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Effect of 9-alkyl derivatives of 6-methylthioguanine on brain specific binding of [^{3}H]diazepam

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Both biochemical and pharmacological evidence support the hypothesis that [^{3}H]benzodiazepine binding to high affinity central benzodiazepine receptors may mediate some of the action of benzodiazepines [1]. Since various findings have suggested purines as putative endogenous ligands for benzodiazepine receptors [2, 3], we have studied the effect of synthetic purines and purine nucleosides on [^{3}H]diazepam binding with rat brain membranes [4, 5]. Among these analogs, 6-*n*-pentyldithioguanine had the highest potency, with a K_i value of 0.92 μM. In the present study we found that some 9-alkyl derivatives of 6-methylthioguanine were capable of inhibiting high affinity brain specific binding of [^{3}H]diazepam in the nanomolar range.

Methods

Male Wistar rats (200–250 g) were obtained from the Animal Unit at the University of British Columbia. The animals were decapitated, brain tissues were homogenized in 20 vol. of ice-cold 0.32 M sucrose in a Teflon–glass homogenizer, and membranes were prepared as described previously [5]. Specific binding of [^{3}H]diazepam was assessed in an incubation volume of 0.2 ml containing 25 mM sodium phosphate buffer, pH 7.4, membrane protein (*ca.* 0.082 mg/assay) and 6.2 nM [*methyl*-^{3}H]diazepam (0.1 μCi/assay). Incubation with or without the addition of purine derivatives was carried out at 0° for 20 min and was terminated by rapid filtration under vacuum through glass-fiber filters (Whatman GF/B). These were washed three times with 5 ml of cold 0.05 M sodium phosphate buffer, pH 7.4, and then counted for radioactivity. Specific binding of [^{3}H]diazepam was defined as that displaced by 3 μM diazepam and represented about 97% of the total binding. Protein content was determined by the method of Lowry *et al.* [6].

IC_{50} Values were assessed graphically at five different concentrations of purine derivatives in duplicate and expressed as means. K_i values were calculated according to the formula: $K_i = IC_{50}/(1 + [L]/K_d)$. $[L]$ equals concentration of ligand.

[*Methyl*-^{3}H]diazepam (sp. act. 80.3 Ci/mmole) was purchased from New England Nuclear. Unlabeled diazepam was a gift from Hoffmann-La Roche Ltd., Vaudreuil, Quebec. Various 9-alkyl derivatives of 6-methylthioguanine were synthesized according to Noell and Robins [7].

Results and discussion

The abilities of various 9-alkyl derivatives of 6-methylthioguanine (structure shown in Fig. 1) to replace specifically bound [^{3}H]diazepam were tested by incubating the

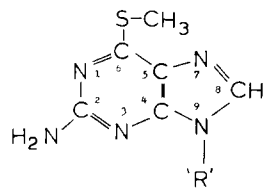

Fig. 1. Structure of 9-alkyl-6-methylthioguanine.

compounds in the standard binding mixture. The results presented in Table 1 show that 9-alkyl derivatives of 6-methylthioguanine were much more active than 6-methylthioguanine which showed a K_i value of 16 μM as an inhibitor of [^{3}H]diazepam binding [4]. The most potent compound, 9-*n*-pentyl-6-methylthioguanine, had an IC_{50} of 0.2 μM with a K_i of 0.082 μM. When the 6-methylthio group of 9-alkyl-6-methylthioguanine was replaced by a 6-thio

Table 1. Inhibition of [^{3}H]diazepam binding to rat cortical membranes by 6-methylthioguanine 9-alkyl derivatives

Compound	IC_{50} (μM)	K_i (μM)
9-Methyl-6-methylthioguanine	10	4.1
9-*n*-Propyl-6-methylthioguanine	0.25	0.10
9-*n*-Pentyl-6-methylthioguanine	0.2	0.082
9-*n*-Propyl-6-thioguanine	5	2.05
9-*n*-Pentyl-6-thioguanine	5	2.05

Data represent the mean values for four to six separate experiments with standard deviations of less than 10%. K_i values were calculated according to the formula: $K_i = IC_{50}/(1 + [L]/K_d)$.

group, the potency in inhibiting [^{3}H]diazepam binding decreased enormously, indicating the need for a methyl group at the 6-position for the inhibition of [^{3}H]diazepam binding. As shown in Fig. 2, the inhibition of [^{3}H]diazepam binding to rat brain membranes by 9-propyl-6-methylthioguanine was competitive in nature.

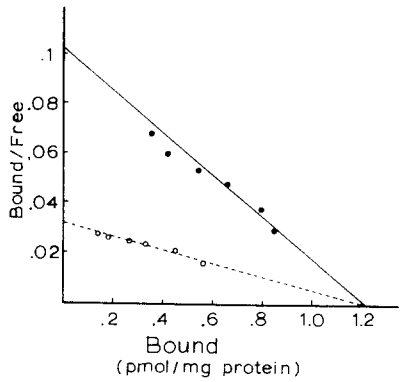

Fig. 2. Binding of [^{3}H]diazepam to rat brain cortical membranes as a function of concentration of [^{3}H]diazepam in the absence (●—●) and presence (○- - -○) of 0.25 μM 9-propyl-6-methylthioguanine.

We found previously that, among various purine derivatives, 6-methylthioguanine is potent in displacing the brain specific binding of [^{3}H]diazepam [4]. Later we observed that some of the more hydrophobic derivatives of 6-methylthioguanine have higher potencies than 6-methylthioguanine [5]. These results suggested that, in addition to hydrophobicity, there seems to be a steric effect by the alkyl derivatives as inhibitors of [^{3}H]diazepam binding.

In the present study we found that 9-alkyl derivatives of 6-methylthioguanine (e.g. 9-*n*-propyl and 9-*n*-pentyl derivatives) competitively inhibited [^{3}H]diazepam binding with K_i values in the nanomolar range. These compounds were several thousand times more potent than inosine and hypoxanthine. Since these 9-alkyl derivatives of 6-methylthioguanine inhibited [^{3}H]diazepam binding at nanomolar concentrations, these compounds could serve as useful tools for behavioral studies related to benzodiazepine function.

In summary, 9-alkyl derivatives of 6-methylthioguanine were tested for their abilities to displace [^{3}H]diazepam binding to rat brain membranes. The most active derivative, 9-*n*-pentyl-6-methylthioguanine, had an IC_{50} of 0.2 μM with a K_i of 0.082 μM. This is the first purine derivative that inhibits [^{3}H]diazepam binding at nanomolar concentrations.

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Formation of adenosine-5′-phosphosulfate from 3′-phosphoadenosine-5′-phosphosulfate in human platelets

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In a previous article [1] from this laboratory, it was demonstrated that ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAPS*) was rapidly metabolized to inorganic sulfate when incubated in the presence of high speed supernatant solutions obtained from human brain. Hydrolysis of PAPS to inorganic sulfate was shown to be more rapid than the rate of sulfation of acceptor substrates by phenol sulfotransferase (PST) and, thus, the concentration of PAPS available for sulfoconjugation became limiting. Breakdown of PAPS to form free sulfate could possibly occur by the initial production of a sulfate-phosphate anhydride from PAPS [2] followed by the hydrolysis of the anhydride to sulfate and inorganic phosphate. Alternatively, PAPS may be broken down by 3′-nucleotidase [3] to form inorganic phosphate and adenosine-5′-phosphosulfate (APS) which may be subsequently hydrolyzed by APS-sulfohydrolase or some other nonspecific hydrolase to form inorganic sulfate and adenosine monophosphate (AMP) [4, 5]. Although the latter pathway is more likely, no APS was detected in the incubation mixtures of PAPS with human brain homogenates.

The presence of these PAPS catabolic enzymes in crude tissue homogenates has the potential to interfere with the assay of phenol and other sulfotransferases that require PAPS as the sulfate donor. For example, in a typical phenol

* Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PST, phenol sulfotransferase; APS, adenosine-5′-phosphosulfate; AMP, adenosine monophosphate; and TEA, tetraethylamine.