PHARMACOLOGY AND CELL METABOLISM

Tryptophan in Alcoholism Treatment II: Inhibition of the Rat Liver Mitochondrial Low \( K_m \) Aldehyde Dehydrogenase Activity, Elevation of Blood Acetaldehyde Concentration and Induction of Aversion to Alcohol by Combined Administration of Tryptophan and Benserazide

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Abstract — Aims: The aims were to provide proofs of mechanism and principle by establishing the ability of the amino acid L-tryptophan (Trp) combined with the kynureninase inhibitor benserazide (BSZ) to inhibit the liver mitochondrial low \( K_m \) aldehyde dehydrogenase (ALDH) activity after administration and in vivo and to induce aversion to alcohol. Methods: Trp, BSZ or both were administered to male Wistar rats and ALDH activity was determined both in vitro in liver homogenates and in vivo (by measuring acetaldehyde accumulation in blood after ethanol administration). Alcohol consumption was studied in an aversion model in rats and in alcohol-prefering C57 mice. Results: Combined administration of Trp + BSZ, but neither compound alone, produced a strong inhibition of ALDH activity and an increase in blood acetaldehyde concentration after ethanol, and induced aversion to alcohol in rats and decreased preference in mice. Another kynureninase inhibitor, carbidopa, induced aversion to alcohol by itself, which was reversed by Trp co-administration. Conclusions: The present results establish a prior art for the use of a combination of Trp plus BSZ in the treatment of alcoholism by aversion, which merits rapid clinical development.

INTRODUCTION

In the preceding paper (Badawy et al., 2011), we have demonstrated the ability of 3 tryptophan (Trp) metabolites of the kynurenine pathway, namely 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and kynurenic acid (KA), to inhibit the rat liver mitochondrial low \( K_m \) aldehyde dehydrogenase (ALDH) activity after administration, to elevate blood acetaldehyde concentration following acute ethanol administration and to induce aversion to alcohol in an experimental rat model. On the basis of these findings, we suggested that the above metabolites may form the basis of a new alcohol aversion therapy. A US patent (to AA-BB) is expected shortly for the use of 3-HK and 3-HAA and suitable derivatives thereof in the treatment of alcoholism by aversion. However, the clinical development of these metabolites and their derivatives requires further studies. A more immediate and potentially superior alternative approach is to use their parent compound Trp. However, Trp acutely administered alone may not produce sufficient sustainable ALDH-inhibitory amounts of the above kynurenine metabolites, but can do so if given under suitable conditions, namely metabolic blockade at the kynureninase step of the hepatic kynurenine pathway. As well as via this major pathway of Trp degradation, Trp is also metabolized by three minor pathways, the serotonin-biosynthetic, the decarboxylation or tryptamine and the transamination, pathways (Fig. 1) (for reviews, see Badawy, 2002, 2005). It is important to emphasize here a number of aspects of the kynurenine pathway (for review, see Bender, 1982) in the context of the present work, namely that: (a) the most rate-limiting enzyme is Trp 2,3-dioxygenase, with the next rate-limiting enzyme being kynureninase and that kynurenine hydroxylase is not rate-limiting under normal conditions. (b) Kynurenine aminotransferase catalyses minor reactions under normal conditions, but these could become quantitatively more significant under conditions of loading with Trp or kynurenine (Bender, 1982). (c) The main kynureninase reaction in the liver is that using 3-HK and not kynurenine as substrate (Bender, 1982) (although in the in vitro assays of this enzyme, kynurenine is usually used). (d) Blockade of the kynureninase reaction will inhibit the formation of 3-HAA, which, in turn, will limit quinolinic acid formation. (e) Kynureninase blockade in the presence of Trp loading could also lead to accumulation of KA. (f) This latter metabolite is the physiological antagonist, whereas quinolinic acid is the physiological agonist, of the \( N \)-methyl-D-aspartate (NMDA) type of glutamate receptors (Stone, 1993) and, although entry of KA into the brain is normally relatively limited, that of kynurenine is not (Fukui et al., 1991). Quinolinic acid entry into the brain has also been suggested to be equally limited (Fukui et al., 1991), although other evidence suggests that peripheral blood plays a significant role in its cerebral uptake (Heyes and Morrison, 1997). It is therefore possible that Trp loading combined with liver kynureninase inhibition could afford dual neuronal protection against NMDA receptor activity. (g) With Trp loading, formation of serotonin in the brain is also expected to be enhanced.

As progress in developing more effective drug therapies is hampered by the multifactorial nature of the alcohol dependence process and the limited capacity of current and potential drugs to influence only one aspect of this process, it has been suggested (Mann, 2004; Johnson, 2008) that progress is more likely to be achieved using combination therapy. Our proposed strategy, based on the above principles, appears to satisfy the requirements for a potentially successful alcoholism therapy, one acting simultaneously on alcohol consumption through aversion, on anxiety and depression, and possibly also reward, through provision of serotonin and on hyperekcitability through decreased NMDA receptor function.

In the present paper, we provide evidence for proof of mechanism (ALDH inhibition) and of principle (induction of...
aversion to alcohol) by the combined administration of Trp with the kynureninase inhibitor benserazide (BSZ). This latter drug is best known for its inhibition of aromatic L-amino acid decarboxylase (ALAAD) activity during therapy of Parkinson’s disease when co-administered with the dopamine precursor 3,4-dihydroxyphenylalanine (L-dopa). The other ALAAD inhibitor carbidopa (also used with L-dopa) is an even-stronger kynureninase inhibitor (Bender and Smith, 1978; Bender, 1980) and was therefore also studied in the present work.

**MATERIALS AND METHODS**

**Chemicals and other materials**

BSZ [DL-serine 2(2,3,4-trihydroxybenzyl) hydrazine hydrochloride] and carbidopa [2(S)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid] were purchased from the Sigma-Aldrich Co Ltd. (Fancy Road, Poole, Dorset, UK). All other chemicals and materials were purchased from this and the other sources listed in the preceding paper (Badawy et al., 2011).

**Animals and treatments**

Adult normal male Wistar rats weighing between 150 and 170 g at the start of experiments were purchased from accredited animal suppliers and were aclimatized to our standard UK Home Office-approved housing conditions (21 ± 2°C, relative humidity of 55 ± 10% and a 12 h/12 h light:dark cycle) for at least one week before experiments. They were housed five per cage, unless stated otherwise, in conventional open-top cages with standard softwood bedding from accredited suppliers, and were allowed free access to standard laboratory RM1 diet and water. Adult male mice of the
alcohol-prefering C57BL/6J strain, weighing between 18 and 21 g at the start of experiments, were also studied. This study was performed under the auspices of Cardiff University and approved and licensed (project licence No: PPL 30/2502) by the UK Home Office under the Animal (Scientific Procedures) Act 1986. All compounds were administered intraperitoneally in 0.9% (w/v) NaCl (physiological saline) in single doses as follows: Trp (50 mg/kg); BSZ (100 mg/kg), carbidopa (50 mg/kg). When administered in combinations, BSZ, carbidopa or an equal volume (1 ml/kg) of saline was injected 15 min, 30 min or 1 h before Trp or an equal volume of saline (2.5 ml/kg). When given repeatedly for up to 7 days, BSZ, carbidopa and Trp were injected in single daily doses either separately or in combinations as above.

**Determination of ALDH activity in vitro and in vivo**

The mitochondrial low $K_m$ ALDH activity was determined in vitro by the method of Tottmar et al. (1973) in liver supernatants prepared according to Mazzanti et al. (1989), and in vivo by measuring the accumulation of acetaldehyde in blood following acute ethanol administration. Both procedures have been described in detail in the preceding paper (Badawy et al., 2011). The blood acetaldehyde concentrations reported in the ‘Results’ section in the present paper have also been corrected for the artificial formation described in the preceding paper, in which pitfalls in acetaldehyde determination have been addressed in detail.

**Alcohol aversion test**

Aversion to alcohol was assessed using the alcohol aversion model of Garver et al. (2000). Briefly, individually housed rats were allowed free access to food and water for 3 days. Trp, BSZ or carbidopa were administered in the above single doses once daily for 4 days, either separately or in combinations wherein BSZ, carbidopa or saline was injected 15 min before Trp or saline. On the evening of the third day, water (but not food) was withdrawn for 18 h, but was then replaced by a 6% (v/v) ethanol solution, 2 h after the injection on the fourth day. Body wt was measured daily and alcohol consumption was monitored hourly for 4 h and levels were expressed cumulatively over this duration in g/kg body wt.

**Alcohol consumption and preference in alcohol-prefering C57BL/6J mice**

As in the preceding paper (Badawy et al., 2011), no attempt was made to enhance alcohol preference by acclimatizing the mice to increasing ethanol concentrations, as the purpose of our study was to investigate aversion, rather than preference. Accordingly, four groups ($n=8$ each) of individually housed mice were given free choice between drinking water and a 10% (v/v) ethanol solution for 16 days to establish their drinking patterns. Thereafter, mice were pretreated once daily with a single intraperitoneal injection of BSZ (100 mg/kg body wt) or an equal volume (1 ml/kg) of saline 0.25 h before a similar injection of Trp (50 mg/kg) or an equal volume (2.5 ml/kg) of saline, for 5 days. Body wt and water and ethanol consumption were recorded daily throughout the whole study duration. Determined per kg body wt, daily alcohol consumption was expressed in absolute amounts (g) and as a percentage of total fluid intake (% preference).

**Determination of liver kynureninase activity**

Liver kynureninase activity was determined by measuring the conversion of kynurenine to anthranilic acid by the method of Chiarugi et al. (1995). In preliminary experiments, we found that kynureninase activity was linear over the kynurenine concentration range of 0–1 mM and that anthranilic acid formation from kynurenine (1 mM) was linear over a 90 min period. Subsequent experiments were therefore performed at a 1 mM kynurenine concentration with a 30 min incubation time. A 1 g piece of frozen liver was homogenized in 4 ml of an ice-cold homogenization buffer consisting of 20 mM sodium phosphate, containing 140 mM KCl at pH 7.0 for 1 min using an ultra-Turrax homogenizer. The homogenate was then centrifuged at 20 000g for 30 min at 4°C. The supernatant was then decanted quantitatively and the volume adjusted to 4 ml with the homogenization buffer. All tests were performed in duplicates with one blank for each rat liver homogenate. To 100 µl of the ice-cold substrate solution (200 mM Tris–HCl buffer, pH 8.0, 100 µM pyridoxal 5’-phosphate and 1 mM kynurenine), 100 µl of the rat liver homogenate was added in a 5 ml plastic tube. The mixture was incubated in a shaking-water bath for 30 min at 37°C. The reaction was stopped by the addition of 200 µl of 24% (v/v) perchloric acid and incubation was continued for a further 2–3 min. The contents of the tubes were centrifuged at 10 000g for 10 min at 4°C, after which the supernatant was decanted carefully into an high-performance liquid-chromatographic (HPLC) autosampler vial. The anthranilic acid produced was separated and quantified both fluorimetrically and by UV as described below for kynurenine metabolites. The protein content of the liver postmitochondrial supernatant was determined by the method of Lowry et al. (1951) using bovine albumin as standard. Enzyme activity was expressed as nmol of anthranilic acid formed/h per mg of protein.

**Assessment of kynureninase activity in vivo**

This can be made by measuring hepatic concentrations of some kynurenine metabolites. The major effect of kynureninase inhibition is elevation of 3-HK concentration (Chiarugi et al., 1995), leading to a decrease in that of 3-HAA. A secondary effect is a potential increase in xanthurenic acid due to elevation of [3-HK]. Hepatic concentrations of the above three and other metabolites were therefore determined in the present experiments, using the HPLC methodology of Badawy and Morgan (2010) under the conditions described in the preceding paper (Badawy et al., 2011).

**Statistical analysis**

Enzymatic and other biochemical test results were compared with those of control groups by the unpaired $t$ test, whereas alcohol consumption results were assessed initially by one-way analysis of variance (ANOVA) and additionally for within-group differences (time factor versus baseline values) by paired $t$-tests, using Sigma Plot (Systat, UK), version 11, with which graphics were prepared. For multiple group comparisons using this programme, the Holm–Šidak test was
applied, as it is more powerful than the Tukey or Bonferroni
tests and can be used for both pairwise comparisons and
those versus a control group. Where the data failed the nor-
mality (Shapiro–Wilk) test, Kruskal–Wallis one-way
ANOVA on ranks was performed. A two-tailed level of
significance ($P$) was set at 0.05.

RESULTS

Effects of administration of BSZ or carbidopa alone or in
combinations with tryptophan on the rat liver mitochondrial
low $K_m$ ALDH activity

The acute effects are depicted in Fig. 2. Neither BSZ alone
(Fig. 2a) nor carbidopa alone (Fig. 2b) exerted any signifi-
cant effects on ALDH activity. Similarly, Trp administered
alone or with carbidopa did not influence ALDH activity
(Fig. 2b). By contrast, combined administration of Trp plus
BSZ caused a remarkable inhibition (Fig. 2a). Thus, ALDH
activity was decreased by this combined treatment by 46% at
1 h ($P = 0.0005$). Inhibition then progressed over time, reach-
ing 92% at 3 h and remaining at this level 1 h later.

Chronic experiments were also performed in which rats
were treated with Trp, BSZ or a combination of the two for
7 days. In data not shown, neither Trp alone nor BSZ alone
exerted any significant effect on ALDH activity, whereas the
combined treatment caused a 55% inhibition ($P = 0.0001$)
Although this could be attributed to an acute effect at 2 h
after the final treatment, it suggests that no tolerance devel-
ops to the acute inhibition after repeated administration of
Trp plus BSZ.

Inhibition of ALDH activity in vivo by combined
administration of tryptophan and BSZ

The accumulation of acetaldehyde and ethanol in blood fol-
lowing acute ethanol administration was studied in rats
treated with Trp, BSZ or a combination of the two (Fig. 3).
Blood acetaldehyde concentration (Fig. 3a) after pretreat-
ment with BSZ did not differ significantly from that in saline-
pretreated controls ($P > 0.0980$). A non-significant trend
towards elevated acetaldehyde concentration after pretreat-
ment with Trp was observed ($P > 0.0971$). By contrast, com-
bined pretreatment with Trp plus BSZ caused a significant
increase in blood ethanaldehyde concentration. The strongest
effect was at 1 h after ethanol (145 μM), which was signifi-
cantly different from all the other three groups ($P = 0.0287–
0.0001$). At 2 h, the increase caused by Trp plus BSZ was
also significant, compared with those in saline or
BSZ-pretreated rats ($P = 0.0029$), but, at 3 h, only the differ-
ence from saline-pretreated controls was still significant ($P =
0.0011$). Acetaldehyde concentration in Trp plus
BSZ-pretreated rats did not differ significantly from that in
Trp-pretreated rats at 2 or 3 h.

The increase in blood ethanol concentration following
ethanol administration to saline-pretreated controls was not
significantly influenced by pretreatment with BSZ, Trp or
both (Fig. 3b). Pretreatment with Trp alone showed a slightly
lower blood ethanol concentrations at 3 h, though not signifi-
cant ($P > 0.1$). The ethanol area under the curve (AUC) was
similar among the saline-, BSZ- and Trp plus BSZ-pretreated
groups (80.35, 80.95 and 81.85 mM, respectively) and
slightly higher than that in the Trp-pretreated group (76.9
mM).

Demonstration of aversion to alcohol after administration of
tryptophan in combination with BSZ, but not with carbidopa

In the preceding paper (Badawy et al., 2011), we have suc-
cessfully applied the alcohol aversion model of Garver et al.
(2000) to demonstrate aversion to alcohol by three kynure-
nine metabolites and to confirm that by the classical ALDH
inhibitor disulfiram. In the present work, we tested the same
model for possible aversion by Trp and the two kynurenine
inhibitors BSZ and carbidopa either alone or in combination
with Trp. As the results in Fig. 4 show, no aversion to
alcohol was observed after administration of Trp alone. BSZ
alone (Fig. 4a) also did not inhibit alcohol consumption,
except only at 2 h. This particular effect appears to be a spur-
ious result, as it did not occur earlier nor was it maintained
thereafter, and it does not fit in with other results described

\[ ALDH \text{ activity (μmol of NADH formed/min per mg of protein)} \]

\[ \text{Time (h)} \]

\[ \text{BSZ, benserazide; CBD, carbidopa; Trp, tryptophan.} \]
in this paper. Carbidopa alone (Fig. 4b) caused aversion to alcohol, decreasing alcohol consumption by 30–33% consistently at 2–4 h ($P = 0.0162–0.0017$), in comparison with saline-treated controls. Combined administration of Trp with carbidopa (Fig. 4b), however, reversed the carbidopa-induced aversion. Here, alcohol consumption resembled that of saline-treated controls. By contrast, combined treatment with Trp plus BSZ (Fig. 4a) induced consistent inhibition of alcohol consumption, of 38–44% at 1–4 h ($P = 0.0127–0.0024$), compared with saline-treated controls. Alcohol consumption with this combined treatment also differed significantly from that with the Trp-treated group at all four time-intervals ($P = 0.0253–0.0030$) and from the BSZ-treated group at 1 and 4 h ($P = 0.0274–0.0106$).

Effects of tryptophan, BSZ or both on alcohol consumption and preference by C57 mice

After a 16-day period of provision of free choice between drinking water and a 10% (v/v) ethanol solution, four groups ($n = 8$ each) of male alcohol-prefering C57BL/6J mice were treated with single daily intraperitoneal injections for 4 days of BSZ (100 mg/kg body wt), carbidopa (50 mg/kg) or an equal volume (1 ml/kg) of saline 1 h before a similar injection of tryptophan (50 mg/kg) or an equal volume (2.5 ml/kg) of saline. All other details are as described in the 'Materials and Methods' section. Consumption of the ethanol solution was measured hourly in all groups for 4 h and is expressed in g/kg body wt cumulatively. Values are means ± SEM (bars) for each group of 5–6 rats. Sal (saline) and other abbreviations are as in Fig. 2a.
Table 1. Inhibition of liver kynureninase activity in vitro by BSZ and carbidopa

| Inhibitor concentration (µM) | Kynureninase activity (nmol of anthranilic acid formed/h per mg of protein) |
|-----------------------------|--------------------------------------------------------------------------|
| 0                           | 1.87 ± 0.11                                                               |
| 10                          | 1.45 ± 0.12*                                                              |
| 25                          | 1.34 ± 0.06*                                                              |
| 100                         | 1.28 ± 0.07*                                                              |
| 250                         | 1.20 ± 0.08*                                                              |
| 500                         | 1.04 ± 0.11*                                                              |

Enzyme activity was determined as described in the ‘Materials and Methods’ section. Values are means ± SEM for each group of liver preparations from four rats. The asterisk denotes a significant difference from the corresponding control value (P<0.0000).

**Fig. 5.** Effects of repeated administration of BSZ, tryptophan or both on alcohol consumption and preference in male alcohol-prefering C57BL/6J mice. Experimental details are as described in the ‘Materials and Methods’ section. Alcohol consumption was monitored daily and is expressed (per kg body wt) for each group from day zero (before the injections) onwards, both in g amounts (a) and as a preference % (b). Values are means ± SEM (bars) for each group of six mice. For abbreviations, see Fig. 4. For statistical comparisons, see the relevant text in the ‘Results’ section.

BSZ group on Days 1, 3 and 5 (P=0.05–0.009). From these data and the graphs, it is clear that, whereas the three single groups exhibited a cyclical alcohol consummatory behaviour, with a high and a lower intake on alternate days, the combined Trp+BSZ group consistently decreased its alcohol intake continuously with time. Accordingly, because of this cyclical behaviour of the single treatment groups, the absence of significant differences between the Trp+BSZ group and the other three was apparent only on days on which mice of these latter groups decreased their alcohol intake.

**Inhibition of liver kynureninase activity by BSZ and carbidopa and the differential effect of combined treatment with tryptophan**

The two most potent inhibitors of kynureninase activity in vitro are carbidopa and BSZ, with the former being a stronger inhibitor (Bender and Smith, 1978; Bender, 1980). This was partially confirmed in the present work. As shown in Table 1, kynureninase inhibition was similar between BSZ and carbidopa at drug concentrations of 10 µM (17–22%), 25 µM (25–28%) and 100 µM (32–33%). However, at larger concentrations, the inhibition by carbidopa was almost twice as strong (61 vs 36% at 250 µM and 80 vs 44% at 500 µM).

Kynurenine aminotransferase activity was also determined simultaneously with that of kynureninase by measuring the increase in KA formation. In data to be reported elsewhere, both BSZ and carbidopa inhibited kynurenine aminotransferase activity, with the inhibition by carbidopa being stronger than that by BSZ at all concentrations tested.

Kynureninase activity was also determined after the acute administration of BSZ, carbidopa, Trp and combinations of Trp with either drug. The results in Fig. 6a show that Trp alone did not inhibit kynureninase activity. Trp administration (30 mg/kg intraperitoneally) has previously been reported (Takeuchi and Shibata, 1984) to enhance kynureninase activity by 15 and 21% at 2 and 3 h, respectively. As no such enhancement was observed in the present work at these time-intervals, a closer examination of a possible early increase was made. At 1.5 h after Trp administration, kynureninase activity (6.08 ± 0.49, expressed as in Fig. 5) was still not significantly different (P>0.1) from the control zero-time value (5.78 ± 0.27). However, a significant increase of 37% was observed at 0.5 h (7.91 ± 0.26; P=0.0005).

As expected, both BSZ and carbidopa inhibited kynureninase activity. As shown in Fig. 6b, BSZ caused a significant inhibition (43%; P=0.0003) only at 1 h, whereas carbidopa (Fig. 6c) caused a similar inhibition (41%; P=0.0013) at 2 h. The inhibition by BSZ was not undermined by combined Trp administration (Fig. 6b). In fact, a constant level of inhibition (of 30–35%; P=0.0196–0.0036) was sustained over the first 3 h, and that at 4 h (23%) was still significant (P=0.0462). As BSZ was administered 30 min before Trp, the latter could be said to have prolonged the BSZ inhibition by 3.5 h. By contrast, the kynureninase inhibition by carbidopa was totally reversed by co-administration of Trp (Fig. 6c).

Kynureninase activity was also determined after chronic administration of Trp, BSZ or both for 7 days. In data not shown, kynureninase activity was decreased by BSZ by 22% and co-administration of Trp did not modify this decrease. In fact, Trp induced a further decrease. Trp alone exerted no effect on kynureninase activity. Under these chronic conditions, BSZ also inhibited kynurenine aminotransferase activity.
activity, by 61% and Trp, which exerted no significant effect when given alone, caused a further significant decrease when co-administered with BSZ (data not shown).

Hepatic kynurenine metabolites after the acute administration of tryptophan, carbidopa, BSZ or various combinations

As stated above, the main effect of kynureninase inhibition (by BSZ and carbidopa) is the elevation of hepatic [3-HK]. However, because these two drugs also inhibit kynurenine aminotransferase activity, the potential increase in xanthurenic acid (due to 3-HK elevation) may be hampered by such latter inhibition. Although hepatic concentrations of Trp and six of its kynurenine metabolites were measured in the present work, only the data on [3-HK] are reported here, as details of other changes and their discussion fall outside the scope of this paper. As shown in Fig. 7, the 3-HK precursor Trp administered alone caused relatively large increases in liver [3-HK] at 3–4 h, with the 190% increase at 3 h being significant, compared with the zero-time control value (P = 0.016). The much smaller increases at 1 and 2 h were not significant. By contrast and as expected, both BSZ (Fig. 7a) and carbidopa (Fig. 7b) induced large increases in [3-HK] (P = 0.004–0.001), which were comparable at 1 and 2 h.
Thereafter, the [3-HK] elevations by carbidopa remained close to the maximum increases, whereas those by BSZ showed a dramatic drop towards normal values.

When BSZ administration was followed 0.5 h later by that of Trp, a dramatic elevation of [3-HK] was observed at 1 h after the Trp injection, with a 1069% increase over the baseline zero-time value (P = 0.008). This increase was a 152% higher than that induced by BSZ alone at this time-interval (P = 0.008). The [3-HK] elevation by the combined BSZ plus Trp treatment continued to be much higher than that by BSZ alone at the 2–4 h time points (P = 0.05–0.001). By contrast, when carbidopa was administered 0.5 h before Trp, there was no potentiation of the carbidopa-induced elevation of [3-HK] by Trp. In fact, none of the [3-HK] values observed with this combination differed significantly from those obtained with carbidopa alone at 1–3 h (P > 0.1) and the value at 4 h was even 26% smaller (P = 0.034) after the combined treatment.

**Animal body weights during repeated administration of tryptophan, BSZ or both**

In the aversion experiments reported in Fig. 4, rats gained weight during the first 3 days of the test. However, as was the case with disulfiram and kynurenine metabolites in the preceding paper (Badawy et al., 2011), a wt loss of 6–7% occurred in all group rats on the morning of the fourth day, following the 18 h-water-deprivation period.

Changes in body wt of rats given single daily injections of saline, Trp, BSZ or a combination of Trp plus BSZ for 7 days in the chronic experiments were also recorded. Rats of all groups gained wt. On Day 7, the wt gain over the starting wt on Day 1 was 19, 21, 21 and 17%, respectively for the above four groups. No significant differences in body wt were observed after analysis of variance in within- or between-group comparisons (P > 0.1).

Body weights of mice in the preference experiments were also recorded. All mice (n = 32) gained wt during the 16-day free choice period (from 19.44 ± 0.16 to 21.85 ± 0.21 g; P < 0.001) (means ± SEM). Body weights remained stable during the 6-day drug administration period, with small rises of 1–3%. No significant differences within- or between-groups were observed (P > 0.1). Both rats and mice appeared healthy and showed no adverse reactions or unusual behaviours.

**DISCUSSION**

**Inhibition of ALDH activity by combined administration of tryptophan plus BSZ**

The present study was based on the assumption that Trp administered alone is metabolized along the kynurenine pathway so rapidly that sufficient levels of its ALDH-inhibitory metabolite 3-HK (Badawy et al., 2011) may not be achieved or sustained to any significant extent, but that this could occur after metabolic blockade of the further degradation of 3-HK to 3-HAA by inhibition of kynureninase activity (Fig. 1). This assumption was borne out by the present findings that the combined administration of Trp plus the kynureninase inhibitor BSZ (but not Trp alone) caused a strong and sustained inhibition of the rat liver mitochondrial low K_m ALDH activity (Fig. 2a). This is almost certainly due to this combination inducing a remarkable and sustained elevation of hepatic [3-HK] (Fig. 7a) resulting from kynureninase inhibition by BSZ [shown here under acute (Fig. 6b) and chronic (see the text) conditions as well as in vitro (Table 1)] superimposed on supply of 3-HK by its Trp precursor. As shown in Fig. 7, Trp alone increased liver [3-HK] significantly only at 3 h, but to a much lesser extent than that observed after kynureninase inhibition by BSZ. The latter drug alone did not influence the mitochondrial low K_m enzyme after acute (Fig. 2a) or chronic (see the text) administration. As carbidopa also inhibits kynureninase activity (Bender and Smith, 1978; Bender, 1980), it was also studied in the present work. Surprisingly, carbidopa did not inhibit ALDH activity after acute administration either alone or in combination with Trp (Fig. 2b), although it inhibited kynureninase activity both in vitro (Table 1) and after acute administration (Fig. 6c) and induced increases in liver [3-HK] (Fig. 7b) comparable with those by BSZ alone (Fig. 7a). Furthermore, Trp did not potentiate the increase in liver [3-HK] caused by carbidopa (Fig. 7b) and this may explain the failure of the combined Trp plus carbidopa treatment to inhibit ALDH activity. As ALDH activity was not inhibited by Trp, BSZ, carbidopa or the latter combined with Trp, it may be suggested that the [3-HK] elevation under these four conditions (up to ~50 μM) (Fig. 7) was not sufficient to cause inhibition of the enzyme when assayed in vitro. In the ALDH assay in vitro, the liver is diluted a 100-fold and the resulting [3-HK] of up to 0.5 μM under the above conditions may have been too small to cause inhibition, whereas a 3-HK concentration of >1 μM in the combined Trp plus BSZ group was more effective. ALDH activity has previously been shown to be inhibited by chronic administration of BSZ (Messiha, 1977) and acute administration of carbidopa (Messiha, 1978a). However, this author used the cytosolic, rather than the mitochondrial low K_m enzyme in both studies.

The kynureninase inhibition by BSZ was prolonged by Trp after acute administration (Fig. 6b) and potentiated after chronic administration (see the text). A possible explanation of these effects of Trp is the greater elevation of [3-HK] after the combined treatment, compared with that after BSZ alone. Evidence from kinetic studies with purified recombinant human kynureninase demonstrating a sigmoidal concentration-activity profile suggests that 3-HK may inhibit kynureninase activity at higher concentrations (Walsh and Botting, 2002). By contrast, Trp prevented the kynureninase inhibition by carbidopa (Fig. 6c). It is well known (Vilter, 1964) that many hydrazine compounds inactivate pyridoxal 5’-phosphate-dependent enzymes by forming inactive hydr- zides with this cofactor. As both BSZ and carbidopa possess a hydrazine group, the above differential effect of Trp on kynureninase inhibition by BSZ and carbidopa is intriguing and requires further investigation. Trp itself stimulated kynureninase activity transiently at 0.5 h, by 37% (Fig. 6a). A15–21% stimulation has previously been observed by Takeuchi and Shibata (1984) at 2–3 h after a 30 mg/kg body wt dose of Trp. The latter authors did not provide a possible explanation of this stimulation in normal rats (as opposed to an inhibition they reported in vitamin B_6-deficient animals due to depletion of the pyridoxal 5’-phosphate cofactor through the flux of Trp down the kynurenine pathway). It is
possible that Trp or a metabolite thereof may interact with carbidopa to undermine its effect on this enzyme, but further work is required to elucidate the nature of such a potential interaction.

**Inhibition of ALDH activity in vivo by combined administration of tryptophan plus BSZ**

The above ALDH inhibition by combined administration of Trp plus BSZ was also observed *in vivo*, as the results in Fig. 3 show. This combined treatment induced a large increase in blood acetaldehyde concentration following acute ethanol administration (Fig. 3a), which mirrored the [3-HK] elevation (Fig. 7a). Whereas BSZ alone exerted no effect on blood acetaldehyde, Trp showed a trend towards elevation, though the increases were not significant. The data in Fig. 3b show Trp to cause small, though not significant, decreases in blood-ethanol concentration at 2 and 3 h. It is possible that significant decreases could occur at subsequent time intervals after Trp administration, possibly due to acceleration of ethanol oxidation through provision of NAD⁺. As this redox cofactor is a terminal metabolite of the kynurenine pathway (Fig. 1), its concentration could be expected to show a greater rise at later time-intervals.

**Induction of aversion to alcohol by combined administration of tryptophan plus BSZ**

The combined treatment of rats with Trp plus BSZ induced a decrease in alcohol consumption in the aversion model of Garver et al. (2000) (Fig. 4a), consistent with the ALDH inhibition observed after their combined administration and in the *in vivo* assay, whereas neither compound caused aversion when administered separately. The effect of Trp on alcohol consumption is a controversial one, with an increase (Myers and Melchior, 1975) and a decrease (Sprince et al., 1972) having both been reported following Trp administration in the rats’ diet. BSZ has also previously been shown not to inhibit alcohol consumption by cats (Baskina and Lapin, 1982) or rats (Messiha, 1978b; Miñano and Myers, 1989) after systemic (intraperitoneal or subcutaneous) administration, but, when given intracerebroventricularly to rats whose alcohol consumption was augmented by prior cycamidate treatment, it decreased it (Miñano and Myers, 1989). While this experimental paradigm may have scientific merit for the purposes of this particular study, it is clearly an artificial one not applicable to standard human pharmacotherapeutic practice. By contrast, carbidopa has been reported to inhibit alcohol consumption (Messiha, 1978b). Our finding (Fig. 4b) that this kynureninase inhibitor induced aversion to alcohol by itself supports this previous finding. An unexpected finding in the present work was the ability of Trp to block the alcohol aversive effect of carbidopa (Fig. 4b). The mechanism of the carbidopa-induced aversion to alcohol cannot be explained at present. Messiha (1978b) suggested ALDH inhibition as a possible mechanism, though as stated above, this inhibition was demonstrated (Messiha, 1978a) using the cytosolic enzyme. An effect of carbidopa on blood acetaldehyde after ethanol administration, however, cannot be ruled out. However, in the absence of such evidence and in view of the failure of carbidopa to inhibit the mitochondrial low Km enzyme (Fig. 2b), other mechanisms must be considered, including central ones. As Trp also prevented the kynureninase inhibition by carbidopa (Fig. 6c), it is possible that this latter effect may be related to the above effects on alcohol consumption. However, a more likely, or additional, explanation for the modulation by Trp of the carbidopa effect on alcohol consumption may be related to carbidopa metabolism. Unlike BSZ, which is metabolised and distributed almost entirely outside the brain (Schwartz et al., 1974), carbidopa is metabolised in rats first by deamination to α-methyl dopa, which can readily cross the blood-brain barrier to form α-methyl dopamine, and this is then followed by β-hydroxylation to α-methyl noradrenaline (Shelton et al., 1985). These latter authors suggested that the main function of α-methyl dopamine formed centrally through carbidopa metabolism is via α-methyl noradrenaline, which is a potent α₂ adrenoceptor agonist, which may influence alcohol consumption. Thus, one α₂-agonist, lofexidine, has been reported (Lê et al., 2005) to attenuate stress-induced reinstatement of alcohol-seeking behaviour and to also decrease alcohol self-administration, whereas the opposite effects were induced by the α₂ antagonist yohimbine. Another α₂ agonist, FLA-57, also reduces alcohol consumption (Socaransky et al., 1985; Daoust et al., 1990). The brain entry of α-methyl dopa formed peripherally from carbidopa could be inhibited through competition by Trp, which shares the same carrier mechanism responsible for the transport of neutral (aromatic and branched-chain) amino acids and also L-dopa itself across the blood-brain barrier (Oldendorf, 1971). For example, administration of L-dopa (200 mg/kg) to rats decreases brain [Trp] and [5-HT] (Algeri and Cerletti, 1974), and it is believed that the incidence of depressive symptoms in patients with Parkinson’s disease is due to serotonin depletion secondarily to exogenous L-dopa inhibiting Trp uptake by the brain, with Trp administration successfully alleviating these symptoms (Miller and Nieberg, 1974). A larger L-dopa dose (500 mg/kg), however, elevates brain [Trp] by increasing plasma [free Trp] secondarily to stimulating the lipolytic release of non-esterified fatty acids (Curzon, 1974). It is therefore possible that aversion to alcohol induced by carbidopa may involve a central mechanism and that Trp blocks it by preventing the cerebral uptake of the carbidopa metabolite α-methyl dopa. Further work will be required to assess this possibility.

**The combined tryptophan-BSZ strategy for alcoholism treatment**

The above results have provided proofs of mechanism (ALDH inhibition after administration and *in vivo*) and principle (induction of aversion to alcohol) by the combined administration of Trp and the kynureninase inhibitor BSZ. A European patent (to AA-BB) is expected shortly for use of this combination in alcoholism treatment by aversion and proof of concept studies demonstrating the ability of this combination to induce aversion to alcohol in human subjects will be an important step in the further development of this intellectual property. This proposed combination is not limited to its aversive property, but can address several other aspects of the alcohol dependence mechanism in addition to alcohol consumption. Thus: (1) the use of Trp can elevate brain serotonin levels to counteract two important determinants of relapse after detoxification, namely anxiety and depression. (2) A third determinant of relapse is the...
hyperexcitability of the acute alcohol-withdrawal syndrome, believed to be due to NMDA receptor activation (Gonzales, 1990; Lovinger, 1995) and suggested to be mediated by the endogenous NMDA receptor agonist quinolinic acid (Morgan, 1991). Minimizing the peripheral formation of this Trp metabolite by kynureninase inhibition (Fig. 1) can only contribute to its decreased entry into the brain (Heyes and Morrison, 1997). (3) Furthermore, cerebral levels of the NMDA receptor antagonist KA are likely to be increased through increased entry of kynurenine into the brain with Trp loading. Thus, plasma and brain kynurenine concentrations have been shown (Gál et al., 1978) to be increased at 1 h after intraperitoneal administration of a 50 mg/kg body wt dose of Trp by 110 and 57% respectively. In the present work, serum Trp and kynurenine concentrations after chronic administration of Trp in the above single daily dose for 7 days were increased by 121 and 131% respectively and joint BSZ administration influenced the kynurenine elevation only moderately (data not shown). Although Trp competes with kynurenine for cerebral uptake (see Møller, 1985 and references cited therein), the above proportionate increases are unlikely to cause an imbalance in entry of either compound into the brain. Although BSZ inhibits liver kynurenine aminotransferase activity (data not shown), a similar effect in the brain is unlikely as BSZ does not cross the blood–brain barrier. Accordingly, the combined Trp plus BSZ treatment is likely to lead to elevation of cerebral KA concentration, leading to NMDA receptor antagonism. The proposed combination could therefore afford dual neuronal protection. (4) As regards reward mechanisms, although the above negative reinforcing properties (anxiety, depression and hyperexcitability) appear to play a more important role in alcohol dependence than the positive rewarding effects, it is also possible that provision of serotonin from Trp in the proposed therapy may modulate the alcohol reward effect. Although dopamine plays a central role in the brain reward mechanisms, other neurotransmitters are also capable of modulating this role, including serotonin, GABA (γ-aminobutyric acid), glutamate and endogenous opioids (Koob and Nestler, 1997) and the importance of the interactions between these systems has been emphasized in relation to alcohol research and treatment (Lê et al., 1996). It is therefore possible that Trp-derived serotonin could also modulate alcohol reward through interacting with dopaminergic and opioidergic, and possibly other, systems. We therefore believe that the Trp–BSZ combination represents a new and unique multiaction approach to combination therapy of alcohol dependence.

There should be little safety issues with the combined use of Trp plus BSZ. In the present work (see the text), test rats and mice gained weight at a normal rate as control animals and showed no side effects. Weight gain and food intake were also reported to be normal after repeated subcutaneous BSZ administration (Miiano and Myers, 1989). Both Trp and BSZ are currently licensed for use in resistant depression and Parkinson’s disease, respectively (the latter in combination with L-dopa). A clinical trial for treatment of schizophrenia with Trp plus BSZ for 6 weeks did not report undue adverse events different from those experienced by a control schizophrenic group treated with chlorpromazine (Chouinard et al., 1978)). The combined use of Trp plus BSZ is also the subject of a US patent (United States Patent, 1993) for the treatment of chronic pain, in which the inventor successfully used this combination in humans with no reported adverse events. BSZ alone has also been used in a trial in schizophrenia (Chouinard et al., 1977) with no reported adverse events.

REFERENCES

Algeri S, Cerletti C. (1974) The role of the adrenergic system in the action of L-dopa on brain serotonin. Adv Biochem Psychopharmacol 10:257–9.

Badawy AA-B. (2002) Tryptophan metabolism in alcoholism. Nutr Res Rev 15:123–52.

Badawy AA-B. (2005) Tryptophan metabolism and alcoholism. In Preedy V, Watson KR (eds), Comprehensive Handbook of Alcohol-Related Pathology, vol.3, chapter 99. London: Elsevier, 1003–22.

Badawy AA-B, Morgan CJ. (2010) Rapid isotopic liquid chromato-graphic separation and quantification of tryptophan and six kynurenine metabolites in biological samples with ultraviolet and fluorimetric detection. Int J Tryptophan Res 3:75–86.

Badawy AA-B, Bano S, Steptoe A. (2011) Tryptophan in alcoholism treatment I. Kynurenine metabolites inhibit the rat liver mitochondrial low Km aldehyde dehydrogenase activity, elevate blood acetaldelyde concentration and induce aversion to alcohol. Alcohol Alcohol 46:651–60.

Baskina NF, Lapin IP. (1982) Changes in alcohol choice by chronically alcoholized cats by tryptophan, its metabolites and preparations affecting its metabolism. Farmakol Toksikol 45, 70–6. (in Russian).

Bender DA. (1980) Inhibition in vitro of the enzymes of the oxidative pathway of tryptophan metabolism and of nicotinamide adenine nucleotide synthesis by benserazide, carbidopa and isoniazid. Biochem Pharmacol 29:707–712.

Bender DA. (1982) Biochemistry of tryptophan in health and disease. Molec Aspects Med 6:101–97.

Bender DA, Smith WRD. (1978) Inhibition of kynurenine hydro-lase by benserazide, carbidopa and other aromatic hydrazine derivatives: evidence for sub-clinical iatrogenic niacin deficiency. Biochem Soc Trans 6:120–2.

Chiarugi A, Carpenedo R, Molina TM et al. (1995) Comparison of the neurochemical and behavioral effects resulting from the inhibition of kynurenine hydroxylase and/or kynureninase. J Neurochem 65:1176–83.

Chouinard G, Annable L, Serrano M et al. (1977) A controlled study of a dopa-decarboxylase inhibitor (benserazide) in the treatment of schizophrenic patients. Int Psychopharmacol 12:1–8.

Chouinard G, Annable L, Young SN et al. (1978) A controlled study of tryptophan-benserazide in schizophrenia. Commun Psychopharmacol 2:21–31.

Curzon G. (1974) Availability of tryptophan to the brain and some hormonal and drug influences on it. Adv Biochem Psychopharmacol 10:263–71.

Daoust M, Protas P, Ladure P. (1990) Noradrenergic system: effect of DSP4 and FLA-57 on ethanol intake in ethanol preferring rats. Pharmacol Biochem Behav 36:133–7.

Fukui S, Schwarz R, Rapoport SI et al. (1991) Blood-brain barrier transport of kynurenines: implications for brain synthesis and metabolism. J Neurochem 58:2007–17.
Gál EM, Young RB, Sherman AD. (1978) Tryptophan loading: subsequent effects on the synthesis of kynurenine and 5-hydroxyindole in rat brain. *J Neurochem* 31:237–44.

Garver E, Ross AD, Tu G-C *et al.* (2000) Paradigm to test drug-induced aversion to ethanol. *Alcohol Alcohol* 35:435–8.

Gonzales RA. (1990) NMDA receptors excite alcohol research. *Trends Pharmacol Sci* 11:137–9.

Heyes MP, Morrison PF. (1997) Quantification of local de novo synthesis versus blood contributions to kynurenic acid concentrations in brain and systemic tissues. *J Neurochem* 68:280–8.

Johnson BA. (2008) Update on neuropharmacological treatments for alcoholism: scientific basis and clinical findings. *Biochem Pharmacol* 75:34–36.

Koob GF, Nestler EJ. (1997) The neurobiology of drug addiction. *J Neuropsychiat Clin Neurosci* 9:482–97.

Lê AD, Tomkins DM, Sellers EM. (1996) Use of serotonin (5-HT) and opiate-based drugs in the pharmacotherapy of alcohol dependence: an overview of the preclinical data. *Alcohol Alcohol* 31 (Suppl 1): 27–32.

Lê AD, Harding S, Juzytis W *et al.* (2005) Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats. *Psychopharmacol* 179:366–73.

Loving DM. (1995) Ethanol and the NMDA receptor: implications for intoxication, tolerance, dependence and alcoholic brain damage. In: Soyka M (ed), *Acamprosate in relapse prevention in alcoholism*, Berlin: Springer, 1–26.

Lowry OH, Rosebrough HJ, Farr AL *et al.* (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–75.

Mann K. (2004) Pharmacotherapy of alcohol dependence: a review. *CNS Drugs* 18:485–504.

Mazzanti R, Moscarella S, Bensi G *et al.* (1989) Hepatic lipid peroxidation and aldehyde dehydrogenase activity in alcoholic and non-alcoholic liver disease. *Alcohol Alcohol* 24:121–8.

Messiha FS. (1977) Possible mechanism of adverse reaction following levodopa plus benserazide treatment. *Br J Pharmacol* 60:55–7.

Messiha FS. (1978a) Modulation of hepatic aldehyde dehydrogenase by carbidopa. *Res Commun Chem Pathol Pharmacol* 20:601–4.

Messiha FS. (1978b) Voluntary drinking of ethanol by rats: biogenic amines and possible underlying mechanism. *Pharmacol Biochem Behav* 9:379–84.

Miller EM, Nieberg HA. (1974) L-Tryptophan in the treatment of levodopa induced psychiatric disorders. *Dis Nerv System* 35:20–3.

Míñano FJ, Myers RD. (1989) Inhibition of brain dopa-decarboxylase by RO 4–4602 infused ICV blocks alcohol drinking and induced in rats by cyanamide. *Psychopharmacol* 98:176–82.

Möller SE. (1985) Tryptophan to competing amino acids ratio in depressive disorder: relation to efficacy of antidepressives treatments. *Acta Psychiat Scand* 72 Suppl 325:1–31.

Morgan PF. (1991) Is quinolinic acid an endogenous excitotoxin in alcohol withdrawal?. *Med Hypotheses* 36:118–21.

Myers RD, Melchior CL. (1975) Dietary tryptophan and the selection of ethyl alcohol in different strains of rats. *Psychopharmacol* 42:109–15.

Oldendorf WH. (1971) Brain uptake of radio-labelled amino acids, amines, and hexoses after arterial injection. *Am J Physiol* 221:1629–39.

Schwartz DE, Jordan JC, Ziegler WH. (1974) Pharmacokinetics of the decarboxylase inhibitor benserazide in man: its tissue distribution in the rat. *Eur J Clin Pharmacol* 7:39–45.

Shelton RC, Karoum F, Chuang LW *et al.* (1985) Metabolism of carbidopa to alpha-methylpseudopine and alpha-methylnorepinephrine in rats. *Biochem. Pharmacol* 34:3623–6.

Socaransky SM, Aragon CMG, Rusk I *et al.* (1985) Norpinespine turnover and voluntary consumption of ethanol in the rat. *Alcohol* 2:339–42.

Sprince H, Parker CM, Smith GG *et al.* (1972) Alcoholism: biochemical and nutritional aspects of brain amines, aldehydes and amino acids. *Nutr Rep Int* 5:185–200.

Stone TW. (1993) Neuropharmacology of quinolinic and kynurenic acids. *Pharmacol Rev* 45:309–85.

Takeuchi F, Shibata Y. (1984) Kynurenine metabolism in vitamin-B-6-deficient rat liver after tryptophan injection. *Biochem J* 220:693–9.

Tottmar SOC, Pettersson H, Kiessling K-H. (1973) The subcellular distribution and properties of aldehyde dehydrogenase in rat liver. *Biochem J* 135:577–86.

United States Patent 5216019. (1993) [http://www.freepatentsonline.com/5216019.html](http://www.freepatentsonline.com/5216019.html)

Vilter RW. (1964) The vitamin B6-hydrazide relationship. *Vit Horm* 22:797–805.

Walsh HA, Botting NP. (2002) Purification and biochemical characterization of some of the properties of recombinant human kynureninase. *Eur J Biochem* 269:2069–74.