Synthesis and pharmacological characterization of $[^{125}I] \text{MRS1898}$, a high-affinity, selective radioligand for the rat A$_3$ adenosine receptor

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Abstract A known selective agonist of the A$_3$ adenosine receptors (AR), MRS1898 [(1′R,2′R,3′S,4′R,5′S)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol], was synthesized in radioactive form and characterized pharmacologically. This agonist ligand series, based on nucleoside analogues containing a rigid, bicyclic ring system in place of the ribose moiety, was selected for radiolabeling due to its high A$_3$AR affinity across species, with nanomolar binding at both rat and human A$_3$ARs. The radioiodination of MRS1898 on its $N^6$–3-iodobenzyl substituent was accomplished in 76% radiochemical yield by iododestannylation of a 3-(trimethylstannyl)benzyl precursor. $[^{125}I]$MRS1898 bound to the rat A$_3$AR with a $K_d$ value of 0.17±0.04 nM and a $B_{\text{max}}$ value of 0.66±0.15 pmol/mg protein. The competition binding profiles for other agonists and antagonists obtained with this radioligand are similar to those previously obtained with other radioligands. The advantages of $[^{125}I]$MRS1898 compared with previously used radioligands are primarily its high selectivity and affinity for the rat A$_3$AR and also its facile synthesis and radiochemical stability; however, a relatively high level of nonspecific binding presents a limitation. Thus, we have introduced the first selective radioligand for the rat A$_3$AR.

Keywords Iodination · G protein-coupled receptor · Binding assay · Purine · Nucleoside · Carbocyclic

Abbreviations

- AR: adenosine receptor
- CHO: Chinese hamster ovary
- IB-MECA: $N^6$-(3-iodobenzyl)-5′-$N$-methylcarboxamidoadenosine
- I-AB-MECA: $N^6$-(4-amino-3-iodobenzyl)-5′-$N$-methylcarboxamidoadenosine
- MRS1191: 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethyl)-3,5-pyridinedicarboxylic acid, 3-ethyl-5-(phenylmethyl) ester
- MRS1220: $N$-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-yl]benzeneacetamide
- MRS1523: 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
- MRS1898: $\text{MRS1898}$, (1′R,2′R,3′S,4′R,5′S)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol
- NECA: 5′-$N$-ethylcarboxamidoadenosine
- CI-IB-MECA: 2-chloro-5′-(3-iodobenzyl)-5′-$N$-methylcarboxamidoadenosine
- MRE 3008F20: 5′-[[4-(methoxyphenyl)amino]carbonyl]amino-8-propyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine

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HEMADO 2-hexyn-1-yl-\(N^6\)-methyladenosine

**Introduction**

Adenosine regulates many physiological functions through specific cell membrane receptors. The \(A_3\) adenosine receptor (\(A_3\)AR) plays an important role in brain ischemia, immunosuppression, and bronchospasm in several animal models [1]. \(A_3\)AR agonists, such as Cl-IB-MECA (2, Chart 1), are currently in clinical trials for various metastatic and inflammatory conditions [2–5]. The \(A_3\)AR is elevated in tumors, and its expression level correlates to tumor responsiveness to therapy, such as the \(A_3\)AR agonist \(N^6\)-(3-iodobenzyl)-5\(^\prime\)-\(N\)-methylcarboxamido-adenosine (IB-MECA 1) [6, 7].

A number of radioligands have been used for the study of the \(A_3\)AR [8–12]. Radioligands for the \(A_3\)AR may be promising diagnostic markers for cancer and possibly other diseases, whereas characterization and quantification of the \(A_3\)AR in vivo in patients may also be a useful tool in both research and therapeutic studies [13]. The nucleoside \(N^6\)-(4-amino-3-iodophenylethyl)-adenosine ([\(^{125}\)I]I-APNEA) showed reasonable affinity for the \(rA_3\)AR (\(K_d=15 \text{ nM}\)), it is even more potent for the \(rA_1\)AR (\(K_d=1.32 \text{ nM}\)) [8,21]; \([^{125}\text{I}]\text{-AB-MECA } 3\) has a similar affinity for \(rA_1\) and \(rA_3\)ARs (\(A_3=1.48 \text{ nM}; A_1=3.42 \text{ nM}\)). 5\(^\prime\)-\(N\)-ethylcarboxamido-adenosine ([\(^\text{H}\)]NECA), showed a reasonable binding affinity at the \(hA_3\)AR (\(K_d=6 \text{ nM}\)) but is nonselective and weak at the \(rA_3\)AR [11]. Recently, a new \(A_3\)AR agonist radioligand, \([^{\text{H}}]\text{HEMADO } 6\), which contains an \(N^6\)-methyl group, showed high affinity (\(K_d=1.1 \text{ nM}\) at the \(hA_3\)AR), selectivity, and low nonspecific

![Chart 1 Structures of nucleoside and nonnucleoside, high-affinity ligands for the \(A_3\) adenosine receptor. Compounds 3–6 were previously prepared in radioactive form for use in receptor labeling and characterization](image-url)
binding. However, it has no effect on the rA3AR [12], which is consistent with previous findings that Nβ-methylsubstituted adenosine derivatives are potent at the hA3AR but not rA3AR [14, 15]. For example, the parent nucleoside N6-methyladenosine has Kᵢ values of 6390 nM at the rA3AR and 9.3 nM at the hA3AR.

Thus, the challenge remains to develop a subtype-selective radioligand for the A3AR in rat and other non-primate species. A new nucleoside derivative, (1'R,2'R,3'S,4'R,5'S)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol) (MRS1898 7), containing the (N)-methanocarba(bicyclo[3.1.0]hexane) ring system as a ribose substitute, displays high potency and selectivity for the hA3AR compared with other A3AR agonists, such as IB-MECA 1 [16–18]. This bicyclic ring system maintains a conformation that is preferred at the A3AR and thus tends to increase selectivity for the hA3AR. MRS1898 7 has previously been shown to bind with high affinity at the rA3AR. Its affinity profile at three AR subtypes is as follows: rA1=83.9 ± 10.3 nM, hA1=136 ± 22 nM; rA2=1660 ± 260 nM, hA2=784 ± 97 nM; rA3=1.1 ± 0.1 nM, hA3=1.51 ± 0.23 nM. In this study, we synthesized a radioiodinated form of this adenosine derivative for preliminary in vitro studies and characterized its binding properties at the rA3AR.

Materials and methods

General All chemical synthetic reagents and pharmacological agents were purchased from Sigma-Aldrich Chemical Company, except where noted. Sodium [125I]iodide (17.4 Ci/mg) in sodium hydroxide (NaOH) (1.0x10⁻⁵ M) was supplied by Perkin–Elmer Life and Analytical Science. Iodogen iodination reagent was purchased from Pierce Biotechnology. High-performance liquid chromatography (HPLC) was performed using an Agilent 1200 Series LC-MS system or a Beckman Gold HPLC system equipped with a Model 126 programmable solvent module, a Model 168 variable wavelength detector, a β–Ram Model 4 radioisotope detector, and Beckman System Gold remote interface module SS420X, using 32 Karat® software. These analyses were performed on Agilent Eclipse XDB-C18 (3.5 μm, 3.0 x 75 mm) and XDB-C18 (5 μm, 4.6 x 250 mm) columns.

Preparation of [125I]MRS1898 [(1′R,2′R,3′Σ,4′R,5′Σ)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol] (10)

MRS1898 (0.018 g, 0.033 mmol), PdCl₂(PPh₃)₂ (5 mg), and hexamethyltin (0.032 g, 0.1 mmol) were mixed together in anhydrous dioxane (3 ml), and the resulting reaction mixture was stirred at 70 °C for 2 h. The mixture was concentrated under reduced pressure. The product was purified by preparative thin layer chromatography by using chloroform (CHCl₃): MeOH as the eluant to afford the stannyl derivative 1 (0.008 g, 45%) as an oil. High-resolution mass spectrometry (HRMS) (M+ 1)⁺ : calculated 593.1090, found 593.1099. HPLC: Rₜ=21.95 min. HPLC system: 5 mM TBAP/CH₃CN from 80/20 to 60/40 in 25 min, then isocratic for 2 min; flow rate of 1 ml/min.

Regeneration of MRS1898 The trimethylstannyl intermediate 10 (0.1 mg) was reconverted to MRS1898 upon dissolving in MeOH (0.1 ml) followed by treatment with I₂ (0.1 M in MeOH, 0.1 ml) for 10 min at room temperature (Scheme 1). The structure was confirmed by HPLC and HRMS. HRMS (M+ 1)⁺ : calculated 555.0408, found 555.0408. HPLC: Rₜ=15.91 min (same system as above).
In a separate experiment that was more predictive of the subsequent radioiodination conditions, the trimethylstannyl precursor 10 (0.2 mg, 0.34 μmol) was dissolved in 50 ul of methanol in an Eppendorf vial, and to this solution was added an iodine–methanol solution (100 ul of 8 mg/ml). The vial contents were shaken for 30 min and the crude product analyzed on an Agilent 1200 Series Liquid-Chromatography Mass Spectrometry (LC-MS). MRS1898 eluted at 7.6 min with a molecular weight of 555.1 that showed [M + H]+.

Radiolabeling of MRS1898: method 1 The radioiodination of MRS1898 was performed using tert-butyl hydroperoxide (TBHP) as an oxidant (Scheme 2) [19]. Next, 15 ul of TBHP (10% solution in chloroform) was added into a v-vial. To this solution were added an acetic acid solution (4 ul, 3% in chloroform) and a sodium [125I]iodide solution (4 ul, 14.8 MBq, 3.7 GBq/ml), followed by the trimethylstannyl precursor 10 (50 ul, 0.2 mg in chloroform). The reaction mixture was mixed under vortex for 30 min at room temperature. The final labeled product was separated using a Beckman System Gold HPLC equipped with an Agilent Eclipse XDB-C18 column (5.0 μm, 4.6×250 mm) under the following conditions: solvent A was 0.1% trifluoroacetic acid (TFA) in water; solvent B was 0.1% TFA in acetonitrile. A linear gradient of solvent B from 20% to 45% over 12 min was used at a flow rate of 1 ml/min; [125I]MRS1898 eluted at 9.0 min. The [125I]MRS1898 was stored in solution (ethanol: water=9:1, v/v) containing 1% ascorbic acid for inhibition of radiolytic processes.

Pharmacology

The binding experiments were done as previously described [8, 14] on Chinese hamster ovary (CHO) cell membranes expressing the recombinant rA3AR and on membranes from rat-brain cortex, mainly expressing the A1AR; and rat striatum, expressing the A2AAR endogenously. In brief, the saturation, displacement and kinetic experiments were performed using membrane preparations from CHO cells expressing rA3AR in a total assay volume of 100 μl, including 25 μl of radioligand, 50 μl membranes, and 25 μl of test compounds or other ingredients. Nonspecific binding was determined in the presence of 10 μM IB-MECA. The mixtures were incubated at 25°C for 60 min, followed by filtration with a 24-well Brandell MT-24 harvester. Radioactivity was determined in a Beckman 5500B γ-counter. For the dissociation experiment, the mixture was first incubated for 60 min, 10 μM IB-MECA was added, and reaction was terminated at various time points, as indicated. Binding parameters were calculated using Prism 4.0 software (GraphPAD, San Diego, CA, USA). IC50 values obtained from competition curves were converted to Ki values using the Cheng-Prusoff equation [20].
expressed as mean ± standard error. cLogP values were calculated using ChemDraw Ultra (Version 11.0).

Results

Chemistry

It was hoped that a new radioligand would permit better analysis of tissue distribution of the A3AR. As MRS1898 already contains an iodine atom that is associated with high receptor affinity, that position was selected for convenient radiolabeling. A common method for rapidly introducing radioactive iodine on an aromatic ring is to use a stannyl precursor. The feasibility of this route was demonstrated through a “cold” iodination reaction (Scheme 1). The trimethylstannyl precursor 10 was generated in one step from MRS1898 using a palladium reagent and hexamethylditin. Protection of the hydroxyl groups or the exocyclic amine of this adenosine analogue was not necessary. Compound 10 was stable upon storage at –8°C for several months. This intermediate 10 rapidly reverted to MRS1898 upon treatment with iodine. The radioiodination of MRS1898 was accomplished through a similar iododestannylation of 10 using sources of $^{125}$I and by two different radioiodination methods (Scheme 2). The tertiary-butyl hydroperoxide (TBHP) method [19] provided a superior yield in comparison with iodogen. The stability of $[^{125}\text{I}]$MRS1898 solution (ethanol:water=9:1, v/v) containing 1% ascorbic acid over 1 month when stored at –20°C was reliably, as reported in Table 1.

Pharmacology

The new radioligand was examined in standard radioreceptor binding experiments. The rA3AR was expressed heterologously in CHO cells [21], from which membranes were prepared for binding experiments. Initially, the nonspecific binding of $[^{125}\text{I}]$MRS1898 to CHO cell membranes expressing the rA3AR was extremely high, almost inseparable from its total binding. After soaking the glass fiber filters with polyethyleneimine (0.1%), the ratio of specific to nonspecific binding (at a concentration of 0.1 nM radioligand) was improved (3–4:1) and acceptable

![Fig. 1 Saturation of binding of $[^{125}\text{I}]$MRS1898 to CHO cell membranes expressing rat A3 adenosine receptor (AR). Experiments were performed using membrane preparations (20 μg protein) from Chinese hamster ovary (CHO) cells expressing rat A3AR in a total assay volume of 100 μl, including 25 μl of radioligand, 50 μl membranes, and 25 μl buffer (total binding) or 25 μl of 10 μM N$^{6}$-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine (IB-MECA) (nonspecific binding). The mixtures were incubated at 25°C for 60 min, followed by filtration with a 24-well harvester](image1)

![Fig. 2 Competition for binding of $[^{125}\text{I}]$MRS1898 (0.1 nM) at the rat A3 adenosine receptor (AR) by A3AR agonists and antagonists. The y-axis shows radioactivity counts corresponding to total binding. The Ki values (nM ± standard error of the mean) were: 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl-5-(phenylmethyl) ester (MRS1191) (1,850 ± 386), N-[9-chloro-2-(2-furanyl)[1,2,4]triazolo-[1,5-c]quinazolin-5-yl]benzeneacetamide (MRS1220) (>10,000), 5-propyl-2-ethyl-4-propyl-3-(ethylaminocarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523) (518 ± 236), MRS1898 (7.8 ± 2.5), N$^{6}$-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine (IB-MECA) (12.9 ± 3.7) and 5'-N-ethylcarboxamidoadenosine (NECA) (872 ± 251)](image2)

Table 1 The radiochemical purities of a (1'R,2'R,3'S,4'R,5'S)-4-[(2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl]-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol ($[^{125}\text{I}]$MRS1898) solution at various time pointsa

| Time (days) | 2  | 8  | 11 | 20 | 25 | 27 |
|-------------|----|----|----|----|----|----|
| Radiochemical purity | 98% | 98.5% | 98.5% | 100% | 100% | 100% |

a Determined by high-performance liquid chromatography (HPLC) with radioactivity detection. $[^{125}\text{I}]$MRS1898 was present at a concentration of 6.9 nM in solution (ethanol:water=9:1, v/v) containing 1% ascorbic acid and stored at –20°C.
for drug screening, although the ratio was still low when the radioligand concentration was raised.

The specific binding of \([^{125}\text{I}]\text{MRS1898}\) to the \(\text{rA}_3\text{AR}\) in CHO cell membranes was saturable (Fig. 1), and Scatchard analysis indicated a \(K_d\) value of 0.17±0.04 nM and a \(B_{\text{max}}\) value of 0.66±0.15 pmol/mg protein.

The ability of various known AR agonists and antagonists to compete for \([^{125}\text{I}]\text{MRS1898}\) binding to the \(\text{rA}_3\text{AR}\) was tested. Figure 2 shows that the rank order of potencies for agonists was \(\text{MRS1898} \geq \text{IB-MECA} > \text{NECA}\), and for antagonists \(5\)-propyl-2-ethyl-4-propyl-3-(ethanesulfonylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523) > 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, \(3\)-ethyl- 5-(phenylmethyl) ester (MRS1191) > \(N(9\)-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-yl]benzeneacetamide (MRS1220), which is similar to those obtained with other radioligands in previous reports.

In the association kinetic experiment (Fig. 3A), \([^{125}\text{I}]\text{MRS1898}\) binding reached a maximum in 30 min, with a \(t_{1/2}\) of 7.4±0.9 min. After a 60 min incubation, the dissociation was initiated with the addition of \(10 \mu\text{M}\) IB-MECA at various time points, as indicated in Fig. 3b.

![Fig. 3](image-url) Kinetics of association (a) and dissociation (b) of \((1'R,2'R,3'S,4'R,5'S)-4\{2\text{-chloro-6}\{3\text{-iodophenylmethyl}\text{amino}[purin-9-yl]-1-\text{(methylaminocarbonyl)}\text{bicyclo}[3.1.0]\text{hexane-2,3-diol}\}^{125}\text{I}\) MRS1898 (0.1 nM) at the rat \(A_3\) adenosine receptor

The binding of \([^{125}\text{I}]\text{MRS1898}\) was further tested using membranes prepared from rat-brain cortex (mainly \(A_1\) adenosine receptor (AR)) and striatum (expressing both \(A_1\)AR and \(A_2\)AR). Two concentrations of \([^{125}\text{I}]\text{MRS1898}\) (0.1 and 1.0 nM) were used. The membrane concentration used in the experiment was 200 \(\mu\text{g/mg protein}\). The \(y\)-axis shows radioactivity counts. The first bar (TB, total binding) in each pair of bars corresponds to total radioligand binding, and the second bar (NSB, nonspecific binding) is in the presence of \(10 \mu\text{M}\) \(6\)-cyclopentyladenosine for \(A_1\)AR and \(10 \mu\text{M}\) 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5\'-\(N\)-ethylcarboxamidoadenosine for \(A_2\)AR. Thus, no specific binding of \([^{125}\text{I}]\text{MRS1898}\) to the \(A_1\)AR and \(A_2\)AR in rat brain was detected.

![Fig. 4](image-url) Binding of \((1'R,2'R,3'S,4'R,5'S)-4\{2\text{-chloro-6}\{3\text{-iodophenylmethyl}\text{amino}[purin-9-yl]-1-\text{(methylaminocarbonyl)}\text{bicyclo}[3.1.0]\text{hexane-2,3-diol}\}^{125}\text{I}\) MRS1898 in membranes from rat-brain cortex ([mainly \(A_1\) adenosine receptor (AR)] and striatum (expressing both \(A_1\)AR and \(A_2\)AR). Two concentrations of \([^{125}\text{I}]\text{MRS1898}\) (0.1 and 1.0 nM) were used. The membrane concentration used in the experiment was 200 \(\mu\text{g/mg protein}\). The \(y\)-axis shows radioactivity counts. The first bar (TB, total binding) in each pair of bars corresponds to total radioligand binding, and the second bar (NSB, nonspecific binding) is in the presence of \(10 \mu\text{M}\) \(6\)-cyclopentyladenosine for \(A_1\)AR and \(10 \mu\text{M}\) 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5\'-\(N\)-ethylcarboxamidoadenosine for \(A_2\)AR. Thus, no specific binding of \([^{125}\text{I}]\text{MRS1898}\) to the \(A_1\)AR and \(A_2\)AR in rat brain was detected.

Discussion

In this study, we synthesized and pharmacologically characterized the new radioligand \([^{125}\text{I}]\text{MRS1898}\) for the \(\text{rA}_3\text{AR}\). The agonist ligand series of which \(\text{MRS1898}\) is representative, based on nucleoside analogues containing a rigid, bicyclic ring system in place of the ribose moiety, was selected for radiolabeling due to its high \(\text{A}_3\)AR affinity across species. The radioiodination of \(\text{MRS1898}\) on its \(N(6)-3\text{-iodobenzyl}\) substituent was accomplished in 76% radiochemical yield by iododestannylation of a \(3\)-(trimethylstannyl)benzyl precursor. This precursor was both synthesized and iodinated without protecting groups present at other sensitive positions of the nucleoside.

As a receptor radioligand, \([^{125}\text{I}]\text{MRS1898}\) may be useful for compound screening, testing of ligand binding affinity, and for studying association and dissociation kinetics at the \(\text{rA}_3\text{AR}\). Although \([^{125}\text{I}]\text{MRS1898}\) could be potentially...
useful for the study of the rA3AR, there are also some potential problems with this radioligand, particularly its high nonspecific binding under the conditions used in this study. The Kd value of the radioligand was somewhat lower than that estimated from the affinity of the nonradiolabeled MRS1898, which may be affected by its hydrophobic nature. The cLogP for MRS1898 was 2.43, which was considerably higher than the cLogP (1.20) for the corresponding 9-riboside, CI-IB-MECA. The frequently used radioligand I-AB-MECA 3 had a cLogP of −0.47. Thus, further efforts are needed toward the design of a similar radioligand with subnanomolar affinity and subtype selectivity, but with lower nonspecific binding, for applications such as autoradiography. Nevertheless, our study demonstrated that [125I]MRS1898 should be useful under certain conditions for the study of the rA3AR. Its A3AR selectivity is clearly superior to the widely used [125I]-AB-MECA.

In summary, a novel selective A3AR radioligand, [125I]MRS1898, was synthesized and pharmacologically characterized in binding to cell membrane receptors. The advantages of this radioligand compared with other previously used radioligands are its facile synthesis and radiochemical stability and its high selectivity and high affinity for the rA3AR, which should be applicable for the study of A3AR in native tissues with mixed AR subtypes. The binding of [125I]MRS1898 to the A3AR in other species and the feasibility of its use in quantification of the A3AR from various tissues may now be studied.

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