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Inhibition of Aspartate Transcarbamylase by a Phenobarbital Derivative

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Mammalian and hepatic aspartate transcarbamylase is inhibited by phenobarbital *p*-nitrophenylhydrazone in a reversible and non-competitive type with K_i values 8.45×10^{-5} and 9.64×10^{-5}M in the reactions toward carbamyl phosphate and aspartate, respectively. *In vivo* inhibition occurred in a dose-dependent manner in which less than 50% of the activity was retained. These observations suggest that this inhibitor may interfere with the *in vivo* regulation of this enzyme and lead to an additional biological effect of phenobarbitals.

Keywords: Aspartate transcarbamylase, Phenobarbital, Inhibition constant

INTRODUCTION

Aspartate transcarbamylase (ATCase, EC 2.1.3.2) catalyzes the carbamoylation of the α-amino group of L-aspartate by carbamyl phosphate (CP) to yield carbamyl aspartate and inorganic phosphate. In eukaryotes, the activities of CP synthetase, ATCase and dihydroorotase, the enzymes catalyzing the first three steps in pyrimidine biosynthesis were reported to be associated in a multienzyme complex (CAD).[1,2] It is well known that uridine monophosphate and 5-bromouridine inhibit ATCase from rat liver.[3] Also, ATCase has been shown to be subject to feedback inhibition in *Escherichia coli*[4,5] and Ehrlich ascites cells.[6] In *E. coli*, only cytidine and CMP exert a potent inhibition,[4] whereas in Ehrlich ascites cells, uracil derivatives are equally effective.[6] The inhibition of ATCase from rat liver by thymidine (**4**, Figure 1) and deoxycytidine was demonstrated to be competitive with respect to aspartate.[7] Phenobarbital (**1**), which is used clinically as a sedative, hypnotic and anticonvulsant,[8] has been known to have a wide range of biological effects. For instance, it induces the expression of a large number of liver-specific genes, mainly those encoding drug- and steroid-metabolizing enzymes.[9,10] This hepatic response plays an important role in the activation and inactivation of many different drugs and environmental agents with toxic, mitogenic and carcinogenic effects.[11,12] The present study examines the action and mechanism of a

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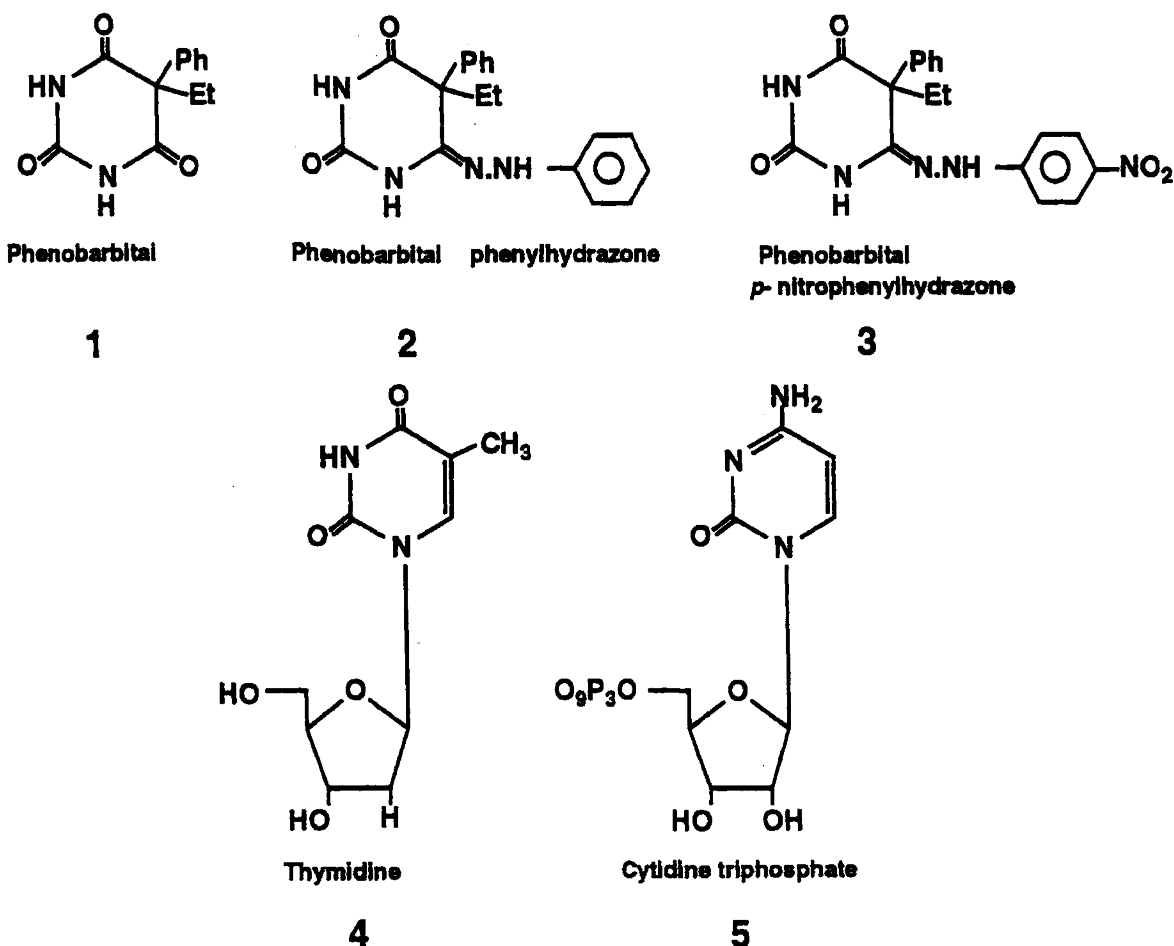

FIGURE 1 Compounds tested for ATCase inhibition.

new and potent inhibitor of ATCase, which is structurally related to pyrimidines. This involved the preparation of (2) and (3) as derivatives of (1) (Figure 1). ATCase enzyme was isolated from mouse liver, purified and then assayed in the absence and presence of various concentrations of (1), (2), (3) and some pyrimidines (4 and 5).

MATERIALS AND METHODS

(A) Animals

Eight-week-old male mice of the BALB/c strain weighing approximately 20 g, were from the animal facility, Institute of Graduate Studies and Research, Alexandria University. The animals were housed under conventional conditions with a 12 h photoperiod. Food and water were supplied *ad lib.*

(B) Materials

Dilithium (CP), carbamyl aspartate, antipyrine, diacetyl-monoxime, **4**, **5** and sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Folin-Ciocalteu's phenol reagent, L-aspartic acid, diphenylamine and mercaptoethanol were purchased from Merck, Hohenbrunn. Orcinol was purchased from

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Aldrich Chemical Co. Ltd, Gillingham, Dorset, England. Bovine serum albumin (Fract V) was purchased from Fisher Biotech (Fisher Scientific Fair Lawn, N.J., USA). Phenobarbital was purchased from Winlab Ltd (Maidenhead, Berkshire, UK). *p*-Nitrophenylhydrazine was purchased from J.T. Baker Chemical Co. Silica gel (G60) was purchased from Cambrian Chemicals.

(C) Synthesis of compounds and elemental analyses

The derivatives of (**1**) namely (**2**) and (**3**) were synthesized as described previously by El-Sadek and Zagzoug.[13] Microanalyses were performed at the Microanalytical Laboratory, Faculty of Science, Cairo University, Giza. IR spectra were obtained in KBr pellets with a Perkin-Elmer 1430 ratio recording IR spectrophotometer and ^{1}H-NMR spectra were obtained with a Varian EM390 apparatus in solvents specified.

In the present study, (**1**) was boiled under reflux with equimolar amounts of phenylhydrazine and *p*-nitrophenylhydrazine to give (**2**) and (**3**) in 90.47 and 91.91% yield, respectively. The purity of the starting material, together with the two prepared compounds (**2** and **3**) was confirmed using TLC which showed different R_f values 0.00, 0.895 and 0.950 respectively. Phenobarbital, (**1**), had white crystals, m.p. 173 °C; ν_{max} (KBr): 1666 (CONH), 1706 and 1769 (CO) and at 3293 (NH) cm^{-1}. ^{1}H NMR ($CDCl_3$), δ, ppm: 0.9 (t, 3H, CH_3), 2.5 (q, 2H, CH_2), and 7.3 (s, 5H, Ph) and 8.8 (s, 2H, 2NH). Found: C, 62.10; H, 5.20; N, 12.4. $C_{12}H_{12}N_2O_3$: C, 62.00; H, 5.16; N, 12.05%. The pure crystals of phenobarbital phenylhydrazone, (**2**), had m.p. 119 °C; ν_{max} (KBr): 1600 (CN), 1650 (CONH), 1700 (CO) and 3295 (NH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.9 (t, 3H, CH_3), 2.4 (q, 2H, CH_2) and 7.2–7.4 (m, 10H, 2Ph). Found: C, 63.30; H, 5.40; N, 17.00. $C_{18}H_{18}N_4O_2{\cdot}H_2O$ requires: C, 63.50; H, 5.80; N, 16.50%. The pure crystals of phenobarbital-*p*-nitrophenylhydrazone, (**3**), had m.p. 161 °C, (KBr), cm^{-1}: 1600 (CN), 1679 (CONH), 1775 (CO) and 3306 (NH). ^{1}H NMR ($CDCl_3$), δ, ppm: 0.95 (t, 3H, CH_3), 2.3 (q, 2H, CH_2), 7.2–8 (m, 9H, Ph). Found: C, 59.10; H, 4.60. $C_{18}H_{17}N_5O_4{\cdot}H_2O$ requires: C, 58.54; H, 5.14; N, 18.50%.

(D) Isolation and purification of ATCase

Fresh mouse liver was homogenized with 9 volume of ice-cold 10 mM sodium phosphate buffer, pH 7.4 containing 250 mM sucrose and 2 mM mercaptoethanol using a Potter-Elvehjem homogenizer with a Teflon pestle. Isolation and purification were as described previously by Inagaki and Tatibana.[14]

(E) Enzyme assay

The activity of ATCase was assayed as previously described[15] with some modifications. Briefly, carbamyl aspartate production was determined in a system containing 40 mM sodium phosphate buffer, pH 8.2, 12.5 mM aspartate and 20–50 µg enzyme in a final volume of 1.0 ml. The mixture was incubated at 30 °C for about 5 min and 3.6 mM dilithium (CP) was then added to initiate the reaction. The reaction was allowed to proceed at 30 °C for 30 min and stopped by the addition of 1.0 ml of 2% $HClO_4$ followed by protein removal by centrifugation. The color reagent was prepared immediately before use by mixing two parts of antipyrine-H_2SO_4 reagent with one part of diacetylmonoxime reagent. The reaction tubes were capped with marbles, covered with aluminum foil, stored in a dark place at room temperature for 24 h and then placed in water bath at 45 °C and exposed to room light for 70 min. The reaction mixtures were then cooled and absorption at 466 nm was measured.

(F) *In vitro* effect on ATCase and kinetic studies

The effect of (**1–5**) on ATCase activity was carried out using 0.1 ml of different concentrations in

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ethanol of (**1**) (0.1–0.5 mM), (**2**) and (**3**) (0.02–0.2 mM). The assay mixture includes 40 mM sodium phosphate buffer, pH 8.2, 12.5 mM aspartate, 20–50 µg of the enzyme and 3.6 mM dilithium (CP) in a final volume of 1.0 ml as mentioned under "Enzyme assay". The reversibility of binding of (**3**) to ATCase was studied as described before for other enzymes.[16,17] Briefly, a solution of 0.2 mM (**3**) or 2.0 mM (**4**) was added to 50 µg enzyme in 0.5 ml of 40 mM sodium phosphate buffer, pH 8.2 for 60 min at room temperature and the mixture was then placed in a cellophane dialysis sac. Dialysis was allowed to proceed for 16 h by keeping the cellophane sac submerged in 10 mM sodium phosphate buffer, pH 7.4 with three changes. A control was also performed by the addition of the same volume of ethanol to the enzyme and the two experiments were carried out in parallel. From each cellophane sac, the mixture was withdrawn and ATCase was assayed. Initial velocities of ATCase were measured from the time course of the reactions in absence and presence of two or three concentrations of (**3**) or (**4**). The kinetic parameters of the enzyme (K_m and V_{max}) and the inhibition constant (K_i) were then determined from a Lineweaver Burk plot of $1/V_o$ *vs.* $1/[S]$.[18] K_i values were calculated from the slopes of the linear plot.

(G) *In vivo* effect of 3 on ATCase

In vivo effects of **3** on ATCase was studied as described previously for other enzymes by Balbaa *et al.*[19] Briefly, mice were allocated randomly into 6 groups, each group contained 4–6 mice. Each group was incubated with a specific dose of (**3**) (200, 400, 600, 800 and 1000 µg in 0.2 ml of corn oil/day) for two days, using gavage. The vehicle-treated group was incubated with 0.2 ml of corn oil/day for two days. Mice were sacrificed 24 h after the final treatment and their livers were removed and prepared for the enzyme assay. Specific and relative enzyme activities were then estimated. The tested doses did not cause death of the mice.

(H) Protein assay

Protein concentration was determined according to the method of Ohnishi and Barr.[20]

All experiments were run on three occasions for reproducibility and all assays were done in triplicate. Student's *t*-test was applied for statistical analyses.

RESULTS

(A) ATCase purification

ATCase was separated from mouse liver by different steps. As shown in Table I, ATCase was purified about 6-fold starting from the crude homogenate to the final step. The post-hydroxylapatite fraction retained approximately 20% of the original total activity (yield).

(B) *In vitro* treatments of ATCase

Compounds (**1**), (**2**) and (**3**) inhibited ATCase activity, and the latter was the most potent in this regard. The IC_{50} values were 0.445 ± 0.018, 0.117 ± 0.003 and 0.076 ± 0.004 mM, respectively. ATCase activity was also inhibited by (**4**) and (**5**), a 50% reduction in enzymatic activity was observed in the presence of 0.7 ± 0.04 and 1.85 ± 0.04 mM of (**4**) and (**5**), respectively indicating that (**4**) was the more effective inhibitor. The different compounds inhibited ATCase in a concentration-dependent manner (Figure 2, panels A&B). The reversibility of (**3**) binding was readily realized when the full activity of the enzyme incubated for 60 min with **3** was recovered after dialysis for 16 h at 4 °C. Full recovery of activity of ATCase incubated with (**3**) or (**4**) occurred on dialysis.

The time course of the reaction of ATCase was studied using CP or aspartate as a substrate.

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TABLE I Purification steps of hepatic ATCase from mice

Step	Total activity (μmol/30 min)	Total protein (mg)	Specific activity (μmol/30 min/mg protein)	Purification (Fold)	Yield (%)
Homogenate	109.62	1710.45	0.064	1	100
13,000 × g supernatant	79.20	1161.60	0.068	1.062	72.22
105,000 × g supernatant	77.42	995.40	0.077	1.20	70.62
$(NH_4)_2SO_4$ precipitation and sephadex G25	38.22	141.44	0.27	4.21	34.86
Hydroxylapatite chromatography	22.41	56.4	0.397	6.20	20.44

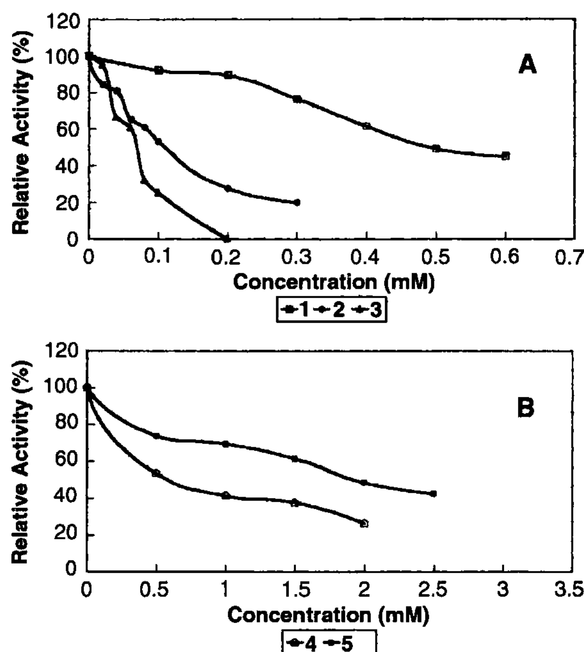

FIGURE 2 *In vitro* effect of different compounds on ATCase. The enzyme is assayed at different concentrations of (**1**), (**2**) and (**3**) (A) and different concentrations of (**4**) and (**5**) (B).

As shown in Figure 3, the untreated enzyme showed a linear increase with respect to time within 25 min in the reactions toward CP (panel A) and aspartate (panel B). On treatment of ATCase with (**3**), the time course of the reaction toward CP (Figure 3, panel A) and aspartate (Figure 3, panel B) showed linearity similar to that of the control.

The Lineweaver Burk plots of 1/v against 1/[S] gave a straight line in the absence and presence of **3**. However, the presence of (**3**) resulted in a change of the slope without altering the intercept on the 1/[CP] (Figure 4A) or 1/[asp] (Figure 4C) axes suggesting that inhibition was non-competitive. The K_m of the enzyme toward CP or aspartate as substrates is 1.1 ± 0.020 and 5.54 ± 0.145 mM, respectively These values agree closely with those reported previously.[7,21] K_i for (**3**) was calculated from the slope of the plots to be 8.45×10^{-5} and 9.64×10^{-5} M in the reactions toward CP and aspartate, respectively (Table II).

The treated enzyme with (**4**) showed a decrease in V_{max} that was 36.43 ± 0.346 μM/min/mg protein. The K_m value (Table II) of the enzyme toward CP was found to be unchanged in the (**4**)-treated enzyme and was equal to

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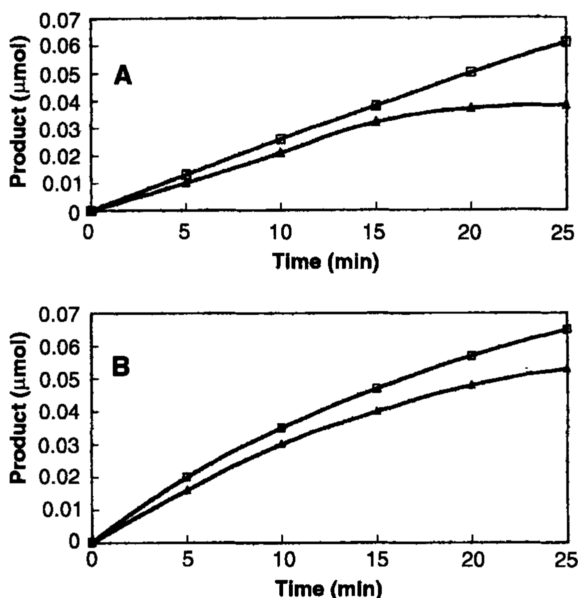

FIGURE 3 Time courses of ATCase. The product (μmol) produced versus time using CP (A) or Asp (B) as a substrate, in absence (□) and presence (▲) of 0.04 mM of (3).

1.1 ± 0.084 mM, a pattern characteristic of non-competitive inhibition (Figure 4B). In the case of ATCase treatment with (4), the K_m of the enzyme toward aspartate was increased and equal to 5.54 ± 0.145 and 6.83 ± 0.157 mM for control and treated enzyme, respectively. The inhibition appeared to be competitive since a comparison between control and treated enzyme with 4 showed no change in V_{max} value, which was 69.40 ± 1.019 μM/min/mg protein in both cases (Figure 4D). The K_i value for (4) was found to be 5.86×10^{-4} and 8.58×10^{-4} M in the reactions toward CP and aspartate, respectively (Table II).

(C) *In vivo* treatment of ATCase

The examined animals treated with different doses of (3) for two days showed a significant inhibition of ATCase at 800 ($p < .05$, $n = 4$) and 1000 μg ($p < .02$, $n = 6$) doses. A relative activity (ratio of the specific activity of treated enzyme to that of the control) of 60.37% and 47.16% were obtained at 800 and 1000 μg doses of 3, respectively. Higher relative activities were observed at

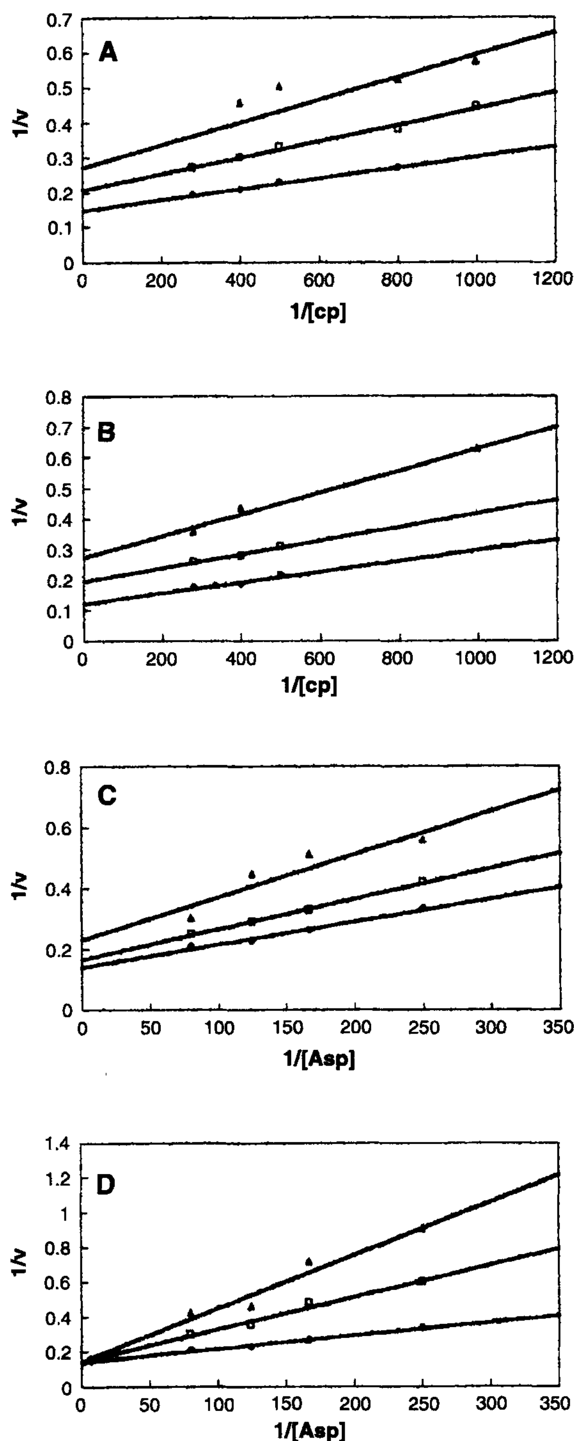

FIGURE 4 Lineweaver-Burk plots of ATCase. (A and B): varying concentrations of CP; (C and D): varying concentrations of Asp. A and C: 0.04 (□) and 0.07 (▲) mM of 3; B and D: 0.2 (□) and 0.5 (▲) mM of (4).

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TABLE II Michaelis and inhibition constants of ATCase toward CP and Asp in absence (control) and presence of (3) or (4) and their K_i values

Substrate	Control K_m (mM)	(3)		(4)	
		K_m (mM)	K_i (M)	K_m (mM)	K_i (M)
CP	1.10 ± 0.020	1.10 ± 0.020*	8.45×10^{-5}	1.10 ± 0.084	5.86×10^{-4}
Asp	5.54 ± 0.145	5.54 ± 0.145	9.64×10^{-5}	6.83 ± 0.157	8.58×10^{-4}

* Mean ± S.E.

TABLE III Specific activities of *in vivo* ATCase after treatment by **3** compared to a vehicle treated control. Mice were treated with different doses of (3) (0–1000 μg) and hepatic ATCase was then purified and assayed at 37 °C.

Dose (μg)	Specific activity (μmol/30 min/mg protein)	p value	Relative activity (%)
0	0.53 ± 0.004*	–	100.00
200	0.46 ± 0.006	< .50	86.79
400	0.44 ± 0.005	< .40	83.01
600	0.32 ± 0.005	< .30	60.37
800	0.30 ± 0.004	< .05	60.37
1000	0.25 ± 0.005	< .02	47.16

* Mean ± S.E.

lower concentrations of (3) (Table III). These relative activities obtained at 200, 400 and 600 μg doses of (3) were not significantly lower than that of the control (86.79, 83.01, 60.37 and 60.37%, respectively, $p < .3$–$.5$ for $n = 4$).

DISCUSSION

The structure of the synthesised compounds (2) and (3) was confirmed by IR and ^{1}H n.m.v. and elemental analysis.

Although CPSase II, ATCase and DHOase exists as a multienzyme complex and remains associated following ammonium sulfate fractionation and hydroxylapatite chromatography,[22,23] the reaction of ATCase is not affected by the presence of the other two enzymes. The equilibrium between carbamyl aspartate and dihydroorotate is pH-dependent with a maximal rate of cyclization of carbamoyl aspartate to dihydroorotate at acidic pH (4.4) which decreases to a very low rate at alkaline pH (9.2).[24] Thus, the ATCase reaction that was carried out at pH 8.2 indicates that the third step reaction of CAD mainly proceeds toward the carbamyl aspartate formation.

The regulation of the ATCase reaction could be critical for pyrimidine nucleotide biosynthesis since it might provide a major source of the pyrimidine supply *in vivo*. ATCase in *E. coli* has been shown to be subject to feedback inhibition by pyrimidines especially cytosine nucleotides such as CDP, CTP (5) and d-CTP which were the best inhibitors of the bacterial enzyme among the pyrimidine derivatives tested.[25] In this respect, the mammalian enzyme was different since (4) is the most potent inhibitor of this enzyme [7]. The data presented in Figure 2 agree with the previously described reports and show that the concentration of (5) that is required for 50% inhibition of ATCase is higher than that required by (4). This emphasizes the weak effect of (5) upon enzyme activity.

A comparison of the effect of structure of the examined compounds on inhibitory potency revealed that the three compounds; (1), (2) and (3) are structurally related to pyrimidines. Compounds (2) and (3) are more potent inhibitors than (4) (Figure 2) which may be due to

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different substitutions at C6. The substitution at C6 by carbonyl and phenylhydrazine groups in (**1**) and (**2**), respectively gave a lower inhibitory potency than that of (**3**) containing a *p*-nitrophenylhydrazine group at C6. In (**3**), the presence of the electron-withdrawing nitro group decreases the availability of the lone pair of electrons located on the hydrazone imino portion of the molecule. However, in an alkaline medium, the nitro group increases the possibility of ionization of the postulated ring enolic structure, which may facilitate the approach between compound (**3**) and the enzyme surface, which mainly has positively charged groups. In fact, basic amino acids represent 11% of the total amino acids of ATCase. Also, acidic amino acids (about 60%) have to be amidated to account for the isoelectric point of ATCase (pI 9.4).[26] In the present study, the inhibitory capacity of (**2**) is lower than that of (**3**) due to the absence of the *p*-nitro group whereas the presence of the keto group at C6 in **1** is likely less effective than the phenylhydrazine group.

The present study revealed that mammalian hepatic ATCase is devoid of the characteristic sigmoidal-dependence of activity on the substrate (aspartate or CP) concentration. This behavior agrees well with those obtained from previous studies.[7,25] A Lineweaver-Burk plot of 1/v against 1/[asp] for the inhibitory effect of **4** suggests competitive inhibition, a finding which is consistent with that obtained in earlier studies.[7] The structural similarity between any competitive inhibitor and its corresponding substrate makes it likely that the two molecules bind to the same site on the enzyme. On the other hand, compound (**3**) acts as a non-competitive inhibitor with respect to CP and aspartate. The inhibition of ATCase by (**3**) can be described as potent in view of the K_i value which is of the order of 10^{-5}.

The effect of (**3**) as an inhibitor is reversible since the complex readily dissociated from the enzyme and the enzyme completely recovered its activity after dialysis.[27]

There are, however, some inhibitors reported to dissociate from EI complexes very slowly i.e. Trehazolin forms a complex with trehalase,[28] castanospermine with sucrase[29] and swainsonine with α-mannosidase.[30]

The *in vivo* effect of (**3**) upon ATCase was studied. The most impressive results of the compounds inhibiting activity were obtained after two successive days of treatment where, the rate of enzymatic reaction was decreased about 52% below the control value following *in vivo* treatment with a 1000 μg dose of (**3**). This finding indicates that (**3**) may interfere with ATCase regulation possibly by binding to the enzyme reversibly as observed from the *in vitro* studies.

The maintenance of ATCase activity at a regulated level controls pyrimidine biosynthesis[2] since the first step uniquely committed to this process is the formation of carbamyl aspartate. Accordingly, the presence of the end product of the pathway or one of the structurally-related compounds to this end product decreased ATCase activity leading to some extent to a decrease in pyrimidine biosynthesis. In conclusion, one of phenobarbital derivatives (**3**) is a potent inhibitor of hepatic ATCase, which exerts a stronger effect than do the pyrimidines (**4**) and (**5**). IC_{50} studies, kinetic parameters, K_i value and *in vivo* studies support this finding. Also, the reversible inhibition of ATCase by (**3**) may be due to abolished regulation of this enzyme.

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