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A NOTE OF CAUTION IN THE USE OF RECEPTOR BINDING ASSAYS TO SCREEN MARINE ORGANISMS: THE ACTION OF HALISTANOL TRISULPHATE ON ADENOSINE RECEPTORS.

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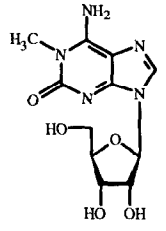
Abstract: The adenosine A_1 receptor binding assay was used to screen marine extracts. Following the isolation of halistanol trisulphate, non-specific interference causing reduction in affinity and the number of binding sites of the radioligand was identified to be associated with this detergent. Methods for detection of non-specific receptor interactions and optimization of the assays for natural product screening are discussed.

We had, some years ago, isolated an adenosine receptor agonist from the marine sponge *Tedania digitata*.[1,2] The aqueous ethanolic extract of *T. digitata* displayed muscle relaxant properties in mice and hypotensive and anti-inflammatory activities in rats. The pharmacologically active constituent, 1-methylisoguanosine (**1**), was shown to be an adenosine receptor agonist.[3] The adenosine radioligand binding assay has been reported to be used to guide isolation of 5-(3-hydroxypropyl)-7-methoxy-2-(3'-methoxy-4'-hydroxyphenyl)-3-benzo[*b*]furan-carbaldehyde (**2**), which has an IC_{50} of 17 nM at the adenosine A_1 receptor, from the roots of *Salvia miltiorrhiza* Bunge. *S. miltiorrhiza* was investigated because of Chinese use to treat myocardial infarction and angina pectoris.[4] Recently we have been exploring the mechanism-based isolation of adenosine agonists and antagonists using radioligand binding assays.[5] We have identified non-specific effects which cause a reduction in radioligand affinity in many of the extracts. This information will be of general value in the application of receptor based screening methods to the evaluation of natural product source material.



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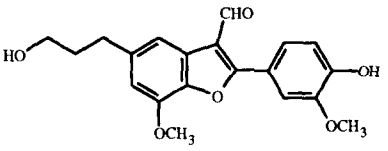

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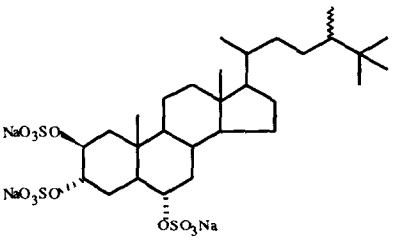

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Marine organisms were freeze-dried and blended with distilled water, centrifuged (26890g, 20 min) and both the extract and pellet lyophilised. Pellets were extracted with 1:1 dichloromethane/methanol. Approximately 20% of the aqueous extracts at 2.5 mg/mL inhibited the specific binding of [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine to rat brain synaptosomal membranes by at least 60%.[6,7] These active extracts had apparent IC_{50} values ranging from 0.041 to 1.07 mg/mL. In order to validate the use of the assay for detecting

adenosine receptor ligands, 1-methylisoguanosine and caffeine were added to one of the inactive extracts. The IC_{50} values determined in the presence of 2.5 mg/mL crude extract were not significantly different to the IC_{50} values of the pure compounds. The organic extracts were less active than the aqueous extracts (apparent IC_{50} values from 0.25 to 1.61 mg/mL).

Chromatography of one of the active aqueous extracts from a sponge collected on the Great Barrier Reef, Australia[8] on Merck Fractogel™ TSK HW-40S in water gave the known compound halistanol trisulphate (**3**)[9] (9.8 % of the aqueous crude extract, 4 % of the sponge dry weight) eluting between 7.8 and 21.3 void volumes. The concentration-inhibition curve of **3** was steep (Hill slope factor >2) and parallel to that of the crude extract (Figure 1). The apparent IC_{50} of the crude extract was 0.26 mg/mL while **3** had an apparent IC_{50} of 0.082 mg/mL or 120 μM (Figures 1 and 2).

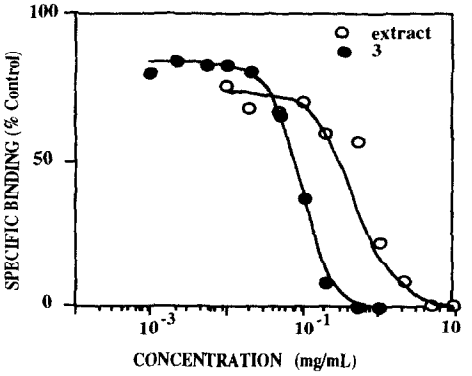

Figure 1. Concentration-inhibition curves of [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine binding by **3** and the crude aqueous extract, n = 3.

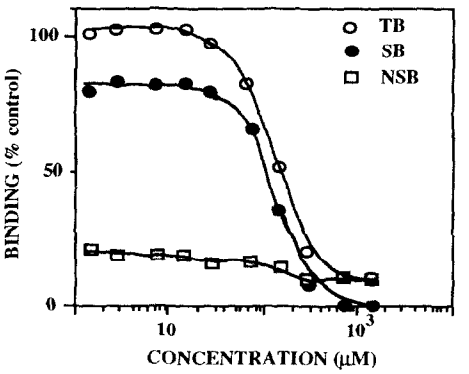

Figure 2. Decrease in total (TB), specific (SB) and nonspecific (NSB) binding of [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine by **3**, n = 3.

With increasing concentrations, halistanol trisulphate caused a decrease in affinity as measured by K_d, the steady state dissociation constant, and the number of binding sites of the radioligand as measured by B_{max}, the density of receptors. K_d (nM) and B_{max} (fmol/mg) were respectively 1.42 and 370 for the **control**, 1.39 and 346 for **3** at 14.5 μM and 10.62 and 319 for **3** at 145 μM (Figure 3). This effect, typical of noncompetitive ligands, was observed even when the glass fibre filters were treated with 0.03 %v/v polyethylenimine, routinely used to filter solubilised receptors.[10] Therefore, reduction of radioligand binding could not be solely due to solubilisation loss of synaptosomes in the assay filtrate.

Halistanol trisulphate might decouple the G_i coupling protein from the A_1 receptor, converting A_1 receptors to a conformation with lower affinity for agonists and therefore reducing specific binding. Antagonist binding is increased when the A_1 receptor is decoupled.[11] Both specific and nonspecific binding of [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine decreased with increasing concentrations of halistanol trisulphate (IC_{50} of 67 μM; Hill slope factor of 1.24, Figure 4) indicating no involvement of the G_i protein in halistanol trisulphate-mediated reduction in ligand binding.[12] Furthermore, halistanol trisulphate decreased [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine specific binding of digitonin-solubilised A_1 receptors in the presence of protease inhibitors (IC_{50} of 145 μM).[13] This suggested a direct, noncompetitive action of halistanol trisulphate on the A_1 receptor.

Adenosine deaminase is required in the binding assays to remove endogenous adenosine. Halistanol trisulphate might inhibit this enzyme and therefore permit high levels of adenosine to compete with the

radioligands. However, halistanol trisulphate caused an activation of adenosine deaminase. At 37 °C, a 90 minute incubation of adenosine deaminase with halistanol trisulphate at 0.1 mg/mL enhanced the V_0 by over two-fold (control, 111 nmol/min/μg; **3**, 237.55 nmol/min/μg).[14]

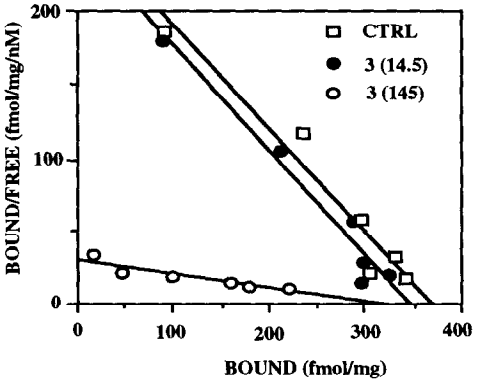

Figure 3. Scatchard plots for [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine binding with **3** at 14.5 μM and 145 μM compared with the control (CTRL), n = 3.

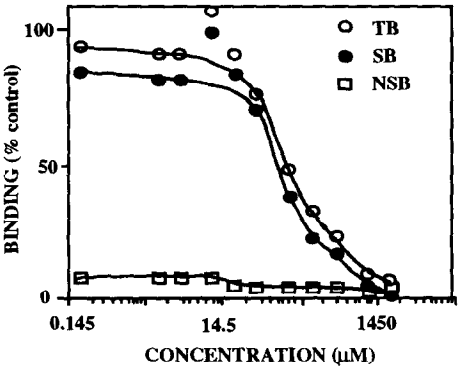

Figure 4. Decrease in total (TB), specific (SB) and nonspecific (NSB) binding of [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine binding by **3**, n = 3.

The potent hemolytic properties of halistanol trisulphate have been previously reported.[8,9] Log-logit transformation gave the EC_{50} for halistanol trisulphate-induced lysis of human erythrocytes as 0.0046 mg/mL, while the EC_{50} of the crude extract was 0.042 mg/mL. Halistanol trisulphate accounts for all the hemolytic activity observed in the aqueous extract of the sponge. The hemolytic activity of halistanol trisulphate results in the destruction of cell membranes at low concentrations.

Halistanol trisulphate is typical of a number of detergents that occur in crude extracts. Extracts with high activity in receptor bindng assays can be subsequently screened for hemolytic activity. In this study, half of the aqueous extracts with activity on the adenosine A_1 binding assay strongly lysed red cells at 2.5 mg/mL. We believe that, with the addition of a hemolytic assay, the adenosine A_1 assay has now been developed to the stage where it can be used as a mechanism-based screen of crude extracts. This approach may serve to identify *bona fide* isosteric ligands from within the vast chemical inventory of crude extracts and to direct attention towards mechanism-based natural product screening in order to identify natural products which mediate biological effects.

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References and Notes

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5. for a description of approaches based on isolated tissue and whole animal pharmacology see:- Quinn, R.J. *Bioorganic Marine Chemistry*, Scheuer, P.J., Ed.; Springer-Verlag: 1988; Vol 2, pp. 1-41.
6. *Preparation of synaptosomal membranes* followed the method of Gray, E. G.; Whittaker V. P. *J. Anat.* **1962**, *96*, 79. Whole brains from Wistar rats (300-350 g) were homogenized in 8 volumes of ice-cold 0.32 M sucrose (Potter-Elvehjem; clearance 0.15 mm; 840 rpm). Homogenates were centrifuged (1000g for 10 min at 4 °C) and the supernatant (S_1) recentrifuged (14500g for 20 min at 4 °C). The crude

mitochondrial fraction (P_2) was resuspended and washed three times in Tris citrate buffer (50 mM, pH 7.1). Aliquots were ultracentrifuged (48000g for 20 min at 4 °C), the pellet resuspended in 8 volumes of ice-cold distilled water and recentrifuged. Pellets were pooled and resuspended in ice-cold incubation buffer (50mM TrisHCl, 1mM $MgCl_2$, pH 7.4, 4 °C). When stored at -30 °C, binding to synaptosomal membranes remained unchanged for several months. The protein concentration was estimated using BSA as a reference and followed the method in Peterson G. L. *Anal. Biochem.* **1977**, *83*, 346.

7. *[^{3}H]-(R)-N^6-(phenylisopropyl)adenosine binding assay* followed the method of Schwabe, U.; Trost, T. *Naunyn Schmiedeberg's Arch. Pharmacol.* **1980**, *313*, 179. Aliquots of synaptosomal membrane were preincubated with adenosine deaminase (1 μg/mL for 30 min at 37 °C) to remove endogenous adenosine. Between 0.3-0.5 mg of synaptosomal protein per replicate was incubated with 5 nM [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine (15 Ci/mmol, New England Nuclear) for 1 h at 37 °C in an incubation volume of 1 mL. Total binding of the radioligand was measured with halistanol trisulphate over a range of concentrations. Nonspecific binding vials also contained unlabelled N^6-cyclopentyladenosine at 10 μM. Reactions were terminated by the addition of 4 mL of ice-cold incubation buffer, filtered over GF/B Whatman filters with two 4 mL buffer washes. Filters were transferred to vials and thoroughly shaken with 4 mL of scintillation fluid. Samples were equilibrated in the dark for a minimum of 6 h. Each vial was counted for 5 min in a Packard tricarb 2000CA analyser at 40% efficiency. Competitive displacement experiments were performed in triplicate. Estimates of IC_{50} and n_H values were obtained from log-logit transforms. Binding was expressed in femtomoles of [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine bound per milligram of total synaptosomal protein (fmol/mg). The standard error of the mean for specific binding (SEM $_{SB}$) was obtained from the expression : $SEM_{SB} = [(SEM_{TB})^2 + (SEM_{NSB})^2]^{0.5}$ with SEM_{TB} and SEM_{NSB} being the standard error of the mean for total binding and nonspecific binding, respectively.

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11. Green, R. D. *J. Neurosci.* **1984**, *4*, 2472.
12. *[^{3}H]-8-cyclopentyl-1,3-dipropylxanthine binding assay* followed the method of Lohse, M. J.; Klotz, K. N.; Lindenborn-Fotinos, J.; Reddington, M.; Schwabe, U.; Olsson, R. A. *Naunyn Schmiedeberg's Arch. Pharmacol.* **1987**, *336*, 204. The assay was performed as in reference 7 using 0.4 nM [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine (109.2 Ci/mmol, New England Nuclear) for 2 h at 25 °C, in an incubation volume of 1 mL.
13. *Solubilised receptor assay.* Synaptosomal protein (4.5 mg) was mixed with detergents to a final volume of 1 mL, and kept on ice for 30 min with gentle inversion every 5 min. The final ratio of detergent to total synaptosomal protein was 2 to 1, by mass. The mixtures were then centrifuged at 105000g for 60 min at 4 °C. For binding studies, 430 μl of supernatant were deaminated, then adjusted to 450 μL total volume with 10 nM [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine. After 2 h at 25 °C, mixtures were filtered over GF/B filters pretreated with 0.3 % PEI, using three 2 mL cold buffer washes. Experiments were conducted using buffer with and without EGTA (1 mM), EDTA (1 mM), DTT (0.1 mM) and soybean trypsin inhibitor (10 μg/mL).
14. *Adenosine deaminase assay* followed the method of Kalckar, H. M. *J. Biol. Chem.* **1947**, *167*, 461. The hydrolysis of adenosine to inosine was monitored kinetically by reading the absorbance at 265 nm. Test solutions were buffered in 50 mM Tris HCl, 1mM $MgCl_2$, pH 7.4 at 37 °C. A standard curve for adenosine from 1 to 100 μM was constructed. Using a 50 μM adenosine solution (approximate K_m), the initial rate of hydrolysis (V_o) was measured for adenosine deaminase concentrations ranging from 10 to 1000 ng/mL. Aliquots of adenosine deaminase (final concentration of 200 ng/mL) were pipetted into a quartz cuvette and reactions initiated by the addition of 990 μl of substrate to a final volume of 1 mL. The V_o values were recorded in triplicate for seven concentrations of adenosine (15 to 80 μM) with and without **3** at a final concentration of 0.1 mg/mL. Additionally, in order to mimic the conditions of the binding assays using [^{3}H]-(R)-N^6-(phenylisopropyl)adenosine, a buffer control and **3** at 0.1 mg/mL, were incubated with adenosine deaminase for 90 min at 37 °C . The initial rate of hydrolysis was then measured by the addition of 50 μM adenosine.