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ANTICHOLINESTERASE ACTIVITY OF THE UNSYMMETRIC BISQUATERNARY 6-AMINOQUINOLINE SALT NSC-176319*

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Abstract—The anticholinesterase activity of the unsymmetric bisquaternary 6-aminoquinoline salt NSC-176319 (QB) was studied *in vitro*. QB proved to be a noncompetitive inhibitor of both acetylcholinesterase (or true cholinesterase) and butyrylcholinesterase (or pseudocholinesterase) having a $K_I = 0.5 \times 10^{-6}$ M for acetylcholinesterase and 1.5×10^{-6} M for butyrylcholinesterase. Further, QB inhibited esterase activity of murine plasma in a noncompetitive manner ($K_I = 4.2 \times 10^{-6}$ M). The inhibition was instantaneous in onset and did not diminish with prolonged incubation of the drug and enzyme. All mice treated intravenously with 2 mg QB/kg died within 5 min. Prior to death, mice developed severe parasympathomimetic effects and convulsions. Although the parasympathomimetic effects were diminished by atropine sulfate pretreatment, death could only be prevented by barbiturate anesthesia.

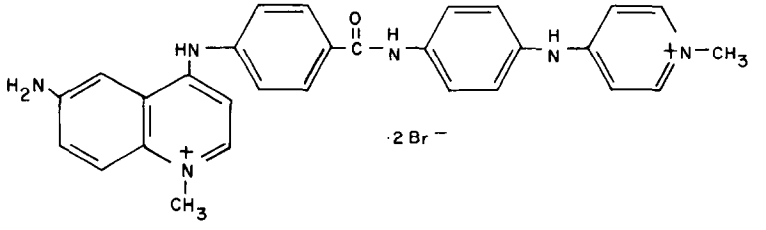

Fig. 1. Structural formula of NSC-176319, 4-[*p*-(*p*-[[pyridylamino]]phenylcarbamoyl) anilino]-quinoline dibromide (QB).

Several bisquaternary ammonium heterocyclic compounds have significant oncolytic activity against the L1210 murine leukemia. Indeed, some compounds produced greater than a 200% increase in the life span of L1210 bearing mice [1]. Unfortunately, most of these compounds also caused a delayed lethal toxicity, resulting in the death of mice approximately 30 days after treatment [2]. A systematic study by Atwell and Cain [3] demonstrated that one could attenuate the chronic toxicity of these compounds by the addition of amino substituents on the quinoline ring. Such substitution resulted in several treated mice surviving 100 days after inoculation of L1210 leukemia.

One such compound, NSC-176319, 4-[*p*-(*p*-[[pyridylamino]]phenycarbamoyl) anilino]-quinoline dibromide (QB), the chemical structure of which is depicted in Fig. 1, has been evaluated experimentally for its toxicity in dogs, monkeys and mice [4, 5]. When administered intravenously at a dose of 5 mg/kg to dogs or 2.1 mg/kg to monkeys, an acute toxicity suggestive of parasympathomimetic activity was observed. Further, mice given QB intravenously at doses as low as 1.5 mg/kg died within 20 min after injection with clinical signs of toxicity similar to those observed in dogs and monkeys.

Several bisquaternary ammonium compounds have been shown to be reversible inhibitors of both acetylcholinesterase (EC 3.1.1.7, AcChE) and butyrylcholinesterase (EC 3.1.1.8, BuChE) [6–8]. It is possible, therefore, that the responses of the dog, monkey and mouse to QB may have resulted, at least in part, from an anticholinesterase activity of QB. This report describes the inhibitory activity of QB *in vitro* and the prevention of acute lethality of QB by barbiturate anesthesia.

MATERIALS AND METHODS

Electric eel acetylcholinesterase, Type IV (EC 3.1.1.7), and horse serum butyrylcholinesterase (EC 3.1.1.8) were purchased from the Sigma Chemical

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Co., St. Louis, MO. Both enzymes were reconstituted with 0.1 M sodium phosphate buffer (pH 8.0) to a stock concentration of 4 units/ml with the phosphate buffer. Acetylthiocholine (ATC) and butyrylthiocholine (BTC, Sigma Chemical Co.) were prepared fresh daily in 0.1 M phosphate buffer (pH 8.0). Ellman's reagent, 5,5-dithio-bis-2-nitrobenzene (DTNB), was prepared fresh daily by dissolving 39.6 mg DTNB and 15 mg $NaHCO_3$ in 10 ml of 0.1 M phosphate buffer (pH 7.0). Physostigmine sulfate (eserine), purchased from CalBiochem, LaJolla, CA, was dissolved in distilled water at a concentration of 1 mg/ml. QB, supplied by the National Cancer Institute, Bethesda, MD, was dissolved in a 5% dextrose solution. Atropine sulfate (Sigma Chemical Co.) was dissolved in distilled water at a concentration of 5 mg/ml. Sodium pentobarbital (Veterinary Laboratories Inc., Lenexa, KS) was diluted with 0.9% NaCl to a concentration of 6.5 mg/ml.

Acetylcholinesterase (AcChE) and butyrylcholinesterase (BuChE) activities were determined by a slight modification of the method of Ellman *et al.* [9]. Briefly, the incubation medium consisted of 3 ml of 0.1 M phosphate buffer (pH 8.0), 100 μl DTNB, 50 μl enzyme, and 20 μl QB or phosphate buffer. After a 5-min preincubation period, 20 μl of substrate was added and the reaction proceeded for 5–8 min at 25°. The reaction was then stopped by the addition of 100 μl eserine, and esterase activity was measured spectrophotometrically by increased absorbance at 412 nm. Control assays demonstrated repeatedly that the system as described yielded a linear increase in absorbance with time for periods exceeding 10 min. Further, esterase activity was completely inhibited immediately upon the addition of eserine. In specific assays, the rate of increase in absorbance at 412 nm was measured directly using a Gilford recording spectrophotometer.

In vivo studies were performed using albino CD mice (20–25 g) purchased from Canadian Breeders, Montreal, Canada. QB was administered intravenously at concentrations such that 0.1 ml/10 g body weight delivered the desired dose. When used, atropine sulfate was administered intraperitoneally at a dose of 50 mg/kg approximately 15 min prior to QB. Sodium pentobarbital was administered intraperitoneally at a dose of 65 mg/kg approximately 15 min prior to QB. The toxicity of QB was then compared by the time required for 50% of the mice to die following treatment (LT_{50}).

To measure the inhibitory effect of QB on mouse esterase activity, mice were bled from the retro-orbital sinus using heparin-treated capillary tubes (Healthco, Burlington, VT). The plasma was separated by centrifugation and 80 μl plasma was added to 110 ml of 0.1 M phosphate buffer (pH 8.0). To 3 ml of diluted plasma was added 100 μl DTNB, 20 μl QB followed 5 min later by 20 μl ATC. Enzyme reaction was inhibited 5–8 min later by the addition of 100 μl eserine.

RESULTS

QB was incubated with AcChE at the final concentrations of 5, 2.5 and 1.25×10^{-6} M. Kinetic analysis of the inhibitory action of QB using the

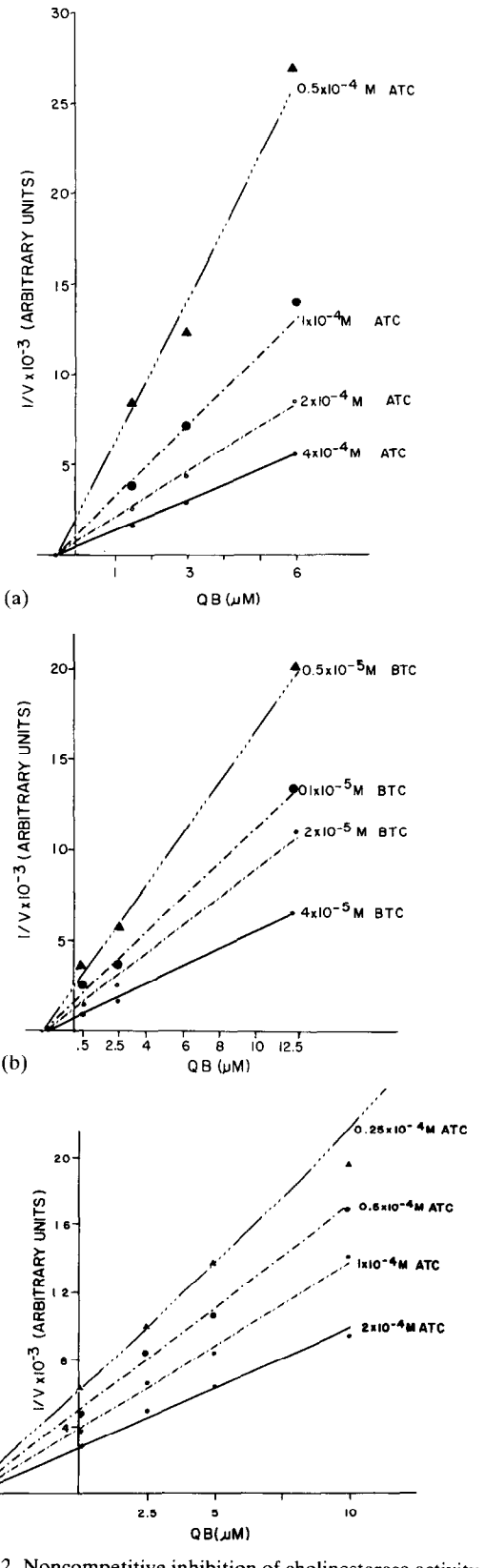

Fig. 2. Noncompetitive inhibition of cholinesterase activity by QB (NSC-176319) as analyzed by the graphical method of Dixon. Key: (a) effect of QB on acetylcholinesterase activity; (b) effect of QB on butyrylcholinesterase activity; and (c) effect of QB on murine plasma esterase activity.

method of Lineweaver–Burk [10] indicated that QB was a noncompetitive inhibitor of AcChE. Graphical representation of the data using the method of Dixon [11] indicated that QB was a noncompetitive inhibitor of the AcChE with a $K_I = 0.5 \times 10^{-6}$ M (Fig. 2a). QB proved to be a slightly less effective noncompetitive inhibitor of BuChE having a $K_I = 1.5 \times 10^{-6}$ (Fig. 2b). When added to mouse plasma, QB also inhibited the esterase activity in a noncompetitive manner with a $K_I = 4.2 \times 10^{-6}$ M (Fig. 2c).

Lalka and Bardos [12] reported that the anticholinesterase activity of alkylating agents increased with increased enzyme contact time, i.e. the length of preincubation of inhibitor and enzyme prior to enzymatic analysis. To determine whether or not similar effects would be observed with QB, two studies were performed. First, the AcChE reaction was initiated without QB and the rate of reaction was measured using a recording spectrophotometer. After 3 min of incubation, QB at a final concentration of 5, 2.5 or 1.25×10^{-6} M was added to the reaction. Inhibition of the enzymatic reaction by QB was almost instantaneous. With no concentration of QB tested, however, was the reaction completely inhibited (data not shown). Second, to determine whether or not preincubation of QB with either AcChE or BuChE would alter its anticholinesterase activity, QB (0.8 ml) was preincubated with 2 ml of either enzyme for periods of 1, 2, 4, 8 or 24 hr prior to enzymatic analysis. The volumes of enzyme and QB used in this study were the same volumetric ratio as used in the standard assay. The anticholinesterase activity of QB against either enzyme remained remarkably constant throughout the entire preincubation period with no significant change in the K_I of QB for either enzyme (data not shown).

To determine whether the acute parasympathomimetic responses of mice to QB were a result of its muscarinic activity, mice were administered atropine sulfate intraperitoneally 15 min prior to an intravenous injection of QB. All mice treated with either QB or atropine sulfate plus QB died (Table 1). Although the mean survival time of the atropinized mice was only increased slightly compared to mice given QB only, the toxic signs in mice given atropine prior to QB were altered. QB administered by itself caused immediate parasympathomimetic responses including defecation, urination and excessive salivation which were followed shortly thereafter by convulsions and death. Atropine-pretreated mice lacked the parasympathomimetic responses but did develop severe convulsions immediately prior to death.

Since the convulsions observed in QB-treated mice were indicative of excessive CNS stimulation, mice were anesthetized with sodium pentobarbital intraperitoneally prior to QB administration. All mice given QB and barbiturate survived the entire 14-day observation period with no episodes of convulsions. There were, however, significant parasympathomimetic responses including defacation, salivation and urination. However, mice treated with pentobarbital and atropine sulfate intraperitoneally approximately 15 min prior to QB survived the entire 14-day observation period without developing any of the acute parasympathomimetic responses associated with QB.

DISCUSSION

QB is a bisquaternary heterocyclic compound that has significant antitumor activity [3]. When given intravenously to dogs, monkeys or mice, a life-threatening parasympathomimetic response was observed [4, 5]. The results of the studies reported in this communication demonstrate that QB has noncompetitive anticholinesterase activity against both true cholinesterase ($K_I = 0.5 \times 10^{-6}$ M) and pseudocholinesterase ($K_I = 1.5 \times 10^{-6}$ M). The inhibitory effect of QB was immediate in onset and was not affected by prolonged periods of incubation of QB with either of the respective cholinesterases. Further, QB proved to be a noncompetitive inhibitor of murine plasma esterase activity ($K_I = 4.2 \times 10^{-6}$ M).

The anticholinesterase activity of QB is most probably related to the bisquaternary structure of the compound. Several other related bisquaternary ammonium compounds have been shown to have similar inhibitory effects on both true and pseudocholinesterase [6–8]. Chen *et al.* [8] reported that bisquaternary ammonium compounds with a flexible bridge linking the two nitrogen atoms were more potent inhibitors of AcChE than of BuChE. Our

Table 1. Survival time of mice treated with QB intravenously with or without pretreatment*

Group No.	Treatment	No. treated	No. of deaths	Mean survival time (min)
1	QB (2 mg/kg)	6	6	3.5 ± 0.6
2	QB (2 mg/kg) Atropine sulfate (50 mg/kg)	6	6	6.3 ± 0.8
3	QB (2 mg/kg) Pentobarbital (65 mg/kg)	6	0	†
4	QB (2 mg/kg) Atropine sulfate (50 mg/kg) Pentobarbital (65 mg/kg)	6	0	†

* Both atropine sulfate and pentobarbital were administered intraperitoneally 15 min before QB. Mean survival time was measured from the time of QB injection until cessation of breathing, and the values are expressed as means ± S.E.M.

† No animal anesthetized with pentobarbital died following QB treatment.

data further substantiate this observation, since QB does indeed have a flexible bridge between nitrogen atoms.

Although QB has significant anticholinesterase activity, the toxicity of QB cannot be solely attributed to a peripheral muscarinic action. This was demonstrated by the fact that despite prior treatment with atropine sulfate, which ablated the muscarinic responses to QB treatment, all mice subsequently developed severe convulsions and died. If, however, the mice were anesthetized prior to QB administration, all animals survived a dose of QB which would be lethal to unanesthetized mice.

It is of interest that Plowman and Adamson [13] while studying the pharmacokinetics of QB in mice and rats administered the drug intravenously to rats at doses of 4 mg/kg without observing any convulsions in the rats. The most probable explanation of this apparent lack of acute toxicity is that rats given QB intravenously in this study were also anesthetized with pentobarbital prior to QB administration. Thus, as with mice, rats were able to survive the acute toxic effects of QB when the rats were anesthetized prior to QB treatment.

REFERENCES

1. B. F. Cain, G. J. Atwell and R. N. Seelye, *J. med. Chem.* **12**, 199 (1969).
2. G. J. Atwell, B. F. Cain and R. N. Seelye, *J. med. Chem.* **11**, 300 (1969).
3. G. J. Atwell and B. F. Cain, *J. med. Chem.* **16**, 673 (1973).
4. PB 257 174/AS *Report to Laboratory of Toxicology*, DCT, NCI (1976).
5. M. P. Hacker, *Proc. Am. Ass. Cancer Res.* **19**, 138 (1978).
6. J. P. Long, in *Handbuch der Experimentellen Pharmakologie* (Ed. G. B. Koelle), p. 374. Springer, Berlin (1963).
7. W. S. Chen, G. H. Cocolas and C. J. Cavallito, *J. pharm. Sci.* **68**, 1025 (1979).
8. W. S. Chen, G. H. Cocolas, C. J. Cavallito and K. J. Chai, *J. med. Chem.* **20**, 1617 (1977).
9. G. L. Ellman, D. Courtney, V. Andres, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1960).
10. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
11. M. Dixon, *Biochem. J.* **55**, 170 (1953).
12. D. Lalka and T. J. Bardos, *Biochem. Pharmac.* **24**, 455 (1975).
13. J. Plowman and R. H. Adamson, *Pharmacology* **17**, 61 (1978).