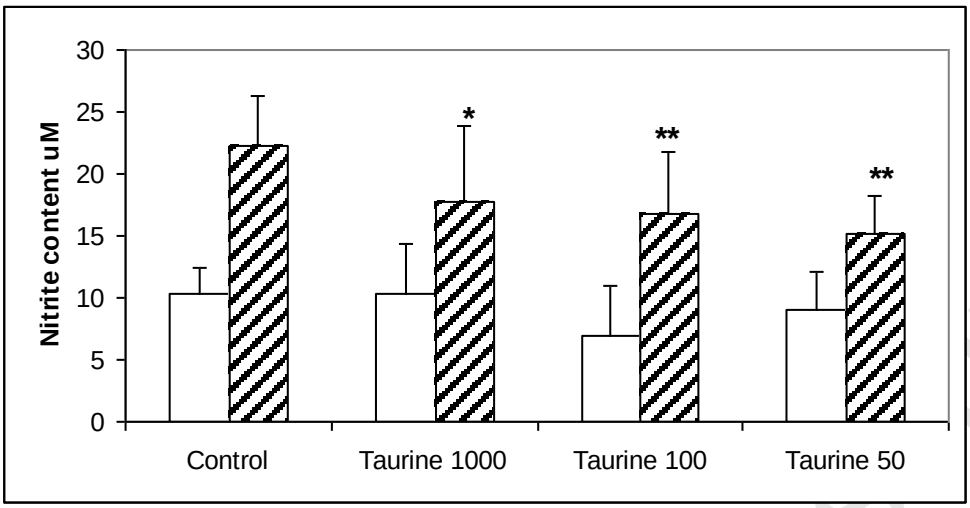


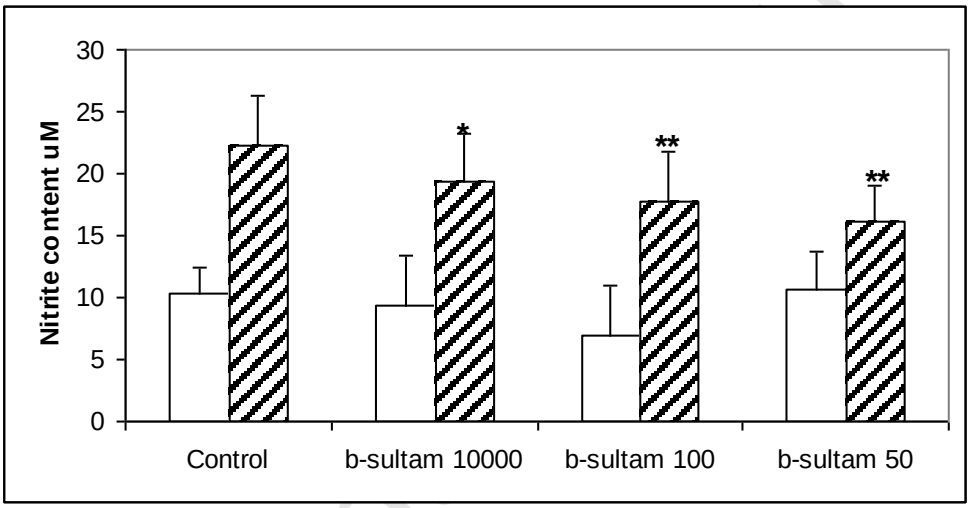

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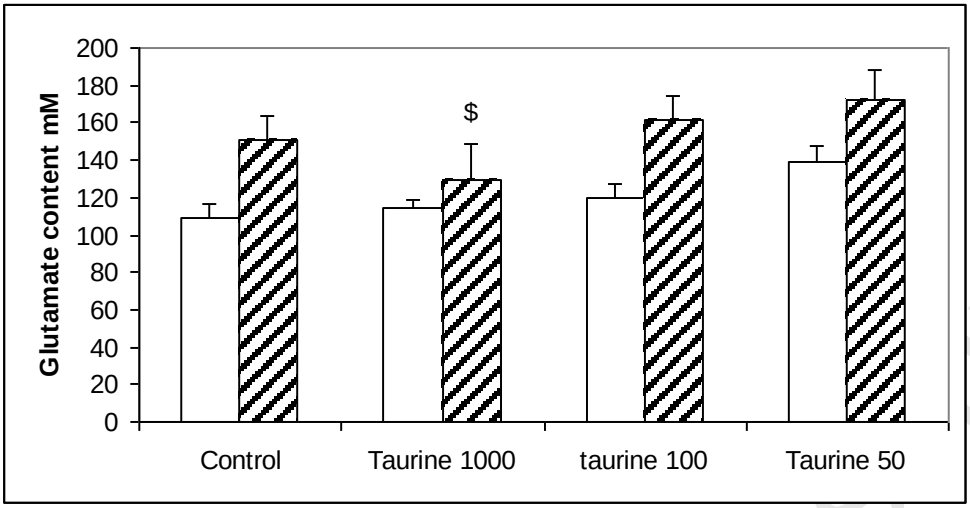


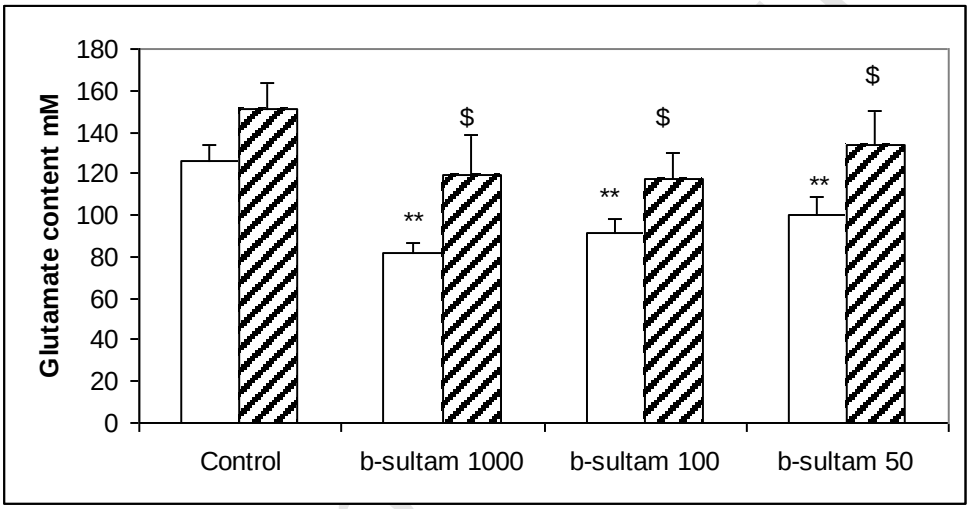

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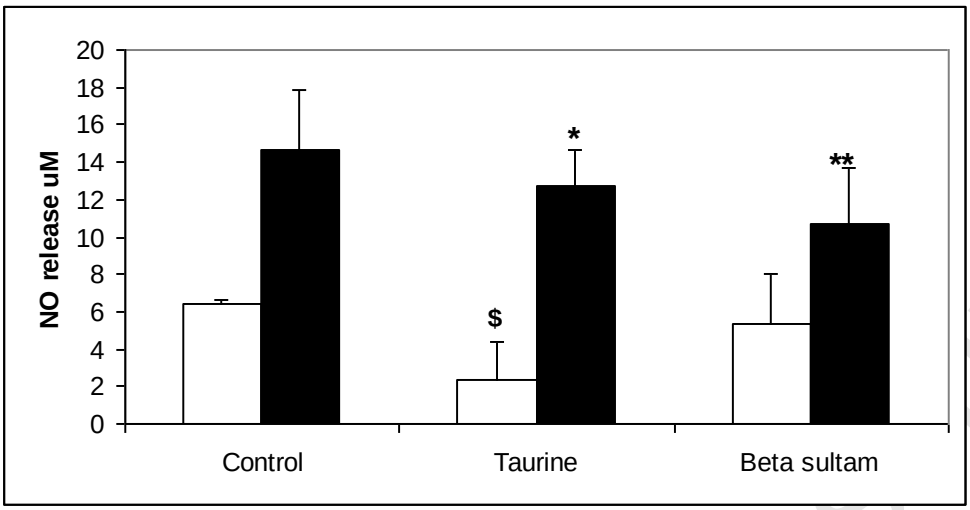

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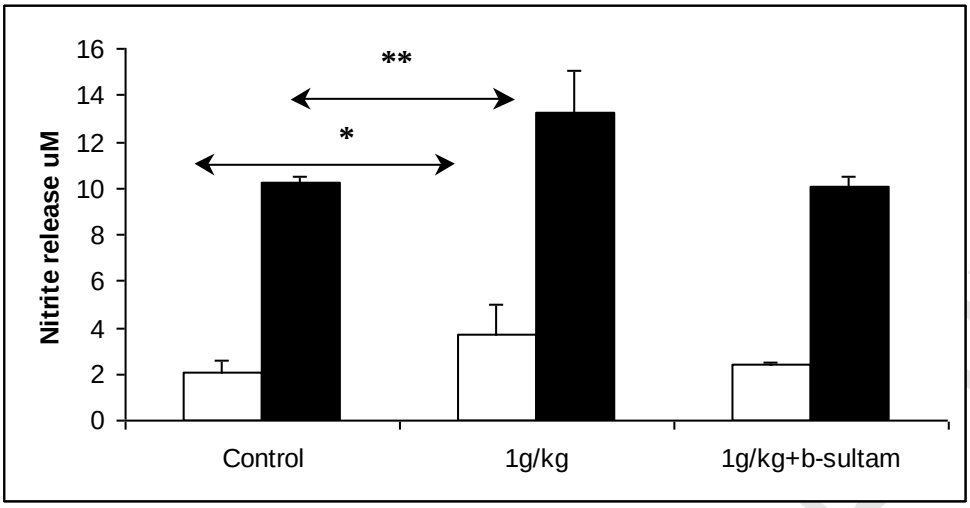



**Figure 6b**

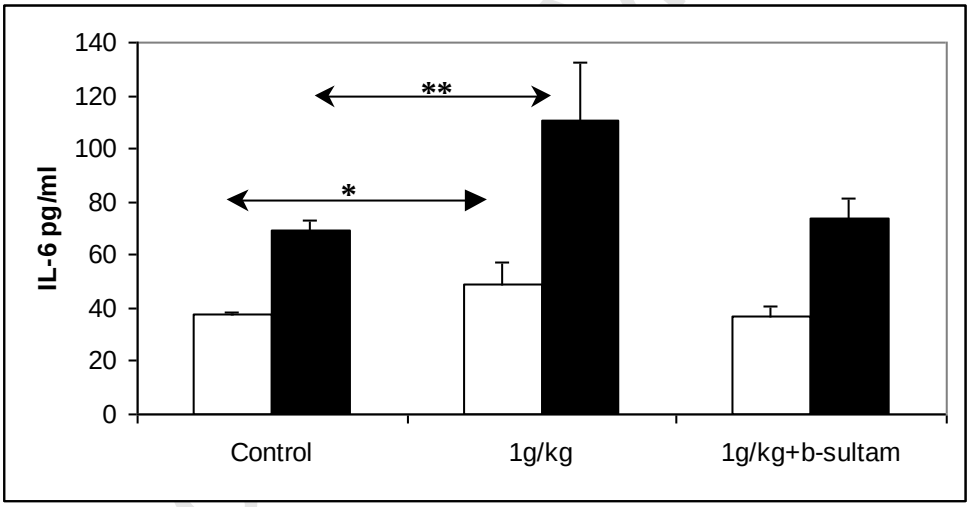
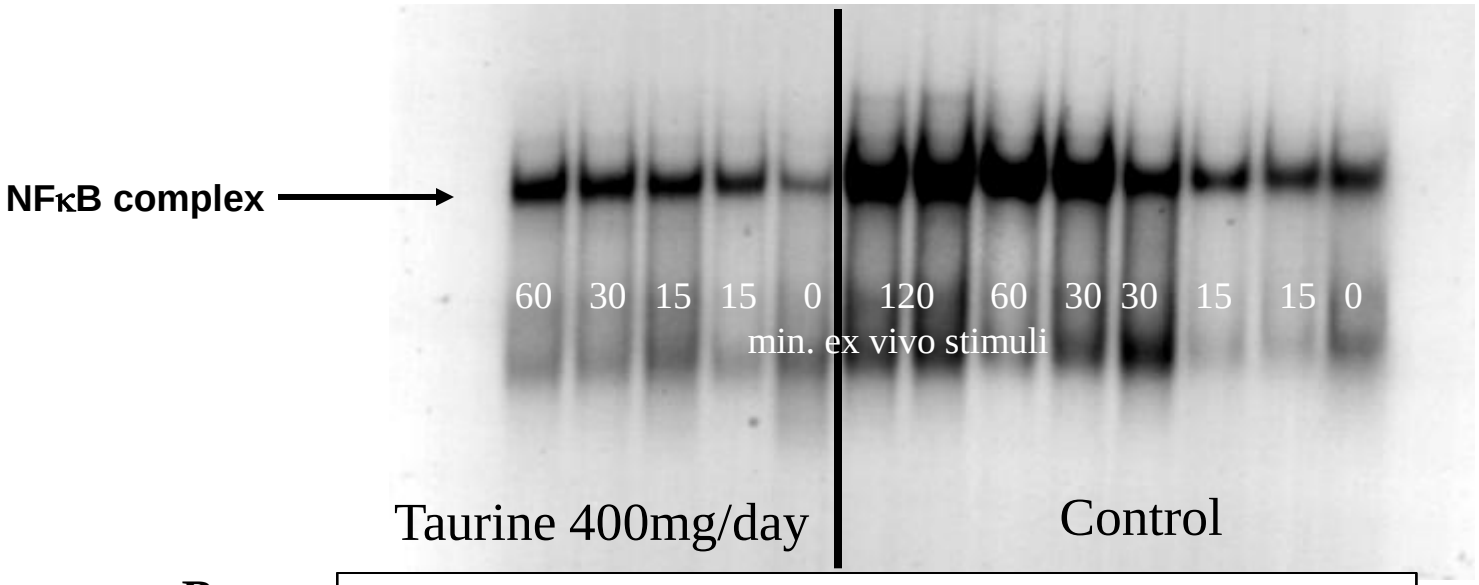


Figure 7

A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Control

B

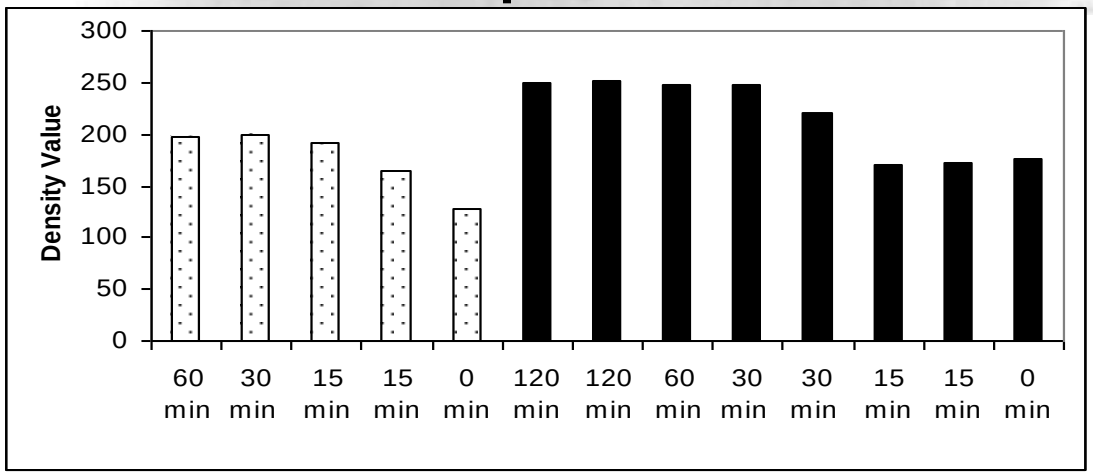
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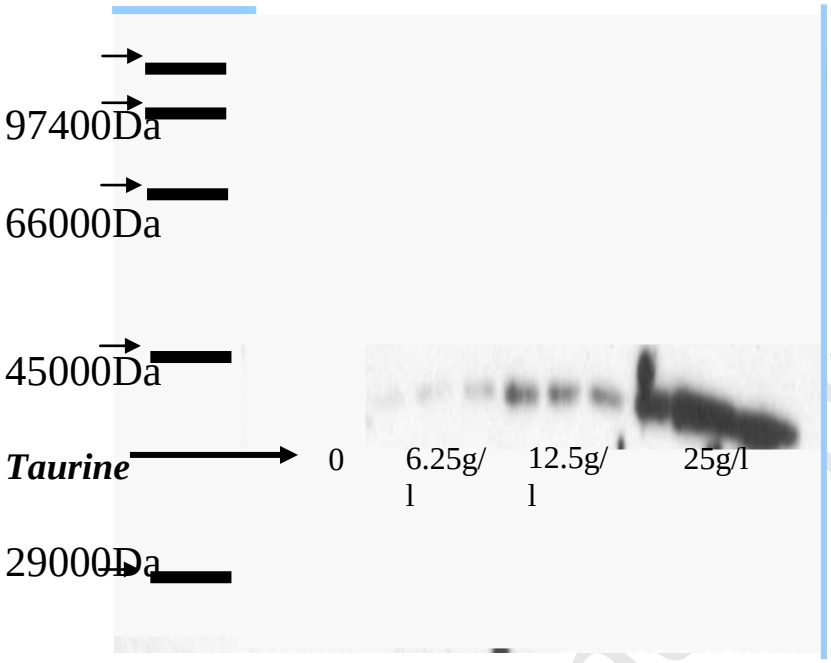
**Figure 8a**

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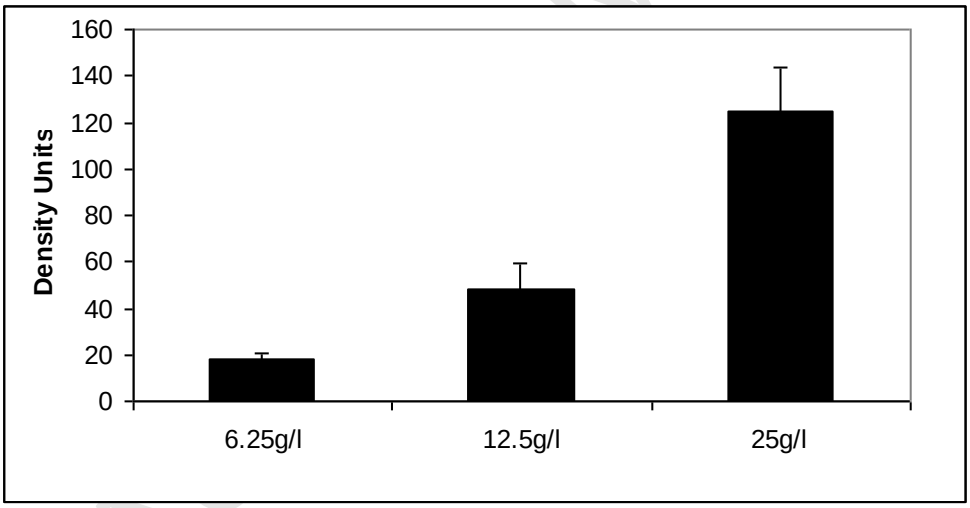

*Graphical Abstract