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Rat Brain Guanosine Binding Site: Biological Studies and Pseudo-Receptor Construction

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Abstract—Rat brain guanosine binding sites were studied by (i) a pharmacological approach to confirm the hypothesis of the existence of specific G-coupled receptors for guanosine (**1**) and, for the first time, delineate a structure–activity relationship for a series of guanosine derivatives; (ii) a molecular modelling approach to design a pseudo-receptor construction. GTP and its non-hydrolysable analogue Gpp[NH]p decreased [^{3}H]-guanosine binding to rat brain membranes. Gpp[NH]p 30 and 100 μM induced a dose-dependent decrease in [^{3}H]-guanosine affinity and PTX pretreatment of rat brain membranes caused a 50% reduction in binding. In slices from rat brain cortex, guanosine induced a dose-dependent increase in intracellular cAMP. This increase is specific for guanosine, since neither the pretreatment with adenosine deaminase nor the A_1 and A_2 adenosine receptor antagonists were able to modify the guanosine-induced cAMP accumulation. The structure–activity relationship showed that the potency order of the best substances able to displace 50 nM [^{3}H]-guanosine was guanosine (**1**) = 6-thioguanosine (**3**) > 8-bromoguanosine (**4**) > inosine (**10**) > 7-methylguanosine (**6**) = 3′-deoxyguanosine (**9**) > 2′-deoxyguanosine (**8**) = guanine (**11**) = 6-thioguanine (**12**) > > N^2-methylguanosine (**5**). The competition studies confirmed that [^{3}H]-guanosine site was distinct from the well characterized ATP and adenosine binding sites. The present results are rationalized in terms of a putative pseudo-receptor construct which includes all the relevant physicochemical interaction between guanosine analogues and their putative binding sites. This construct will be useful for the in silico screening of compound libraries in search for new potent and structurally diverse pharmacological tools.

Introduction

Guanosine (**1**, Chart 1), like its adenine-based counterpart adenosine (**2**), is a primitive molecule which produces a wide spectrum of biological activities.[1,2]

Guanine-based purines, similarly to the adenine-based ones, are released from neurons and/or glia both under basal conditions and after various types of stimulation, including stress conditions.[3–5] Extracellular guanosine has many trophic effects, including the promotion of the division of astrocytes[6–8] and other cell types,[9] the stimulation of the synthesis and release of various trophic factors,[8,10–12] and the promotion of neurite outgrowth from neurons and neuron-like cells.[13,14] Although some of these actions of guanosine (**1**) may be intracellularly mediated after its uptake, the inefficiency of nucleoside uptake inhibitors to affect the trophic actions of guanosine (**1**)[13] seems to rule out this possibility. Moreover, several effects of guanosine (**1**) may be mediated through G-protein dependent signalling pathways involving cyclic nucleotides or MAP kinase pathway.[11,15,16] Recent binding and functional studies have shown the presence of specific binding sites for guanosine in rat brain and primary cultured rat astrocytes, apparently G-protein coupled.[17,18] Indeed, the displacement studies have shown that some commercially available guanine-based purines were weak displacers of [^{3}H]-guanosine; their very low affinity, however, prevents their use as pharmacological tools. In this paper we report experiments showing that guanosine binding sites belong to the class of G-protein coupled receptors involving cAMP as a possible intracellular signalling pathway. Furthermore, in order to gain more insight

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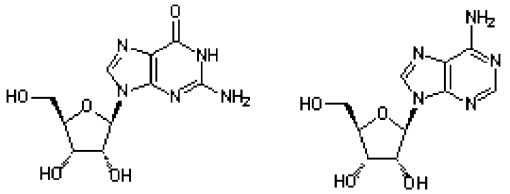


Guanosine (**1**) Adenosine (**2**)

Chart 1.

into the physical–chemical and structural requirements of the putative guanosine binding sites, studies were aimed at a thorough pharmacological characterization of the binding sites and at a detailed interpretation of the thereby obtained data by means of computational approaches.

Results and Discussion

Biology

Effects of guanine nucleotides on [^{3}H]-guanosine binding. GMP and GDP were unable to modify [^{3}H]-guanosine binding, whereas GTP and Gpp[NH]p caused a dose-dependent decrease reaching about 40 and 80% reduction at 100 μM concentration, respectively (Fig. 1).

The saturation isotherm experiments in the absence or in the presence of 30 and 100 μM Gpp[NH]p are shown in Figure 2. The binding, performed in all conditions became saturable at 150–200 nM [^{3}H]-guanosine concentrations. The pooled data were fitted by a computerized non-linear regression analysis and resolved for the presence of a single high affinity binding site. In the absence of Gpp[NH]p the $K_D = 42.42 \pm 4.46$ nM and $B_{max} = 1.13 \pm 0.04$ pmol/mg prot. The binding parameters were significantly changed in the presence of Gpp[NH]p. Gpp[NH]p 30 μM significantly decreased the affinity ($K_D = 74.35 \pm 8.86$ nM) and reduced the B_{max} value (0.85 ± 0.042 pmol/mg prot). Gpp[NH]p 100 μM decreased more the affinity ($K_D = 137.9 \pm 39.47$ nM) without causing a further decrease in the B_{max} value (0.71 ± 0.12 pmol/mg prot).

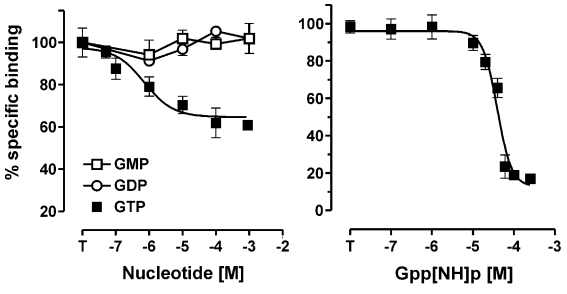


Figure 1. Inhibiting effects of guanosine nucleotides. Rat brain membranes (200 μg) were incubated in standard assay conditions with [^{3}H]-guanosine 50 nM in the presence of different concentrations of the nucleotides. The values are the mean ± SE of 3–4 experiments each dose in triplicate.

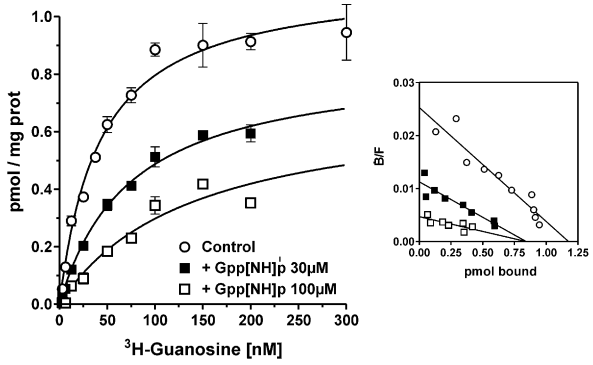


Figure. 2. Effects of Gpp[NH]p on saturation. Rat brain membranes (200 μg) were incubated with increasing concentrations (3.125–300 nM) of [^{3}H]-guanosine under standard assay conditions. The points represent means ± SE of data obtained from 2–3 experiments, each dose in triplicate. The data were fitted by a computerized non-linear regression analysis and resolved with a one site model. The significant differences between saturation curves were evaluated by *F*-test. The curves obtained in the presence of 30 and 100 μM Gpp[NH]p were significantly different from control ($F = 124.4$, $p < 0.0001$ and $F = 199.52$, $p < 0.0001$, respectively). In addition, the curve obtained with 100 μM Gpp[NH]p was significantly different from the one obtained with 30 μM Gpp[NH]p ($F = 94.46$, $p < 0.0001$). Inset: Scatchard analysis of the data.

Effects of PTX pretreatment. The pretreatment of the rat brain membranes with PTX 8.3 μg /mg prot for 30 min at 37 °C induced about 45% decrease in the specific binding of 50 nM [^{3}H]-guanosine. Control and PTX pretreatment values were 0.271 ± 0.019 and 0.148 ± 0.016 pmol/mg prot, respectively (Fig. 3).

Effect of guanosine in cAMP accumulation. Guanosine (**1**) caused a dose-dependent increase in intracellular cAMP accumulation in slices of rat cerebral cortex. The mean basal value was 21.47 ± 5.04 pmol/mg prot, and those of 75, 150 and 300 μM guanosine (**1**) were 23.17 ± 4.2, 38.98 ± 5.5, 57.45 ± 8.9 pmol/mg prot, respectively. The ANOVA analysis for data is significant ($F = 4.991$; $p = 0.0124$) as well as the post test for linear trend ($p = 0.0017$). Since an inter-day variability of basal values appeared, to emphasize the increasing effects of guanosine, in Figure 4b the data are expressed as number of fold increase in cAMP over the basal values of the day. To determine if the observed cAMP increase is co-mediated by the guanosine-induced

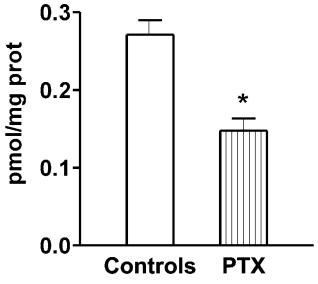


Figure 3. Effects of PTX pretreatment of rat brain membranes on [^{3}H]-guanosine 50 nM specific binding.. The data are the mean ± SE of two experiments in triplicate. *$p < 0.001$, Student's *t*-test.

release of adenosine (**2**),[11] experiments with guanosine (**1**) 300 μM were performed in the presence of adenosine deaminase (ADA). With respect to the condition without ADA, no variations of basal values were observed and the fold number increase of cAMP accumulation induced by guanosine 300 μM were not changed. The specific A_1 and A_2 adenosine receptor antagonists DPCPX (**32**, 30 nM) and DMPX (**34**, 100 μM), used alone or in combination with guanosine (**1**), were unable to modify cAMP increase induced by guanosine 300 μM both in the absence or presence of ADA (Fig. 4b). IBMX, inhibitor of PDEs, reduced guanosine-induced cAMP accumulation (data not shown).

Displacement studies. In order to obtain a better pharmacological characterization of the binding sites and to understand which are the important groups of guanosine (**1**) likely to be involved in the receptor binding, a series of compounds belonging to the groups of guanine- and adenine-based purines were tested. The results are shown in Table 1.

The potency order of the best substances able to displace 50 nM [^{3}H]-guanosine was guanosine (**1**) = 6-thioguanosine (**3**) > 8-bromoguanosine (**4**) > inosine (**10**) > 7-methylguanosine (**6**) = 3′-deoxyguanosine (**9**) > 2′-deoxyguanosine (**8**) = guanine (**11**) = 6-thioguanine (**12**) > > N^2-methylguanosine (**5**). The displacement curves of these compounds were resolved by non-linear regression analysis with a one-site model.

Hypoxanthine (**15**) and xanthine (**16**) were unable to significantly displace [^{3}H]-guanosine from its binding sites, whereas xanthosine displayed a flat displacement curve, reaching about 50% at 1 mM concentration. Also the guanosine derivatives, acyclovir (**13**) and AIT-082 (**14**), did not modify [^{3}H]-guanosine binding. Adenine nucleotides **23**–**25**, adenosine as well as the agonists and antagonists analogues for A_1, A_2 and A_3 adenosine receptors **26**–**34** were unable to displace [^{3}H]-guanosine from its binding sites even at very high concentrations. Neither of the non-specific adenosine receptor antagonists, caffeine (**18**) or theophylline (**19**) displaced [^{3}H]-guanosine from its binding site. Nitrobenzylthioguanosine (NBTG, **20**), nitrobenzyl thioinosine (NTBI, **21**) and propentophylline, which inhibit the nucleoside transporter systems, did not alter [^{3}H]-guanosine binding.

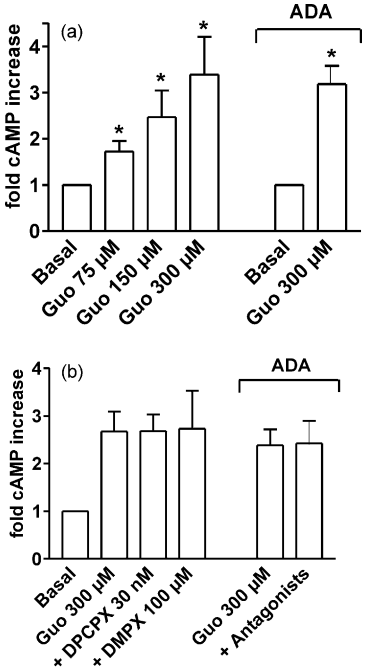


Figure 4. cAMP accumulation induced by guanosine in rat brain cortex slices. (a) Dose effect of guanosine (**1**) (b) effects of A_1/A_2 receptor antagonists on cAMP accumulation elicited by 300 μM guanosine (**1**). The data are expressed as number of fold increase over the basal values of the day and are the mean ± SE of 2–3 experiments in duplicate. *$p < 0.05$, one-sample t-test.

Molecular modeling

The ability of the studied compounds to displace the binding of [^{3}H]-guanosine can be interpreted in terms of interaction energies with a putative binding pocket herein referred to as pseudo-receptor. In order to delineate a possible pharmacophore scheme for the

Table 1. Effects of purines and various agents in displacing 50 nM [^{3}H]-guanosine binding to rat brain membranes

Compounds	K_i, μM (95% C.L.)[a]	c, μM (% inibition)
Guanosine (**1**)	0.031 (0.024–0.04)	
Adenosine (**2**)		1000 (15%)
6-Thioguanosine (**3**)	0.032 (0.024–0.043)	
8-Br-guanosine (**4**)	0.88 (0.64–0.12)	
6-Cl-guanosine (**5**)	253.1 (192.9–333.9)	
7-Methylguanosine (**6**)	166.5 (104.1–266.5)	
N_2-Methylguanosine (**7**)	1360 (514–3608)	
2′-Deoxyguanosine (**8**)	299.2 (223.9–399.7)	
3′-Deoxyguanosine (**9**)	159.4 (126.7–200.5)	
Inosine (**10**)	5.5 (3.72–8.16)	
Guanine (**11**)	219.4 (133.9–359.5)	
6-Thioguanine (**12**)	251.0 (151.2–416.9)	
Acyclovir (**13**)		> 1000 (0%)
AIT-082 (**14**)		> 1000 (10%)
Hypoxantine (**15**)		> 100 (12%)
Xanthine (**16**)		> 1000 (15%)
Xanthosine (**17**)		> 1000 (50%)
Caffeine (**18**)		> 1000 (6%)
Theophylline (**19**)		> 1000 (0%)
NBTG (**20**)		> 10 (0%)
NBTI (**21**)		> 10 (0%)
Propentophylline (**22**)		> 10 (10%)
ATP (**23**)		1000 (11%)
ADP (**24**)		1000 (13%)
AMP (**25**)		1000 (15%)
CPA (**26**)		1000 (25%)
CCPA (**27**)		1000 (4%)
NECA (**28**)		1000 (16%)
CGS21680 (**29**)		1000 (18%)
CPCA (**30**)		1000 (20%)
ABMECA (**31**)		1000 (17%)
DPCPX (**32**)		1000 (9%)
PACPX (**33**)		1000 (0%)
DMPX (**34**)		1000 (4%)

[a]The displacement curves were performed with 9–12 concentrations, each point in triplicate. The data from at least three separate experiments were pooled and resolved by non-linear regression analysis with a one-site model. The K_i values were calculated from competition curves using the equation of Cheng and Prusoff.[19] Values given in parentheses indicate the percent of inhibition when the inhibition is less than 50%.

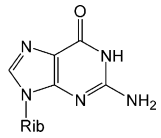
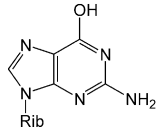
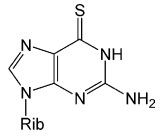
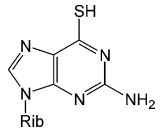
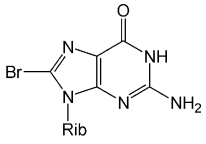
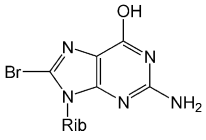
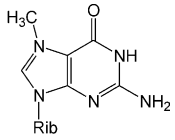
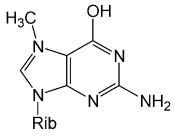
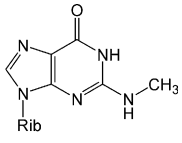
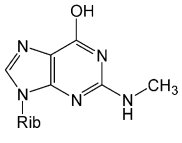
active ligands, a computational approach based on the following points was followed. As a first step, semi-empirical calculations were carried out to identify, where present, the most stable tautomer of studied compounds (**1**, **3**, **4**, **6–12**). Results of the AM-1 and AM1-SM2 hamiltonian are reported in Table 2. A clear preference for the 6-keto tautomer (lactame form) is shown in all purine derivatives with respect to the 6-hydroxy one (lactime form) with the exception of the 6-thioderivatives if calculated in vacuo[20] (AM1). However this difference is less pronounced if the aqueous environment is implicitly considered (AM1-SM2), thus all purines were analyzed in their lactame form.

In order to identify a putative bioactive conformation, a conformational analysis was carried out on all the rotable bonds of the most active compound, guanosine (**1**). All the obtained conformations were energetically minimized and compared to remove duplicates. A total of 13 minima conformations were found in a range of 3.64 kcal/mol from the global minima confomer. Since no experimental observations are available about the bioactive conformation of guanosine (**1**) to our binding site, we made the assumption that the binding pocket is somehow optimized for the most stable conformer of guanosine (**1**) and should contain all the necessary structural elements for a tight binding (pharmacophore points). Accordingly, all active compounds should match the bioactive conformation of guanosine (**1**) on the necessary pharmacophoric points and weaker or inactive compounds are, therefore, those which either do not match all the pharmacopohore points or possess additional, disallowed volumes.

Thus, using a common subgraph match routine, seven pharmacophoric points were identified on guanosine, four hydrogen bond donor atoms and three hydrogen bond acceptor atoms (Fig. 5a). All compound were aligned by fitting these points. To generate a pseudo-receptor model, guanosine (**1**), 6-thioguanosine (**3**) and 8-bromoguanosine (**4**) were used as template molecules. Their contribution for the pseudo-receptor generation was weighted by taking into account their respective activity. Electrostatic, hydrophobic, and hydrogen bond interaction features of the pseudo-receptor model (Fig. 5) were used to calculate the interaction energies of all aligned compounds with the binding pocket (Table 3).

Indeed, the pseudo-receptor model is intended to provide a continuous description of the chemical environment of the binding pocket, based on its shape- and electrostatic complementarity with the most active compounds (**1**, **3**, **4**), which are thought to achive an 'optimal' fit. Projection of the interaction energies over the pseudo-receptor map yields favourable (magenta colored) and unfavourable (green colored) regions which can be considered estimations of the complentarity of the given molecule with the 'true' binding pocket (Fig. 6). Inspection of the interaction energies and their respective projection over the pseudo-receptor map suggests qualitative structure activity relationship.

Table 2. Relative stabilities in vacuo (AM1) and in aqueous solvent (AM1-SM2) of the main tautomeric forms in guanine-derivatives (**1**, **3–4**, **6–12**)

No.	Tautomers	ΔE (kcal/mol) AM1	ΔE (kcal/mol) AM1-SM2
1		0.0	0.0
		4.2	7.0
3		8.0	0.2
		0.0	0.0
4		0.0	0.0
		4.2	7.9
6		0.0	0.0
		4.3	8.8
7		0.0	0.0
		4.5	6.9

(*Continued on next page.*)

Table 2 (*continued*)

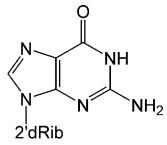
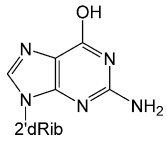
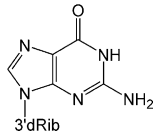
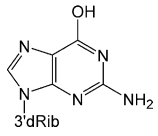
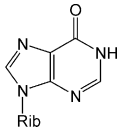
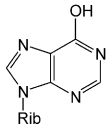
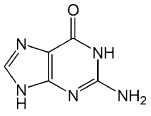
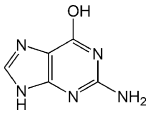
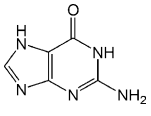
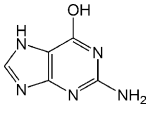
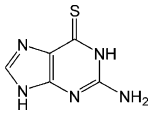
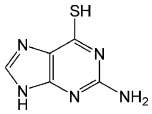
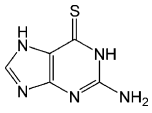
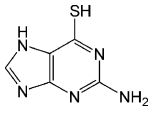
No.	Tautomers	ΔE (kcal/mol) AM1	ΔE (kcal/mol) AM1-SM2
8		0.0	0.0
		4.5	6.5
9		0.0	0.0
		4.4	7.1
10		0.0	0.0
		5.5	9.7
11		0.0	0.0
		2.8	5.7
		1.9	3.1
		6.3	8.2

Table 2 (*continued*)

No.	Tautomers	ΔE (kcal/mol) AM1	ΔE (kcal/mol) AM1-SM2
12		9.1	0.1
		0.0	0.0
		8.9	1.0
		4.3	1.9

Rib = ribose, 2′Rib = 2′deoxyribose, 3′Rib = 3′deoxyribose.

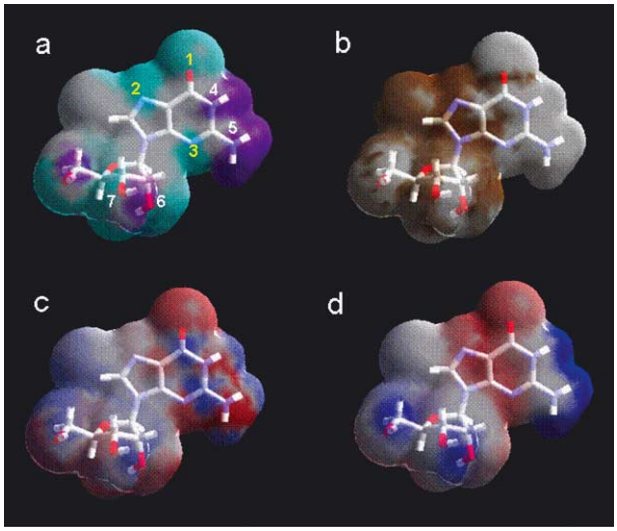


Figure 5. Physicochemical properties of the pseudo-receptor model: (a) pharmacophoric points (1–7) and hydrogen bonding properties (purple = donor; cyan = acceptor); (b) hydrophobic properties (brown); (c) charge properties (red = negative charges; blue = positive charges); (d) electrostatic properties (red = negative elec. potential; blue = positive elec. potential).

Thus, the weak inactivity of some compounds can be interpreted as follows. 6-Chloro-guanosine (**5**) shows an unfavourable interaction energy located around the chlorine moiety (Fig. 6i). Indeed, the non-isosteric substitution of the 6-oxo moiety with the chlorine atom results into the loss of the hydrogen acceptor group in the 6 position and in the change of pK_a of the nitrogen in the 2 position. The explanation of much lower affinity

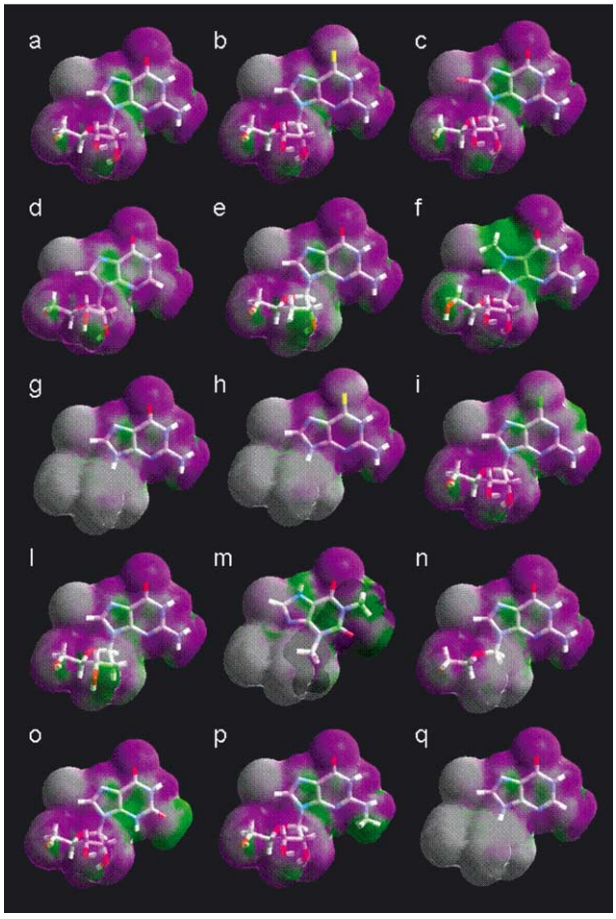


Figure 6. Plot of favorable (purple) and unfavorable (green) interaction energies of compounds of Table 3 and the pseudo-receptor model (a = **1**; b = **3**; c = **4**; d = **10**; e = **9**; f = **6**; g = **11**; h = **12**; i = **5**; l = **8**; m = **19**; n = **13**; o = **17**; p = **7**; q = **15**).

of 7-methylguanosine (**6**) and N^2-methylguanosine (**8**) is connected to their unfavourable steric interactions (Fig. 6f, p) in the areas of the pseudo-receptor surrounding the methyl groups.

Although the inactivity of compounds such as guanine (**11**), thioguanine (**12**), 2′- or 3′-deoxyguanosine and xanthine derivatives can be qualitatively explained on the basis of the lack of any of the four hydrogen bond donating groups (Fig. 6e, g–h, l–o, q), from the inspection of their electrostatic interaction energies this is less evident at least for 3′-deoxyguanosine and xanthosine.

More interesting is the analysis of the activity of inosine (Fig. 6d). Indeed, the inspection of the calculated electrostatic interaction energies (Table 3) reveals that inosine is predicted to be among the less active derivative of guanosine analogues, due to the lack of the amino group in the 2-position. Nevertheless, inosine (**10**) is endowed with an acceptable potency (5.5 μM) thus indicating that the presence of an hydrogen donating group in the 2-position is not strictly required.

Previous studies had suggested that the guanosine binding sites found in membranes from rat brain or primary astrocytes in culture could belong to the class of G-protein coupled receptors.[17,18] It is known that

Table 3. Activity and binding energies of purines with the pseudo-receptor

Compounds	pK_i	Intra-energy[a]	Inter-ELE[b]	Inter-VdW[c]
Guanosine (Guo, **1**)	1.52	48.78	−7.74	−6.68
6-Thio-Guo (**3**)	1.49	43.55	−6.64	−6.87
8-Br-Guanosine (**4**)	0.06	54.84	−7.31	−7.13
Inosine (**10**)	−0.74	32.46	−3.17	−5.95
3-Deoxy-Guo (**9**)	−2.20	43.63	−6.61	−6.28
7-Met-Guo (**6**)	−2.22	113.94	−7.44	−2.58
Guanine (**11**)	−2.34	21.59	−2.58	−3.51
6-Thio-Guanine (**12**)	−2.40	16.88	−1.49	−3.72
6-Cl-Guanosine (**5**)	−2.40	50.55	−5.88	−6.68
2-Deoxy-Guo (**8**)	−2.47	47.69	−3.67	−6.20
Theophylline (**19**)	−3.00	69.81	−1.63	−0.49
Acyclovir (**13**)	−3.00	28.70	−4.64	−5.21
Xanthosine (**17**)	−3.00	49.41	−6.25	−5.88
N-2-Met-Guo (**7**)	−3.13	107.53	−7.16	2.30
Ipoxantine (**15**)	−2.00	19.47	−2.37	−2.89

[a]Intramolecular energy (the molecular strain induced by the pseudo-receptor model).
[b]Electrostatic intermolecular energy.
[c]Intermolecular VdW energy.

GTP and its nonhydrolyzable analogue Gpp[NH]p, are the most effective in inhibiting the ligand binding to receptor coupled to their effectors by GTP-binding protein. Consistent with this action, both GTP and Gpp[NH]p caused an inhibition of [^{3}H]-guanosine binding to rat brain membranes which occurred by decreasing the affinity of the receptor. Furthermore, pertussis toxin (PTX) has been shown to ADP-ribosylate Gi- or Go-proteins resulting in an uncoupling of the receptors from G-protein which, in turn, reduces receptor's affinity for ligand and decreases the efficiency of the second messenger pathways. PTX-pre-treatment of rat brain membranes reduced [^{3}H]-guanosine binding. These data agree with the results obtained using astrocyte membranes. In these preparations [^{3}H]-guanosine binding showed the same kinetic characteristics of those observed on rat brain membranes and the pre-treatment of astrocyte cultures with PTX caused about 3-fold decrease in [^{3}H]-guanosine affinity.[17,18] These evidences suggest that guanosine binding sites are linked to G-proteins, and experiments to evaluate the effects of guanosine on the cAMP intracellular transduction pathway have been performed on rat brain cortex slices to support this hypothesis. In slices from rat brain cortex, guanosine (**1**) induced a dose-dependent increase in intracellular cAMP. This increase is specific for guanosine, since neither the pre-treatment with adenosine deaminase nor the A_1 and A_2 adenosine receptor antagonists were able to modify the guanosine-induced cAMP accumulation. On the contrary, the cAMP guanosine-induced increase in PC12 cells was considered to be indirectly mediated by endogenous extracellular adenosine (**2**)[16] released by guanosine (**1**) itself.[7]

It is not surprising that the activation of a PTX sensible receptor can cause an activation of adenylate cyclase. In fact, several reports suggested that free Gβγ subunit can inhibit or stimulate adenylate cyclase activity as well as activate the mitogen-activated protein kinase (MAPK) pathway.[21–23] It has been previously shown that guanosine (**1**) was able to stimulate the MAPK pathway as well as the activity of adenylate cyclase in primary rat

astrocyte cultures. The phosphorylation of ERK1/2 and the increased cAMP levels were strongly reduced by PTX pre-treatment.[11,18]

The competition curves of guanosine (**1**) and related compounds, at concentrations ranging over several orders of magnitude, demonstrate the specificity of the [^{3}H]-guanosine binding site. The data confirm that this site is distinct from the well characterized ATP and adenosine binding sites. Neither adenine nucleotides, nor adenosine and the agonist and antagonist analogues for A_1, A_2 and A_3 adenosine receptors, nor the non-specific adenosine receptor antagonists, caffeine (**18**) and theophylline (**19**), reduced [^{3}H]-guanosine binding. Moreover, the [^{3}H]-guanosine binding sites are different from other possible cell membrane binding sites, like nucleoside transporter systems, for the following reasons. First, the binding of [^{3}H]-guanosine to the membranes was unaffected by co-incubation with the inhibitors of the nucleoside transporter: NBTI (**20**), NBTG (**21**) and propentophylline (**22**). Second, the inability of other purine nucleosides to displace guanosine rules out the possibility that the binding can be due to a non-specific purine nucleoside transporter. Finally, a mammalian guanosine-specific transporter has been described,[24] but the concentrations of guanosine (**1**) tested were four orders of magnitude higher than those used in the binding studies. Moreover, inosine (**10**) which inhibited the transporter only at a concentration of 1 mM, in our experiments showed a K_i of 5.5 μM.

The relative abilities of analogues to displace [^{3}H]-guanosine provide insight into the characteristics of the binding site which have been analysed by a computational approach. Thus, 6-thio-guanosine (**3**) and guanosine (**1**) were equally effective in displacing [^{3}H]-guanosine, whereas adenosine (**2**) which has an amino group in C6 was ineffective. These data indicate that a 6-keto or 6-thio group is important, and that a 6-amino group greatly reduces the binding affinity. Also the substitution in C6 with an electronegative atom as 6-chloroguanosine (**5**) reduces the affinity of about 8500 fold. Otherwise, the substitution in C8 with bromine conferred to 8-bromoguanosine (**4**) a diplacement potency lower only to that of guanosine (**1**). A reduction in affinity was produced also by the introduction of a methyl group in N7 as in compound **6**. The 2-amino group appears to be important for the binding but not mandatory, since inosine (**10**), which lacks this group, resulted in 200-fold reduction in affinity with respect to the parent derivative **1**, but anyway keeps a reasonable activity. More dramatic is the effect of the mono-substitution of $-NH_2$ (N^2-methylguanosine, **8**) which caused a substantial decrease in affinity. Molecular modelling suggests that this reduction should be ascribed to the occupancy of an extravolume with respect to guanosine. Also the presence of a 2-keto rather than a 2-amino group, as in xanthine derivatives, resulted in a loss of activity. The presence of the ribofuranosyl group in N9 is relevant: the bases, guanine (**10**) and 6-thio-guanine (**11**) were three orders of magnitude less effective than guanosine in displacing [^{3}H]-guanosine. Moreover, the guanine analogue acyclovir (9-(2-hydroxyethoxymethyl) guanine, **13**), in which the ribose is replaced, was ineffective. Also 2′-deoxyguanosine (**8**) and 3′-deoxyguanosine (**9**) were less effective than guanosine. The ribose moiety also appears important in the displacement capacity of xanthine derivatives. In fact only xanthosine (**17**), which maintain the ribose group, was able to displace [^{3}H]-guanosine, although the displacement curve was flat.

Conclusion

In conclusion, the reported data confirm the hypothesis of the existence of specific G-coupled receptors for guanosine (**1**) and, for the first time, allow to delineate a structure–activity relationship for a series of guanosine derivatives. Application of molecular modelling techniques have also allowed to define a pseudo-receptor construct that will be instrumental for the in silico screening of novel, structurally diverse chemical entities to be employed as more potent and selective pharmacological tools.

Experimental

Guanosine [8-^{3}H], specific activity 20 Ci/mmol, was supplied by ICN Pharmaceuticals, Irvine, CA, USA. Cyclic AMP (^{3}H) assay system was purchased from Amersham (UK); All other chemicals used and compounds tested **1**–**13**, **15**–**34**, if not stated, were supplied by Sigma-Aldrich S.r.l. Italy. AIT-082 (**14**) was a generous gift of Dr. Alvin Glasky, NeoTherapeutics Inc, Irvine, CA, USA.

Membrane preparations. Crude rat membranes were prepared under conditions similar to those previously used to identify specific binding of ^{3}H-adenosine.[25,26] Male Wistar rats (200–250 g) were used, the brains without cerebellum were quickly removed and homogenized in 9 vol of ice-cold buffer (0.32 M sucrose, 20 mM HEPES pH 7.4) by motor driven teflon-glass homogenizer at 250–350 rpm., 7 strokes up and down. The homogenate was centrifuged at 1000×*g* for 10 min at 4 °C. The pellets were resuspended in 10 mL of ice-cold buffer and centrifuged as above; then the supernatants were pooled and centrifuged at 10,000×*g* for 20 min at 4 °C. The pellet was resuspended in about half the volume used in the first resuspension of ice-cold 20 mM HEPES pH 7.4 and centrifuged at 100,000×*g* for 30 min at 4 °C. The final pellet was resuspended in the same buffer at a final protein concentration of 3–5 mg per mL. Aliquots were stored at −80 °C. Membranes before being used in binding experiments were washed twice with PBS buffer 8 mM, pH 7.4. The protein concentration was measured by the Lowry et al. method[27]

Binding assay. The measurement of [^{3}H]-guanosine binding in rat brain membranes was performed by the centrifugation technique. Briefly, aliquots of 200 μg of proteins were incubated with [^{3}H]-guanosine (20 Ci/mmol) in 0.5 mL PBS buffer 8 mM pH 7.4, for 10 min at 25 °C in 1.5 mL Eppendorf Safe-Lock tubes. 1 mM

cold guanosine was used to detect non specific binding. The reaction was stopped by sedimentation (21,900×*g* at 4 °C for 10 min) and bound radioligand was recovered after removal of supernatant with three gentle consecutive washings in ice-cold buffer. The pellets were solubilized with 50 μL Soluene-350. After adding 1.2 mL of Hionic-Fluor scintillation cocktail, the Eppendorf tubes were inserted in polyethylene vials to be counted by a liquid scintillation counter (Tri-Card 2100TR, Packard, Meridien, CT, USA). For saturation experiments a concentration range (3.125–200 nM) of [^{3}H]-guanosine was used under standard assay conditions. In competition experiments displacing agents and 50 nM [^{3}H]-guanosine were added under standard assay conditions and the reaction was started by adding the membranes.

Pertussis toxin pre-treatment. PTX pre-treatment was performed according the conditions used by Monahan et al.[28] incubating for 30 min at 37 °C rat brain membrane preparations in a buffer containing Tris 50 mM, NAD 1 mM, EDTA 1 mM, DTT 5 mM, ATP 1 mM, tymidine 10 mM, $MgCl_2$ 2.5 mM pH 7.4 and PTX 8.3 μg/g prot. PTX was removed by two consecutives washings with PBS buffer 8 mM pH 7.4 and then binding assays were performed as previously described.

Preparation of rat cortex slices. Cortex slices were obtained from adult male Wistar rats (180–200 g). The brains, immediately removed on ice, were transferred to Krebs buffer. Cortex slices (400 μm) were pre-incubated for 5 min in Krebs buffer at 20 °C, under an atmosphere of 95% O_2 and 5% CO_2 at pH 7.4 to wash the tissue.

Radioassay of camp. Cortex slices were incubated for 15 min with fresh medium containing different treatments. The reaction was ended by transferring each slice in $HClO_4$ 0.4 M, kept in an ice/bath; the samples were homogenized at 0 °C and centrifuged at 4500 rpm for 15 min at 0 °C. The supernatants were collected and after neutralization with NaOH, the samples were centrifuged at 7000 rpm for 2 min at 0 °C to remove insoluble salts. The cellular cyclic AMP was quantified by a radioassay kit.

Computational approach. All compounds were constructed starting from fragment dictionary and geometry optimized using the Universal force-field v.1.2 and the Smart Minimizer protocol of OFF.[29] Atomic charges and tautomers were computed using the semi-empirical AM1 method[30] with a convergence criteria below 0.5×10^{-3} kcal/mol and implicit treatment of water (AM1-SM2).[31] Conformational analysis was carried out using the Grid Search routine with an increment value for the torsional angles of 30° in a range from −180 to 150°. The pseudo-receptor model was generated using the Receptor approach.[32] Briefly, the pseudo-receptor model represents the essential features of the binding site by assuming complementarity between the shape and properties of the binding pocket of the putative receptor and the set of purine derivatives. 3-D surface of the binding site encloses the most active members (after appropriate alignment) of the starting set of compounds and represent the pseudo-receptor. The surface is generated from a 'Shape Field'. The atomic coordinates of the contributing molecules are used to compute field values on each point of a 3-D grid using a van der Waals function (1).

$$V(r) = r - \mathrm{VdWr} \tag{1}$$

where r is the distance from the atomic coordinate to the grid point (called *surface fit* and set here to 0.1) and VdWr is the van der Waals radius of the atom.

The contribution of compounds for surface generation was weighted taking into account their respective activity (Table 1). The interaction energies were calculated for all aligned molecules inside the pseudo-receptor. A solvation correction term and the electrostatic charge complementarity method were used for energy evaluation. The solvation energy correction term is a penalty function that attempts to account the loss of solvation when polar atoms are forced into hydrophobic regions of the receptor surface.

All the computations have been carried out by using the Cerius2 software,[33] distributed by Accelyrs Inc, and MacSpartan v.1.2,[34] distributed by Wavefunction Inc., running on a O2 R5000 SGI workstation and a MacIntosh PowerPC, respectively.

Statistical analysis. All the statistical analysis and the evaluation of binding data were performed using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA, USA). The significant differences between saturation curves performed in the presence or absence of Gpp[NH]p were evaluated by *F*-test, which compare the fit of one equation to different sets of data. The following formula was used:

$$F = \frac{\left(SS_{\mathrm{combined}} - SS_{\mathrm{separate}}\right)/\left(DF_{\mathrm{combined}} - DF_{\mathrm{separate}}\right)}{SS_{\mathrm{separate}}/DF_{\mathrm{separate}}}$$

SS_{separate} is the total sum of squares obtained from two data sets fit separately. DF_{separate} is the sum of DF of two fits. SS_{combined} is sum-of-squares obtained from the fit of the two data sets combined into one. DF_{combined} is degree of freedom (number of data points minus three variables).

The statistical analysis of cAMP data, expressed as pmol/mg prot, was performed using ANOVA followed by post test for linear trend, and that of cAMP data, expressed as number of fold increase over the basal values of the day, were performed using a one-sample *t*-test.

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