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SHORT COMMUNICATIONS

Antimuscarinic activity of aprophen

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Aprophen [α-methyl-α-phenylbenzeneacetic acid 2-(diethylamino)ethyl ester] is an antispasmodic and cholinolytic agent [1]. Because of its high cholinolytic potency and ability to penetrate the blood–brain barrier, it is administered prophylactically and therapeutically as an antidote to nerve agents [2]. We report here that aprophen is an antimuscarinic agent that competes for binding to the muscarinic receptors. Additionally, it is a cytostatic as well as cytotoxic agent.

Materials and methods

Materials. Aprophen was synthesized at Walter Reed [3]. L-[Benzilic-4,4′-^{3}H(N)]quinuclidinyl benzilate ([^{3}H]QNB; 33.1 Ci/mmole) and *d*[13-^{3}H]tubocurarine (15.8 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA).

Methods. N4TG1 neuroblastoma cells and NG108–15 neuroblastoma × glioma hybrid cells were grown as described [4–6]. 3T3-C2 fibroblasts were cultured as reported for 3T3-L1 cells [7, 8]. Clone 9 liver cells, H9c2 myoblasts and C6 glioma cells were grown as described [9].

For the binding studies, cells in log phase of growth were detached from flasks by tapping, washed twice with phosphate-buffered saline, and resuspended in Hanks' media containing 25 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (pH 7.3). Typically, 0.5×10^6 cells, representing about 1 mg of protein, were incubated in the same media containing [^{3}H]QNB for the study of the muscarinic receptors, or [^{3}H]tubocurarine for the nicotinic receptors. The concentration of each radioactive ligand used was 2 nM in assessing the ability of aprophen to compete for binding to the muscarinic receptors or nicotinic receptors. After 20 min of incubation at 22°, the cells were layered over 500 μl of silicone oil (General Electric Versilube F50) in an Eppendorf tube and pelleted by centrifugation for 30 sec at 10,000 *g*. After removal of the aqueous (top) and oil (bottom) layers, the cell pellet in the Eppendorf tip was cut off and solubilized in 1.0 ml of 1% Triton X-100; 10 ml of scintillation fluid was added and the radioactivity was then determined. To measure the nonspecific binding of the radioactive antagonists, the cells were incubated with 100 μM atropine for the muscarinic receptor assay [10] or 100 μM tubocurarine for the nicotinic receptor assay [11], for 10 min prior to the addition of the radioactive antagonists. The nonspecific binding counts were subtracted from total binding counts to obtain the specific binding values. By using the present method, lower background counts were observed in comparison to the conventional method of using filters. It was also found that equilibrium in binding was observed within 2 min, and linear binding was observed with the cell concentrations ranging from 0.2 to 1×10^6 cells.

Results and discussion

The antimuscarinic activity of aprophen was assayed by the inhibition of binding of the muscarinic antagonist [^{3}H]QNB to the cells. As shown in Fig. 1, aprophen blocked the binding of [^{3}H]QNB to the N4TG1 neuroblastoma cells and the NG108–15 neuroblastoma × glioma cells in a dose-dependent manner. The I_{50} (minimal concentration that inhibited 50% of the binding) was 5 μM for the N4TG1 cells and 0.8 μM for the NG108–15 cells. There were about 1.7×10^5 muscarinic sites/cell (290 fmoles/10^6 cells) in the N4TG1 cells, with a K_D of 13 nM. In the NG108–15 cells, there were 2.0×10^5 muscarinic sites/cell (340 fmoles/10^6 cells), with a K_D of 10 nM. The K_D values obtained for the two cell lines (≃10 nM) were in close agreement with 8 nM for human neutrophils [12], 1 nM for rat cortex homogen-

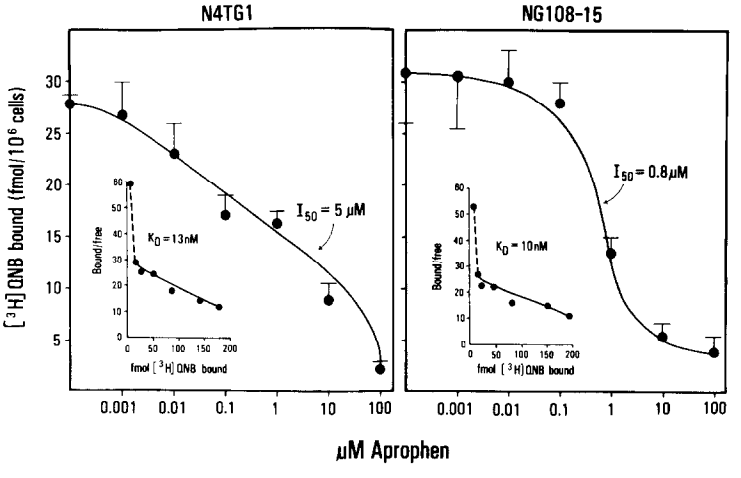


Fig. 1. Inhibition of [^{3}H]QNB binding by aprophen to the muscarinic receptor of N4TG1 neuroblastoma cells (left) and NG108-15 neuroblastoma × glioma hybrid cells (right). Insets are Scatchard plots of [^{3}H]QNB binding; data represent mean ± S.E.M. of triplicate samples. A 2 nM concentration of [^{3}H]QNB was used for the inhibition assay; assay duration was 20 min.

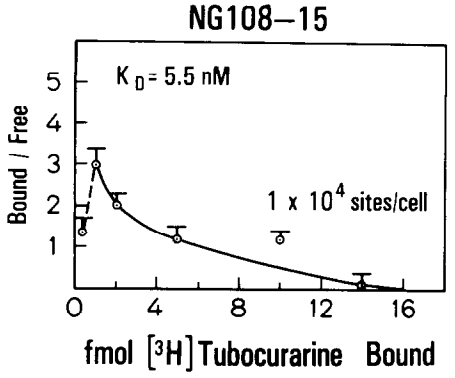


Fig. 2. Scatchard plot of the binding of [^{3}H]tubocurarine to NG108-15 neuroblastoma × glioma hybrid cells.

ates [13], and 0.2 to 0.5 nM for solubilized atrial membranes [10]. If the binding of [^{3}H]QNB were calculated on a mg protein basis, both cell lines would bind approximately 300 fmoles/mg, a value comparable to 366–558 fmoles of [^{3}H]QNB bound per mg of synaptosomal membranes [14], and 450 fmoles/mg for solubilized atrial membranes [10]. An estimation of 5×10^4 muscarinic sites per neutrophil cell has been reported [12].

In contrast to its inhibitory effect on the muscarinic receptors, aprophen has no discernable effect on the nicotinic receptors of NG108–15 cells when assayed with [^{3}H]tubocurarine (not shown). There are about 1×10^4 nicotinic sites/cell (16 fmoles/10^6 cells) in the NG108–15 cells, with a K_D of 5.5 nM (Fig. 2). The K_D value for [^{3}H]tubocurarine compared favorably with a K_D of 12 nM for the binding of [^{3}H]acetylcholine to nicotinic receptors of rat brain [15]. Moreover, the amount of [^{3}H]tubocurarine bound to the NG108–15 cells (16 fmoles/mg) was close to the finding of 4.6 fmoles of [^{3}H]acetylcholine bound to the nicotinic receptors of rat brain [15]. Virtually no nicotinic receptors could be detected in the N4TG1 cells, and this is in agreement with another observation in which α-[^{125}I]-bungarotoxin was used (G. J. Gwyn and E. Costa, personal communication).

Table 1. Cytoxicity of aprophen

Cell type	I_{50}* (μM)
NG108-15 neuroblastoma × glioma	0.1
H9c2 myoblasts	2.0
C6 glioma	2.0
N4TG1 neuroblastoma	3.0
3T3-C2 fibroblasts	18.0
Clone 9 liver cells	20.0

* The I_{50} values were determined after 48 hr of incubation with various concentrations of aprophen.

Aprophen was next tested for cytotoxicity. The cells that were most sensitive to the adverse effect of aprophen are those of neuronal or muscular origin (Fig. 3; Table 1). At 0.1 μM, aprophen was cytostatic to the NG108-15 neuroblastoma × glioma cells. Between 0.5 and 5.0 μM, the cytotoxicity of aprophen became obvious. The I_{50} (minimal concentration causing a 50% inhibition in cell growth after 48 hr) was 0.1 μM for the NG108-15 cells (Table 1). The I_{50} for both the H9c2 myoblasts and C6 glioma cells was 2.0 μM and for the N4TG1 neuroblastoma cells was 3.0 μM. The cells least affected by aprophen were 3T3-C2 fibroblasts and Clone 9 liver cells, with I_{50} values of 18.0 and 20.0 μM respectively.

The ability of aprophen to compete with [^{3}H]QNB for binding to the muscarinic sites is most likely due to the fact that aprophen is a benzilate analog, thus sharing a common steric conformation at the muscarinic site (Fig. 4). The present investigation also agrees with the suggestion that there is no direct correlation between cellular levels of acetylcholinesterase and the number of muscarinic receptors in cells [16, 17]. The cytostatic and cytotoxic effects of aprophen may be mediated by the occupancy of the muscarinic receptors, leading probably to cellular imbalances of Ca^{2+} and Na^+ across the channels involved [18, 19]. With respect to cell division, muscarinic receptor-mediated cyclic GMP formation may be involved [20].

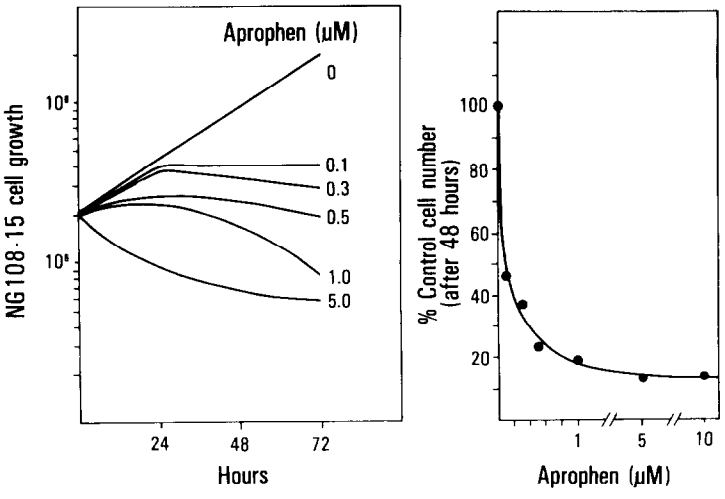
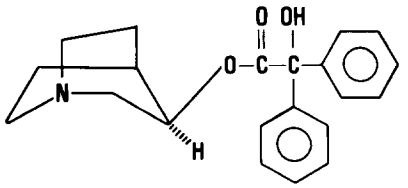
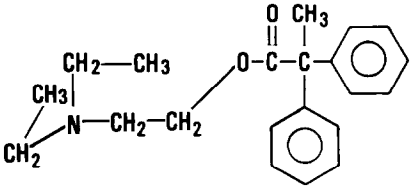


Fig. 3. Growth of NG108-15 neuroblastoma × glioma cells in the presence of various concentrations of aprophen.



QNB



APROPHEN

Fig. 4. Chemical structures of QNB and aprophen.

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The effect of lithium on rat erythrocyte choline, glycine and glutathione levels

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The concentration of red blood cell (RBC) choline increases in patients as a result of lithium treatment [1, 2]. This accumulation of choline reaches steady-state levels 8 to 10-fold above baseline after 4–6 weeks of lithium treatment [1, 3, 4]. Lithium enhances brain acetylcholine (ACh) levels in rats following a pulsed dose of [$^2H_4$]choline [5]. Since choline is a precursor of ACh, and both RBC and neuronal membranes share many similarities, changes in RBC choline may parallel increased neuronal availability of ACh during lithium treatment.

Lithium has also been shown to increase the concentration of glycine in the RBC of manic-depressive patients [3, 6, 7] and the RBC and brain of rats [8]. Glycine is an inhibitory neurotransmitter and is also a precursor of RBC glutathione (GSH). If lithium increases RBC glycine, then these changes may affect RBC GSH levels. GSH is important for cell survival because it maintains the reducing potential within erythrocytes.

Free choline, glycine and GSH levels were measured in rat erythrocytes after lithium treatment with a view to develop an animal model in which to extend previous findings of lithium-induced changes in human erythrocytes. RBC metabolite concentrations were measured using proton magnetic resonance (^{1}H NMR) spectroscopy in conjunction with the spin-echo pulse sequence.

Materials and methods

Fourteen adult male Sprague–Dawley rats (390–480 g)