Inhibition of Muscarinic Receptor Binding and Acetylcholine-Induced Contraction of Guinea Pig Ileum by Analogues of 5′-(Isobutylthio)adenosine

Marvin C. Pankaskie,*† James F. Kachur,‡ Tokuo Itoh,§ Richard K. Gordon,‖ and Peter K. Chiang‖

Department of Biomedicinal Chemistry, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68105, Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, and Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307. *Received March 15, 1984*

(Isobutylthio)adenosine (SIBA, 1) and its derivatives have been shown to produce a variety of biological effects on the basis of the hypothesis that such agents act directly as inhibitors of transmethylation reactions, as inhibitors of *S*-adenosylhomocysteine hydrolase, or as inhibitors of polyamine biosynthesis. We report here the ability of selected analogues of SIBA to inhibit the binding of the muscarinic antagonist quinuclidinyl benzilate (QNB) to cultured N4TG1 neuroblastoma cells and to antagonize the acetylcholine-induced contraction of guinea pig ileum. The most potent inhibitors were 5′-deoxy-5′-(isobutylthio)-1-deazaadenosine (1-deaza-SIBA, **5**) and 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine (3-deaza-SIBA, **3**), while the parent nucleoside SIBA and the carbocyclic derivative 5′-(isobutylthio)-3-deazaaristeromycin were less active. The same agents had no effect on the nicotinic receptors of NG108-15 neuroblastoma × glioma hybrid cells. The acyclic derivative 9-[[2-(isobutylthio)ethoxy]methyl]adenine, 3-deazaadenosine, 5′-(isobutylthio)tubercidin, and 5′-(isobutylamino)adenosine were inactive at the 1-mM level. These results suggest that SIBA and 3-deaza-SIBA may have profound effects on membrane-mediated phenomenon, including inhibition of muscarinic receptor binding.

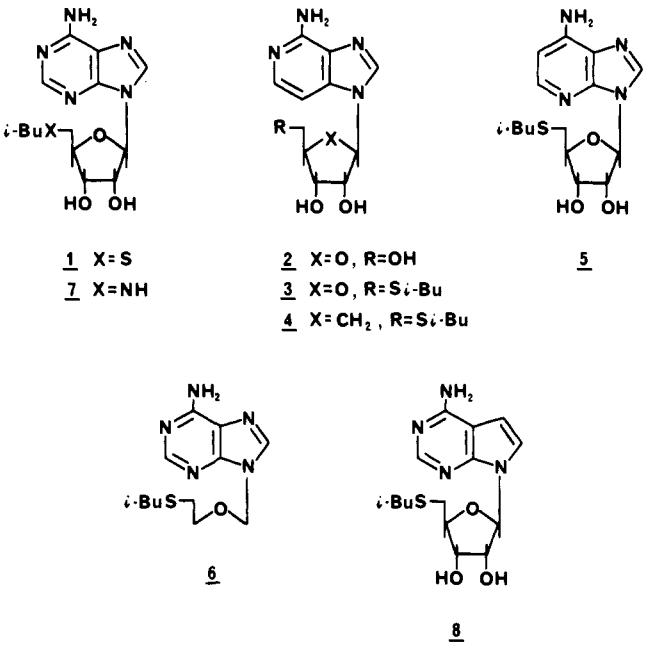
During the last decade, numerous studies have shown that *S*-adenosylmethionine (AdoMet) mediated transmethylation reactions are involved in the regulation of various physiological processes.[1] Inhibitors of such reactions, including derivatives of *S*-adenosylhomocysteine (AdoHcy), adenosine, and 5′-(methylthio)adenosine (MeSAdo), have been shown to produce profound effects on protein synthesis,[2] nucleic acid synthesis,[3] viral replication,[4] cellular differentiation[5] and chemotaxis,[6] and polyamine metabolism.[7] All of these effects have been attributed to a perturbation of intracellular ratios of AdoMet/AdoHcy via the direct inhibition of various transmethylases[8] or more frequently the inhibition of AdoHcy hydrolase. The latter enzyme catalyzes the metabolism of AdoHcy, which is known to be a potent inhibitor of numerous transmethylation reactions.[9]

The purine-modified nucleoside 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine (3-deaza-SIBA, **3**), a moderate noncompetitive inhibitor of AdoHcy hydrolase (K_i = 0.4 mM),[10] has been shown to produce inhibition of Rous sarcoma virus replication at 5–10 times lower concentrations than those required for the inhibition of AdoHcy hydrolase (72% inhibition at 0.03 mM).[10] It has recently been shown that 3-deaza-SIBA (**3**) as well as several other known transmethylase inhibitors can affect physiological processes unrelated to transmethylation, including inhibition of choline uptake by RBL-2H3 leukemia cells,[11] inhibition of Ca^{2+} transport, CDP-choline biosynthesis, and acetylcholine secretion in NG108-15 neuroblastoma cells,[12] and the inhibition of histamine release from peritoneal mast cells.[11] These studies suggest that 3-deaza-SIBA (**3**) and its derivatives may perturb membrane function and integrity and consequently affect other membrane-mediated phenomenon as well. In this report, selected analogues of SIBA (**1**) and 3-deaza-SIBA (**3**) are shown to inhibit the binding of quinuclidinyl benzilate (QNB), a potent muscarinic antagonist, to cultured N4TG1 neuroblastoma cells. In addition, SIBA and 3-deaza-SIBA are shown to block the contraction of guinea pig ileum induced by acetylcholine (ACh), albeit in a noncompetitive fashion.

Results

Chemistry. 5′-Isobutylthio nucleosides chosen for this study are shown in Chart I. Compounds **1**, **3**, and **5** were prepared according to previously published methods,[10,13–15]

Chart I



† University of Nebraska Medical Center.
‡ Virginia Commonwealth University.
§ Hokkaido University.
‖ Walter Reed Army Institute of Research.

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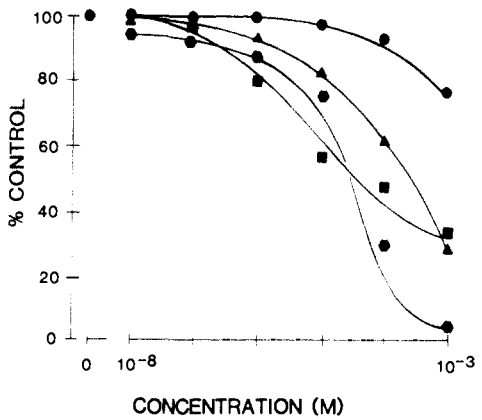

Figure 1. Dose–response curves for [^{3}H]QNB binding to N4TG1 neuroblastoma cells in the presence of target compounds 1 (●), 3 (●), 4 (▲), and 5 (■) over the concentration range indicated.

via chlorination of the parent nucleoside with $SOCl_2$/HMPA followed by displacement of the 5′-chloro group with isobutyl mercaptan in $NaOCH_3$/methanol. The 3-deazaaristeromycin analogue **4**, the acyclic analogue **6**, the isobutylamino analogue **7**, and the tubercidin analogue **8** were all prepared in a similar fashion in 60–80% yields, starting from the appropriate nucleoside or in the case of **6**, 9-[(2-hydroxyethoxy)methyl]adenine, and are herein described for the first time.

Muscarinic Receptor Studies. In order to evaluate the effects of 3-deaza-SIBA and its derivatives on membrane-mediated responses, target compounds **1–6** were tested for their ability to inhibit the binding of the cholinergic ligand quinuclidinyl benzilate (QNB) to muscarinic receptors of cultured N4TG1 neuroblastoma cells.[16] As shown in Figure 1, the binding of [^{3}H]QNB was inhibited by compounds **1**, **3**, **4**, and **5** over a concentration range of 10^{-8}–10^{-3} M, with I_{50} values of 1500 ± 900, 39 ± 8, 210 ± 30, and 52 ± 10 μM, respectively. Aprophen, a potent antimuscarinic agent previously evaluated by Gordon et al.,[16] was found to have an I_{50} of 5.0 ± 0.2 μM in this same cell system. Increasing the incubation time for these compounds failed to further decrease QNB binding. No effect was observed with **2**, **6**, **7**, or **8**, even at 10^{-3} M.

Nicotinic Receptor Studies. The same agents that were effective in blocking the binding of [^{3}H]QNB had no effect on the binding of [^{3}H]tubocurarine to the nicotinic receptors of NG108-15 neuroblastoma × glioma hybrid cells (not shown).

Guinea Pig Ileum Studies. Target compounds 1 and **3**, exhibiting two extremes in inhibition of QNB binding (I_{50} values of 1500 and 39 μM, respectively), were then evaluated for their ability to antagonize ACh-induced contractions of isolated guinea pig ileum. Availability of sufficient quantities of other target compounds precluded their evaluation at this time, although their qualitative effect would be expected to be similar. Figures 2 and 3 show that SIBA (**1**) and 3-deaza-SIBA (**3**), respectively, blocked the ACh-induced contraction of guinea pig ileum. The pattern of inhibition was noncompetitive in nature.

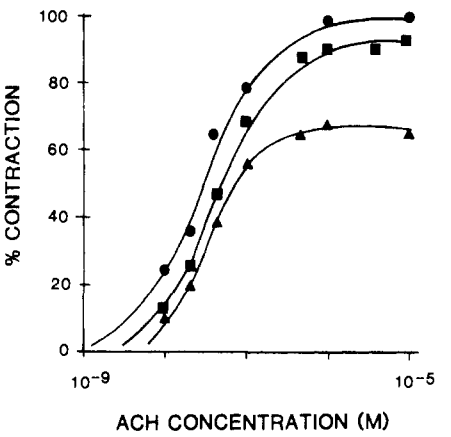

Figure 2. Dose–response curves for ACh-induced ileal contractions in the presence of (●) ACh alone, (■) ACh plus 15 μM SIBA (1) and (▲) ACh plus 75 μM SIBA (1). Each data point is the average of two independent observations.

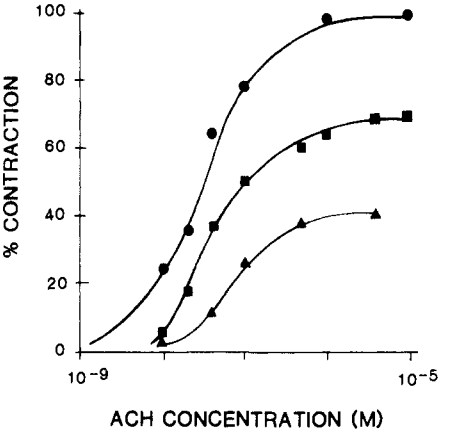

Figure 3. Dose–response curves for ACh-induced ileal contractions in the presence of (●) ACh alone, (■) ACh plus 35 μM 3-deaza-SIBA (**3**), and (▲) ACh plus 100 μM 3-deaza-SIBA (**3**). Each data point represents the average of two independent observations.

Discussion

The existence of several structural requirements in the present series of SIBA analogues suggests that the observed activity is not strictly nonspecific. The relatively low I_{50} values compared with more classical ligands such as aprophen or atropine indicate that these compounds may not be competing for the QNB binding site per se but are rather exerting some noncompetitive or allosteric effects. The lack of any structural similarity between 3-deaza-SIBA and atropine-like or ACh-like ligands supports this hypothesis. The fact that several analogues, including **2**, **6**, **7**, and **8**, are unable to affect QNB binding indicates, however, that some structural features are critical and that the observed activity is not strictly nonspecific. For example, the activities of **1**, **2**, and **7** suggest that hydrophobicity at the 5′-position is important. Hydrophobic influence may also explain the differences in activities between **1**, **3**, and **5**, all of which differ only in the nature of the purine ring. The lower activity of **4** compared to **3** and the inactivity of **6** point to the importance of an intact ribofuranose ring, while the inactivity of **8** supports the importance of a basic nitrogen at position 7 of the purine ring. Additional analogues are being prepared and

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tested to compile a more complete structure–activity relationship.

Although the noncompetitive inhibition of ACh-induced contraction of guinea pig ileum also suggests that SIBA (1) and 3-deaza-SIBA (3) may exert their effect on the muscarinic receptor at site(s) distant from the active binding site of ACh, their failure to affect the nicotinic receptors of NG108-15 neuroblastoma × glioma hybrid cells points to a specific influence on muscarinic receptor binding rather than some nonspecific membrane disruption. Recent studies showing the inhibitory effect of SIBA and 3-deaza-SIBA on Ca^{2+} uptake in NG108-15 hybrid cells plus the known effects of Ca^{2+} ions on muscarinic receptor integrity suggest that the observed inhibition of QNB binding may be due directly or indirectly to alterations in Ca^{2+} transport. Other Ca^{2+}-mediated phenomenon are currently being studied to assess the extent of this effect.

Experimental Section

Melting points were determined on a Mel-Temp apparatus and are uncorrected. Routine NMR, IR, and UV spectra were consistent with assigned structures and were obtained on a Varian EM360, a Beckman IR18A, and a Beckman DU-7 spectrometer, respectively. HPLC analyses were performed on a μBondapak C_{18} column (Waters Associates, Inc.) using 15% methanol/50 mM KH_2PO_4 (pH 4.5) as the mobile phase and a 254- or 280-nm UV detector (Laboratory Data Control). All chemicals were reagent grade or better and were obtained from Aldrich Chemical Co. or Sigma Chemical Co.

5′-Deoxy-5′-(isobutylthio)-3-deazaaristeromycin (4). To a cooled suspension of 0.5 g (1.9 mmol) of 3-deazaaristeromycin[17] in 7 mL of HMPA was added 1.0 mL of $SOCl_2$ and the solution stirred overnight at room temperature. Ice water (25 mL) was added slowly to decompose excess $SOCl_2$, and after an additional period of cooling (0–5 °C), the resulting product, 5′-deoxy-5′-chloro-3-deazaaristeromycin, was filtered off and briefly air-dried. The crude product was then dissolved in 10 mL of 2 N $NaOCH_3$/methanol containing 1.5 mL (12 mmol) of isobutyl mercaptan and stirred at room temperature for 24 h. After neutralization with glacial acetic acid, the crude reaction product was isolated by filtration and recrystallized from water to give pure 4 (0.4 g, 63%), mp 119–121 °C. Anal. ($C_{16}H_{24}N_4O_2S$) C, H, N.

9-[[2-(Isobutylthio)ethoxy]methyl]adenine (6). To a cooled suspension of 1.0 g (4.4 mmol) of 9-[(2-hydroxyethoxy)methyl]-adenine[18] in 15 mL of HMPA was added 2.0 mL of $SOCl_2$, and the solution was allowed to stir at room temperature overnight. Ice water (25 mL) was added during which time a precipitate began to form. After additional cooling, the precipitate was filtered off and air-dried to give 9-[(2-chloroethoxy)methyl]adenine. This intermediate was not further purified but was dissolved in 20 mL of 2 N NaOH and treated with 2.5 mL (20 mmol) of isobutyl mercaptan. After the mixture was stirred overnight at room temperature, the desired product was precipitated by neutralization of the cooled reaction mixture with glacial acetic acid; a single recrystallization from water yielded 6 as transparent plates (0.9 g, 74%), mp 166–168 °C. Anal. ($C_{12}H_{19}N_5OS$) C, H, N.

5′-Deoxy-5′-(isobutylamino)adenosine Hydrochloride (7). To 1.4 g (5.0 mmol) of 5′-deoxy-5′-chloroadenosine[19] was added 25 mL of isobutylamine and the mixture kept at room temperature for 3 days during which time excess amine was allowed to evaporate off. The resulting off-white solid was crystallized twice from absolute ethanol to give 7 as a fluffy white solid (1.4 g, 88%), mp 155–158 °C. Anal. ($C_{14}H_{22}N_6O_3HCl$) C, H, N.

5′-Deoxy-5′-(isobutylthio)tubercidin (8). To 0.5 g (1.8 mmol) of 5′-deoxy-5′-chlorotubercidin[20] dissolved in 5 mL of 2 N NaOH was added 1.5 mL (12 mmol) of isobutyl mercaptan. After the mixture was stirred at room temperature for 24 h, the product was precipitated by neutralizing the reaction mixture with cold glacial acetic acid. After two recrystallizations from water, 8 was obtained as an off-white solid (0.4 g, 64%), mp 186–187 °C. Anal. ($C_{15}H_{22}N_4O_3S$) C, H, N.

Muscarinic Receptor Assay. [^{3}H]QNB binding and displacement measurements were performed as previously described with use of N4TG1 neuroblastoma cells.[16] Cells were incubated with 100 μM atropine and/or varied concentrations of the SIBA analogues for 10 min prior to the addition of 2 nM [^{3}H]QNB. I_{50} values were determined with ALLFIT, a computer program for analysis of sigmoidal curves.[21]

Nicotinic Receptor Assay. NG108-15 neuroblastoma × glioma hybrid cells were grown as described.[12] Cells were incubated with 100 μM tubocurarine for 10 min before the addition of 2 nM [^{3}H]tubocurarine to assay for specific binding to the nicotinic receptors.[16]

Guinea Pig Ileum Assay. Distal ileum was obtained from male albino guinea pigs (350–500 g). All animals were allowed free access to food and water prior to sacrifice by cervical dislocation. A segment of distal ileum approximately 20 cm in length was excised 5 cm above the ileocaecal junction and immediately placed in oxygenated Krebs–Ringer solution containing 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 0.93 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 11 mM glucose. Segments 2.5 cm in length were suspended in a 10-mL organ bath, which was continuously aerated with 5% CO_2/O_2 and maintained at 37 °C. Isometric contractions were recorded by means of a free-displacement transducer on a Grass Model 7 multichannel polygraph. After a stabilization period of 45 min, test compounds were added to the bath by pipette, allowed to act for 1 min, and then washed out. The tissue was allowed 8–10 min to recover prior to the next addition. The concentration response curve for ACh was obtained by using a series of single doses of increasing concentration, with the maximal contractile response designated as 100% and other responses reported as a percentage of that maximum response.

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