# Chapter 7
# Medicinal Chemistry of the $A_3$ Adenosine Receptor


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**Abstract** Numerous structure-activity relationship (SAR) studies of ligands of the $A_3$ adenosine receptor (AR) have generated selective agonists, antagonists, partial agonists, and allosteric modulators. The efficacy of nucleoside agonists may be reduced, while retaining affinity, by successive structural changes. Subnanomolar affinity and selectivity of >10,000-fold have been achieved for various compound classes, but often with a pronounced species dependence, especially for diverse heterocyclic antagonists. Two prototypical $A_3$AR agonists, IB-MECA and Cl-IB-MECA, are being evaluated clinically for treating autoimmune inflammatory disorders and liver diseases. The design of $A_3$AR orthosteric ligands is now largely guided by computational approaches, in which the receptor is modeled by homology to X-ray structures of the $A_{2A}$AR and other G protein-coupled receptors (GPCRs). Thus, we have amassed a large body of data concerning the relationship of receptor structure and function and have supported many of the hypotheses generated with experimental data.

**Keywords** $A_3$ adenosine receptors · $A_3$ agonists · $A_3$ antagonists · $A_3$ allosteric modulators · Structure-activity relationship

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## 7.1 Introduction

Since its identification as one of the four subtypes of adenosine receptors (ARs) in human (Zhou et al. 1992; Salvatore et al. 1993), the $A_3$AR has been well studied by medicinal chemists in search of selective agonists, antagonists, and allosteric modulators. The $A_3$AR has become a target for the design of drugs for treating chronic diseases, including cancer, stroke, glaucoma, chronic neuropathic pain, inflammatory diseases, and cardiovascular diseases (Jacobson et al. 2017; Janes et al. 2016). Initial findings suggested that a selective $A_3$AR antagonist might have anti-inflammatory or anticancer effects (Gessi et al. 2011; Torres et al. 2016; Borea et al. 2017), but upon further delving into the biology, particularly in vivo, it appears that $A_3$AR agonists also produce effects that are predictive of their therapeutic potential (Fishman et al. 2001, 2012; Borea et al. 2016). Two of the $A_3$AR agonists are entering advanced clinical trials for psoriasis, rheumatoid arthritis, and liver diseases (David et al. 2016; Stemmer et al. 2013; Fishman and Cohen 2016; Jacobson et al. 2017).

There is not yet an X-ray crystallographic structure of the $A_3$AR, but considerable modeling has been performed based on its homology to the human (h) $A_{2A}$AR, for which both agonist- and antagonist-bound structures have been determined (Jespers et al. 2018). The $A_{2A}$AR structures can serve as templates for the modeling of the $A_3$AR, in which many of the key residues involved in ligand recognition are conserved. Thus, ligand design for the $A_3$AR is increasingly structure-guided, and many of the newer agonists and antagonists reported have been docked in homology models in an effort to understand the structure-activity relationship (SAR). Virtual (in silico) screening to discover both $A_3$AR agonists and antagonists is now feasible.

The effects on $A_3$AR affinity and efficacy of structural changes at specific sites to adenosine and diverse antagonists are discussed below. It is noteworthy that there are species differences in the affinities of $A_3$AR ligands, particularly nonnucleoside antagonists, which often are weak or inactive at the rodent homologues. This is consistent with a low sequence identity among rodent vs. primate $A_3$ARs, which for mouse (m) $A_3$AR vs. human (h) $A_3$AR is only 73% (Paoletta et al. 2013).

## 7.2 Nucleosides as $A_3$AR Agonists

The rat (r) $A_3$AR sequence was first identified in a cDNA library prepared from rat testes (Meyerhof et al. 1991), but only later was identified as a pharmacologically novel AR (Zhou et al. 1992). Soon thereafter, the cloned h$A_3$AR was validated as an AR (Salvatore et al. 1993), at which [$^{125}$I]I-ABA **3** (Fig. 7.1) bound with high affinity (10 nM) and functioned as a partial agonist. The order of affinity in agonist binding at the h$A_3$AR ($K_i$, nM) was NECA **8** (26) ~ R-PIA **1** (34) > CPA **2** (89). This indicated that nucleosides previously considered to be $A_1$AR–selective displayed considerable affinity at this new receptor. The levels of expression were highest in

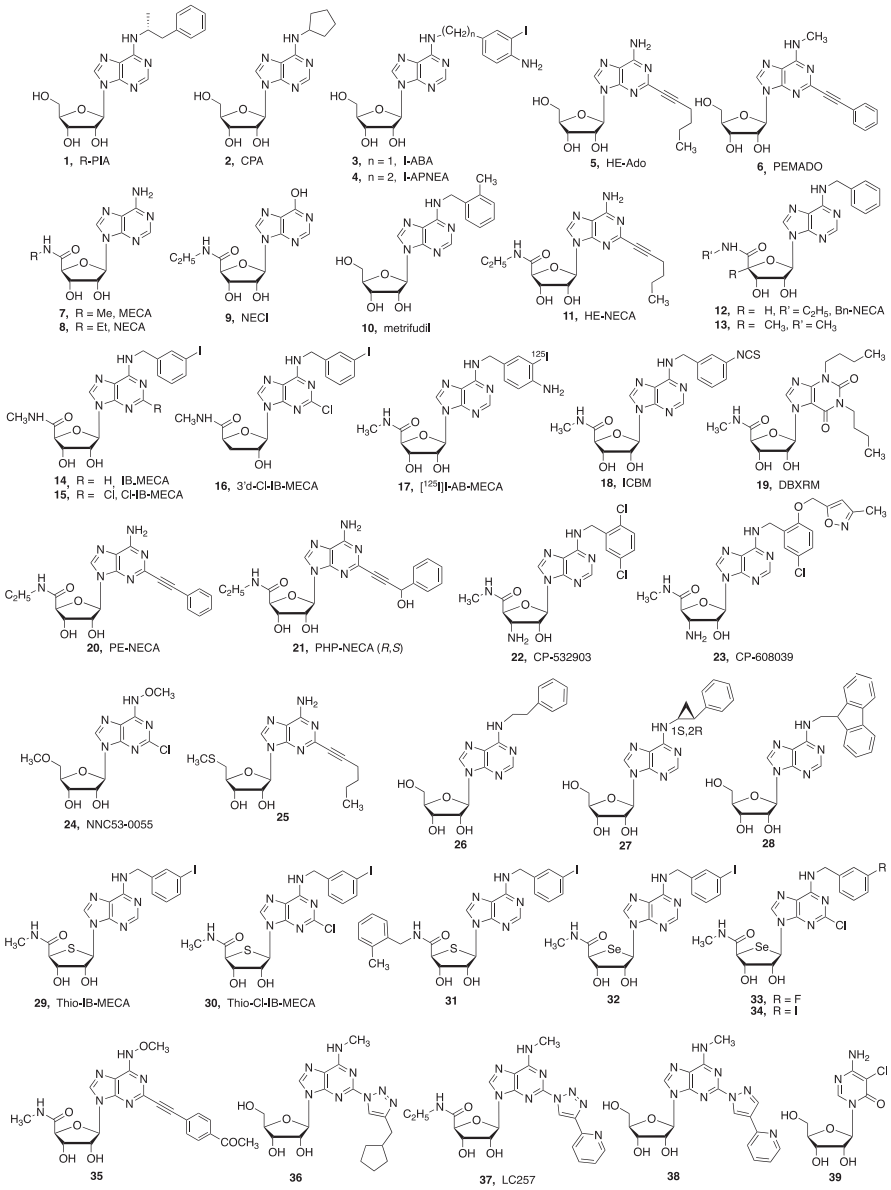


**Fig. 7.1** Ribose-containing $A_3$AR agonists

human lung and liver, which was unlike the distribution of other AR subtypes. Research was initiated at NIH to computationally model this atypical AR and to identify structural features of known AR agonists that increased $A_3$AR affinity or selectivity (van Galen et al. 1994). Initially, affinity at the r$A_3$AR was used as a criterion (Gallo-Rodriguez et al. 1994), and only in later SAR studies was screening performed at the human homologue (Gao et al. 2003a).

## *7.2.1 Nucleobase Substitutions*

### 7.2.1.1 Purine 6-Position Substitutions

The initial reports on radioligand binding at the $rA_3AR$ by Stiles and coworkers utilized [$^{125}$I]APNEA **4** as a radioligand having a $K_d$ value of 15.5 nM (Zhou et al. 1992). Also, the widely used nonselective 5′-modified AR agonist NECA **8** was a potent activator of the $A_3AR$ with a binding $IC_{50}$ value of 74 nM. Thus, it was evident that both $N^6$-arylalkyl and 5′-*N*-alkyluronamide modifications were possible. The combination of these two modification sites was reported by Jacobson and coworkers (van Galen et al. 1994; Gallo-Rodriguez et al. 1994), leading to the first slightly selective (7-fold) $A_3AR$ agonist $N^6$-benzyl-NECA **12** and later to more selective agonists. A comparison of various $N^6$-arylalkyl modifications of adenosine determined the following rank order of affinity at the $rA_3AR$: 2-(phenyl)ethyl- **26** = benzyl- > phenyl-adenosine. The choice between $N^6$–2-(phenyl)ethyl and $N^6$-benzyl substituents was informed by the selectivity ratios of the corresponding adenosine derivatives. Although both were associated with high affinity at the $A_3AR$, the latter group was much weaker than the former at $A_1$ and $A_{2A}$ARs. Thus, an $N^6$-benzyl group was deemed optimal in the series to provide $A_3AR$ selectivity. A survey of the affinity of diverse AR ligands and related purines at the $rA_3AR$, accompanied by molecular modeling of the receptor and its binding site, was also performed.

An $N^6$-benzyl derivative of adenosine, metrifudil **10** (Table 7.1.), was administered orally in a preliminary clinical trial for glomerulonephritis in the 1970s (Wildbrandt et al. 1972), and it demonstrated a trend to reduce proteinuria. It displays a $K_i$ value of 360 nM at the $rA_3AR$, although it is roughly an order of magnitude more potent at the $rA_1AR$ and the $rA_{2A}AR$ (Siddiqi et al. 1995). Metrifudil was later shown to be a nonselective, full agonist at the $hA_3AR$ (Gao et al. 2003a). Thus, metrifudil was the first $A_3AR$ agonist with moderate affinity to be administered in humans.

Subsequently, other $N^6$ modifications were explored for achieving selectivity at the $A_3AR$. For example, $N^6$-methyl, e.g., **6** and **36**–**38**, and $N^6$-ethyl groups were found to be suitable for $hA_3AR$ selectivity (Volpini et al. 2002; Zhu et al. 2006). However, these small $N^6$-alkyl groups did not maintain the degree of selectivity at the mouse or $rA_3AR$ seen with the $N^6$-benzyl derivatives, which was considered an important feature for animal model studies. The $N^6$-methoxy group as in **35** was also reported to be suitable for binding at the $A_3AR$ (Volpini et al. 2007).

$N^6$-Monoalkyl derivatives are more potent at the $A_3AR$ than corresponding dialkyl derivatives. $N^6$-Acyl and urea groups were evaluated as modifications of known $A_3AR$ agonists, but these derivatives displayed only moderate affinity (Baraldi et al. 1998).

$N^6$–2-Phenylcyclopropyl groups were explored at the $hA_3AR$ as sterically constrained analogues of the $N^6$-phenylethyl group, which is known to afford high affinity. In that series, it was found that the (1*S*,2*R*) stereoisomer, e.g., **27**, provided

**Table 7.1** Affinity of selected nucleoside derivatives as $A_3$AR agonists, partial agonists, and antagonists

| Compound | $pK_i$ value | | | Ref. |
|---|---|---|---|---|
| | $A_1$AR | $A_{2A}$AR | $A_3$AR | |
| **6** | 4.48 (h) | 4.38 (h) | 8.52 (h) | Volpini et al. (2002) |
| **10**, metrifudil | 7.22 (r) | 7.62 (r) | 7.33 (h) | Gao et al. (2003a) |
| **11** | 7.22 (h) | 8.19 (h) | 8.62 (h) | Volpini et al. (2002) |
| **14**, IB-MECA | 7.29 (h) | 5.50 (h) | 8.74 (h) | Melman et al. (2008) |
| | 7.27 (r) | 7.25 (r) | 8.96 (r) | " |
| | 8.23 (m) | ~6 (m) | 10.1 (m) | " |
| **15**, Cl-IB-MECA | 6.66 (h) | 5.27 (h) | 8.85 (h) | Melman et al. (2008) |
| | 6.09 (r) | 6.33 (r) | 9.48 (r) | " |
| | 8.14 (m) | 5.27 (m) | 9.10 (m) | " |
| **21** | 8.57 (h) | 8.51 (h) | 9.38 (h) | Volpini et al. (2002) |
| **23** | 5.14 (h) | <4.3 (h) | 8.24 (h) | DeNinno et al. (2003) |
| **29** | <5 (h) | <5 (h) | 7.81 (h) | Jeong et al. (2006) |
| **30** | 6.71 (h) | 5.36 (h) | 9.42 (h) | Jeong et al. (2006) |
| **35** | 4.27 (h) | 4.98 (h) | 8.60 (h) | Volpini et al. (2007) |
| **37**, LC-257 | 5.79 (h) | <4 (h) | 8.74 (h) | Cosyn et al. (2006) |
| **38** | 5.42 (h) | <5.3 (h) | 8.70 (h) | Cosyn et al. (2006) |
| **43** | 7.74 (h) | 5.49 (h) | 8.43 (h) | Jacobson et al. (2005) |
| **46**, MRS3558 | 6.59 (h) | 5.64 (h) | 9.54 (h) | Tchilibon et al. (2005) |
| **48**, MRS3609 | 5.66 (h) | <5 (h) | 8.44 (h) | Tchilibon et al. (2005) |
| **49**, MRS3611 | 6.21 (h) | ~5 (h) | 8.82 (h) | Tchilibon et al. (2005) |
| **50**, MRS5151 | 4.83 (h) | ~5 (h) | 8.62 (h) | Tosh et al. (2009) |
| **53**, MRS5698 | <5 (h) | <5 (h) | 8.46 (h) | Tosh et al. (2014) |
| | <5 (m) | <5 (m) | 8.51 (m) | " |
| **54**, MRS5679 | <5 (h) | <5 (h) | 8.51 (h) | Tosh et al. (2014) |
| **55**, MRS5980 | <5 (h) | <5 (h) | 9.15 (h) | Tosh et al. (2014) |
| **58**, MRS5841 | <5 (h) | <5 (h) | 8.72 (h) | Paoletta et al. (2013) |
| **64**, MRS5919 | <5 (h) | <5 (h) | 8.22 (h) | Tosh et al. (2016) |
| **65** | <4 (h) | <4 (h) | 6.19 (h) | Volpini et al. (2001) |
| **68**, MRS1292 | ND | ND | 7.53 (h) | Gao et al. (2002a) |
| **74** | 5.60 (h) | 6.47 (h) | 8.38 (h) | Jeong et al. (2007) |
| **76** | <4 (h) | 8.14 (h) | 7.93 (h) | Hou et al. (2012) |
| **77**, MRS5127 | 5.75 (h) | 5.80 (h) | 9.14 (h) | Müller and Jacobson (2011) |
| **78**, MRS5147[a] | 5.52 (h) | 5.97 (h) | 8.84 (h) | Müller and Jacobson (2011) |
| **79** | 5.23 (h) | <5 (h) | 7.54 (h) | Perreira et al. (2005) |
| **80** | <5 (h) | <5 (h) | 8.03 (h) | Jeong et al. (2008) |
| **81**, MRS5776 | <5 (h) | <5 (h) | 7.70 (h) | Tosh et al. (2012b) |
| **82** | <5 (h) | 5.13 (h) | 8.31 (h) | Nayak et al. (2014) |
| **85** | 9.34 (h) | 6.48 (h) | 9.50 (h) | Petrelli et al. (2017) |

*h* human, *r* rat, *m* mouse, *ND* not determined

[a]as stable Br isotope

38-fold higher $hA_3AR$ affinity than the corresponding (1*R*,2*S*) diastereoisomer (Tchilibon et al. 2004).

In addition to NECA **8**, the corresponding inosine derivative, i.e., NECI **9**, was found to bind to the $rA_3AR$ with a $K_i$ value of 5 μM (van Galen et al. 1994). This was the first indication that inosine ($K_i$ at $rA_3AR$ 45 μM) and its derivatives could serve as $A_3AR$ ligands, although adenosine-like effects of inosine on rat mast cells were previously reported (Marquardt et al. 1978). Inosine was later shown to be a weak partial agonist of the $hA_3AR$ (Jin et al. 1997; Gao et al. 2011), and due to its generation in vivo from the action of ubiquitous adenosine deaminase on adenosine, it could be considered an alternate endogenous $A_3AR$ agonist under stress conditions. Inosine derivatives, such as **42**, were later explored as potential $A_3AR$ agonists (Ravi et al. 2001; Tosh et al. 2016).

#### 7.2.1.2 Alternate Nucleobases

One of the early characteristics of the $rA_3AR$ observed is that the conventional AR antagonists, i.e., alkylxanthines, were much weaker than at the $rA_1AR$. However, by appending a ribose moiety to the 7-position, they were able to bind to the $rA_3AR$, in some cases with selectivity. 1,3-Dibutylxanthine-7-ribosides, e.g., **66**, were shown to be the optimal alkyl chain length for binding to the $rA_3AR$ (Park et al. 1998). The corresponding 5′-*N*-methyluronamide DBXRM **19** is a selective agonist, either partial or full, at the $rA_3AR$. The 7-riboside series was later expanded to the replacement with bicyclic ribose substitutes, e.g., **44**, but the observed $A_3AR$ affinity was reduced compared to ribose analogues.

Virtual screening for AR agonists identified 6-amino-5-chloropyrimidin-4(1H)-one riboside **39** as a novel $A_3AR$ full agonist, although it also activated the $A_1AR$ (Rodriguez et al. 2016). The screening utilized the structure of an agonist-bound $A_{2A}AR$ as a template, but this required a specially devised routine for virtually screening the commercially available nucleobases. These ring NH-containing bases were first converted computationally to their ribosides and then chemically adding the ribose moiety to the hit molecules.

#### 7.2.1.3 Purine C2-Position Substitutions

Another position of substitution was added to the growing list of $A_3AR$ agonist modifications with the observation that elongation of groups at the C2-position was compatible with receptor binding (Kim et al. 1994; Volpini et al. 2002; Gao et al. 2004). Thus, the $A_{2A}AR$ agonist 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680, structure not shown), reported in 1990, was found to be only 2.5-fold less potent at the $hA_3AR$ than at the $hA_{2A}AR$. Within the range of C2 substitutions, the 2-chloro group in Cl-IB-MECA **15** was shown to increase selectivity in binding to the $rA_3AR$ to >1000-fold (Kim et al. 1994). Thus, Cl-IB-MECA **15** became a widely used selective $A_3AR$ agonist tool molecule,

although with less selectivity for the $hA_3AR$. However, even with moderate selectivity in $A_3AR$ binding, there are examples in the literature that IB-MECA and Cl-IB-MECA might activate the $A_1AR$, $A_{2A}AR$ or even the $A_{2B}AR$, depending on the model used and the dose range (Murphree et al. 2002; Tian et al. 2015). Thus, agonists with even greater $A_3AR$ selectivity were sought as pharmacological probes. Nevertheless, clinical trials of these two prototypical $A_3AR$ agonists for treating autoimmune inflammatory disorders (**14**, entering Phase III) and liver diseases (**15**, entering Phase II) are continuing and appear encouraging (Jacobson et al. 2017).

Adenosine C2-alkynyl homologues were introduced by the Matsuda (Homma et al. 1992) and Cristalli (Cristalli et al. 1994) groups as $A_{2A}AR$ agonists of increased affinity, but they were later found to be $A_3AR$ agonists as well (reviewed in Dal Ben et al. 2011). In particular, a C2-(2-hexynyl) group in HE-Ado **5** was studied initially at the $A_{2A}AR$ and later shown to be tolerated in potent binding at the $A_3AR$ (Baraldi et al. 1998). The combination of a C2-alkynyl group with a 5′-*N*-ethyluronamide group, i.e., HE-NECA **11**, also resulted in high $A_3AR$ binding affinity, but it lacked selectivity (Jacobson et al. 1995; Volpini et al. 2002). Many adenosine analogues in the riboside series containing C2-phenyl-ethynyl or phenyl-alkylethynyl groups, e.g., **20** and **35**, have been reported to be highly selective agonists (Volpini et al. 2002, 2007, 2009; Dal Ben et al. 2014). Thus, the combination of extended 2-ethynyl groups with other $A_3AR$-enhancing modifications of adenosine proved to be additive.

Agonists with heterocyclic groups, such as triazoles (Cosyn et al. 2006), attached directly at the C2-position have been introduced as $A_3AR$ agonists. Adenosine derivative **38** containing a C2-pyrazole group was found to be highly selective in binding to the $hA_3AR$ ($K_i$ 2 nM, Elzein et al. 2004), but its functional activity was not presented.

## *7.2.2 Ribose Group Modifications*

### 7.2.2.1 5′-Position

Optimization of $N^6$-arylalkyl and 5′-uronamide substitutions was reported by Gallo-Rodriguez et al. (1994). The smaller 5′-*N*-methyluronamide in MECA **7** was more conducive to $A_3AR$ selectivity than the corresponding *N*-ethyl group, and the substitution pattern of the $N^6$-benzyl group favored *m*-substituted halogens and other groups. Thus, IB-MECA **14** was identified as the first useful $A_3AR$ agonist probe, displaying ~50-fold selectivity for the $rA_3AR$ in comparison to $A_1$ and $A_{2A}ARs$. Alternative small amides at the 5′-position were explored by Tosh et al. (2012a), and *N*-propyl and *N*-cyclopentyl groups were found to be tolerated at the $hA_3AR$.

When the cloned $hA_3AR$ became available for compound screening, it was noted that the $A_3AR$ selectivity and nM affinity of IB-MECA and many of its 5′-*N*-alkyluronamide derivatives generalized to this species (Gao et al. 2003a). An alternative to the use of nonselective AR agonist I-APNEA as an $A_3AR$ radioligand

was needed, and the $N^6$–4-amino-3-iodobenzyl derivative I-AB-MECA **17** with a $K_d$ value at the cloned $rA_3AR$ of 1.48 nM fulfilled this need (Olah et al. 1994). Among other affinity reagents for studying the $A_3AR$ introduced early, a 3-isothiocyanatobenzyl 5′-*N*-methyluronamide derivative **18** was shown to irreversibly label the $rA_3AR$ and was presumed to be covalently binding to the receptor because of the presence of the electrophilic group and the inability to restore $A_3AR$ radioligand binding (Ji et al. 1994).

Knutsen and coworkers modified the 5′-position with ethylene, methyl ether NNC53-0055 **24**, and chloromethyl groups and found significant $hA_3AR$ selectivity (Mogensen et al. 1998). IJzerman and coworkers explored 5′-alkylthioether modifications, such as in **25**, that still allowed $A_3AR$ selectivity (van Tilburg et al. 2002).

As stated above, the 5′-amides with small alkyl groups enhance $A_3AR$ affinity and functional efficacy compared to 5′-$CH_2OH$. Nevertheless, certain bulky groups present on the amide nitrogen are still compatible with high affinity at the $A_3AR$. For example, a 5′-*N*-(2-methylbenzyl)-amide group in **31** provided a $K_i$ value of 31 nM at the $hA_3AR$, and this compound was inactive at $A_1AR$ and $A_{2A}AR$ (Choi et al. 2009).

#### 7.2.2.2 4′-Position

The 4′-methyl derivative **13** of $N^6$-benzyl-MECA displayed selectivity for the $rA_3AR$ with a $K_i$ value of 604 nM. Thus, steric bulk at this ribose carbon is tolerated at the $A_3AR$ (Siddiqi et al. 1995), although with reduced affinity.

The ribose ring oxygen can be substituted with sulfur or selenium, with retention of $A_3AR$ selectivity. 4′-Thio derivatives **29** and **30** of prototypical $A_3AR$ agonists display high affinity. 4′-Seleno derivatives **32**–**34** were recently reported as potent $A_3AR$ agonists by Yu et al. (2017). The oxo- and thio- analogues were predicted in receptor docking to attain an *anti*-conformation of the glycosidic bond, as was found for adenosine derivatives in the $A_{2A}AR$ X-ray structures. However, an X-ray structure of compound **34** alone ($K_i$ 4.2 nM; maximal efficacy ($E_{max}$) 94% of 10 μM NECA) indicated a *syn*-conformation; presumably, the energetic stabilization of the $A_3AR$ interaction of this nucleoside converts it to an *anti*-conformation as required to fit the binding site.

#### 7.2.2.3 Ribose 2′ and 3′ Hydroxyl Group Modifications

The 2′ and 3′ hydroxyl groups of adenosine are considered positions that are not tolerant of extensive modification in AR agonists (Siddiqi et al. 1995). We now know the structural explanation for this finding; the ribose resides in a sterically limited sub-pocket of the receptor and is surrounded by hydrophilic residues, which coordinates it through H-bonding (Ciancetta and Jacobson 2017). Nevertheless, there are isolated examples of modification of these two hydroxyl groups that maintain $A_3AR$

selectivity. For example, 3′-deoxy Cl-IB-MECA **16** displayed an affinity of 33 nM at the $rA_3AR$, which it fully activated in a measure of cAMP inhibition (Jacobson et al. 1995). Cordycepin (3′-deoxyadenosine, structure not shown) was found to exert an antitumor effect in mouse by activation of the $A_3AR$ (Nakamura et al. 2006). However, the affinity of this compound at the $rA_3AR$ was shown to be weak with 33% binding inhibition at 100 μM (van Galen et al. 1994). Some 3′-amino-3′-deoxy adenosine derivatives are potent $hA_3AR$ agonists, e.g., the anti-ischemic agents **22** and **23** (DeNinno et al. 2006), but the preservation of $A_3AR$ affinity in 3′-amino derivatives does not generalize across the range of adenosine modifications.

### *7.2.3 Methanocarba Analogues*

The rigid methanocarba modification of nucleosides features a rigid bicyclo[3.1.0] hexane ring system replacing the tetrahydrofuryl group of ribose. There are two isomeric methanocarba modifications of ribose that result in locking the conformation as either a North (N)- or South (S)-envelope conformation, i.e., adenosine analogues **40** and **41**, respectively (Fig. 7.2). These modifications were applied in earlier studies of antiviral nucleosides, and Jacobson et al. (2000) first applied this pair of isomeric modifications to nucleosides acting at cell surface receptors. There was a consistent increase of $hA_3AR$ affinity and selectivity, across a variety of adenosine derivatives, associated with the (N)-methanocarba analogue compared to both the (S) analogue and the native riboside. (N)-methanocarba analogues were also more potent at the $A_3AR$ than the simple carbocyclic (cyclopentane) analogues. For the simple adenosine analogues, $K_i$ values in binding to the $hA_3AR$ were determined to be 404 nM (**40**) and 62.5 μM (**41**), respectively. The $A_3AR$, among all of the ARs, most benefitted from a locked (N)-methanocarba conformation. This suggested that the (N)-methanocarba modification achieve a pre-locking of the $A_3AR$-preferred conformation of the ribose ring. Although the three other ARs also likely require a (N)-conformation of ribose, as is now known from X-ray crystallographic structures of agonist-bound $A_{2A}AR$, the (N)-methanocarba modification is most suited structurally to binding at the $A_3AR$.

Functionality that is known to enhance $A_3AR$ affinity and selectivity was combined with the (N)-methanocarba modification, and this combination was shown to be general for the range of SAR at this receptor (Tchilibon et al. 2005). Direct replacement of Cl-IB-MECA and its $N^6$-(3-halobenzyl) congeners with (N)-methanocarba provided potent and selective $A_3AR$ agonists **45** and **46**. Compound **45** and its bromo analogue **47** were also radiolabeled, and these radiotracers were shown to have low nonspecific binding and to be useful in receptor characterization (Gao et al. 2009; Kiesewetter et al. 2009). Alternative functionality at the 2-position was allowed, e.g., 2-iodo **48** and 2-methylthio **49** analogues. The enhancement of $A_3AR$ selectivity by this modification is so robust that even combination with the $A_1AR$-enhancing $N^6$-cyclopentyl group led to a balanced

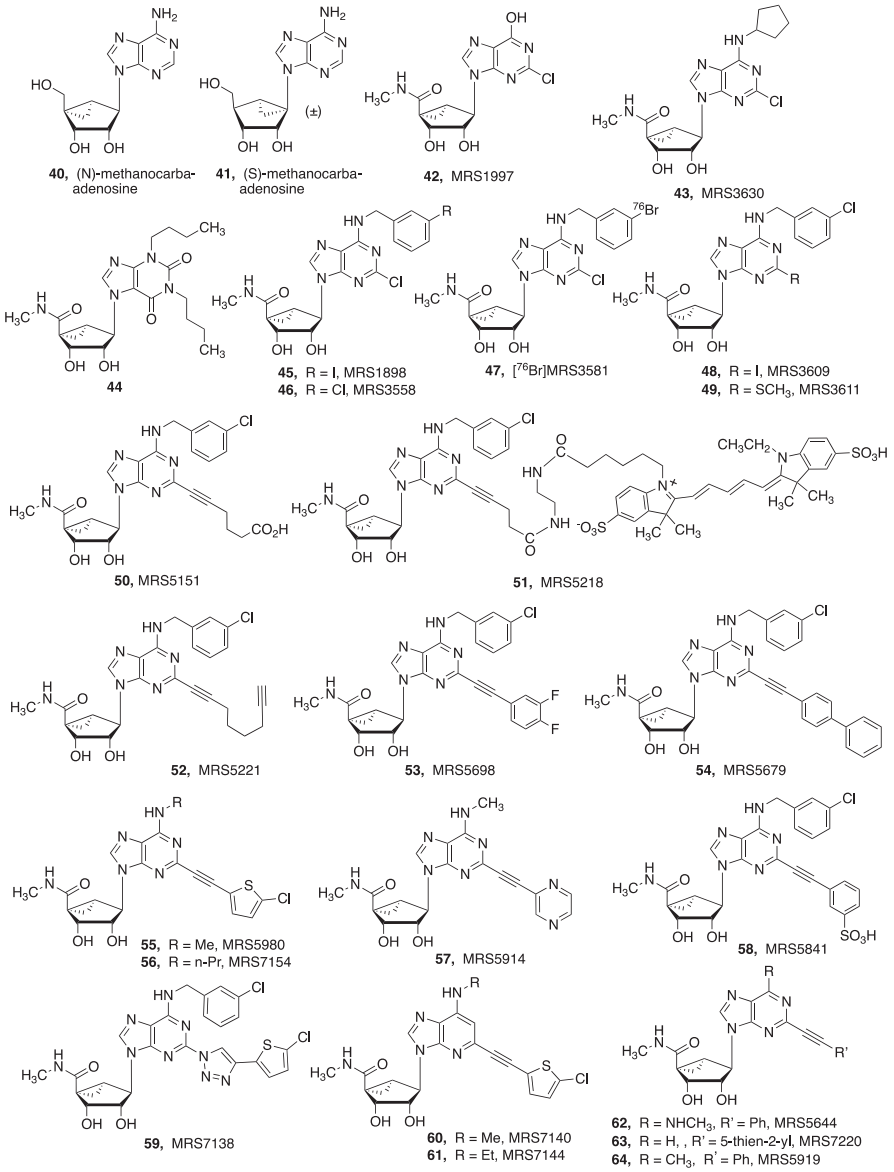


**Fig. 7.2** Methanocarba analogues of $A_3$AR agonists

$A_1$AR/$A_3$AR mixed agonist **43**, which was shown to have anti-ischemic properties in the isolated mouse heart (Jacobson et al. 2005).

The $N^6$ group can be eliminated entirely, but this applies only when other affinity-enhancing groups, such as C2-extended substituents (Tosh et al. 2016), are present on the molecule, e.g., in 6-H derivative MRS7220 **63** ($K_i$ h$A_3$AR, 60 nM) and 6-methylpurine derivative MRS5919 **64** (6.0 nM). Nevertheless, **64** was

sevenfold less potent in binding to the $A_3$AR than the corresponding 6-methylamino analogue **62**.

The (N)-methanocarba modification was suitable for functionalized congeners of $A_3$AR agonists (Tosh et al. 2009), such as an affinity-optimized carboxylic acid congener **50** containing a three-methylene spacer. The shorter two-methylene carboxylic acid homologue could be labeled by coupling to an amine-functionalized cyanine5 (Cy5) fluorophore to provide the high affinity fluorescent $A_3$AR agonist MRS5218 **51** which was shown to be a useful tracer for characterizing the receptor on whole cells or for use in drug screening (Kozma et al. 2013). For coupling to reporter groups or polymeric carriers, terminal alkyne **52** served as an intermediate for efficient click reactions rather than coupling by amide bond formation (Tosh et al. 2009). Conjugates of both the carboxylic acid and terminal alkyne functionalized congeners tended to retain $A_3$AR affinity.

C2-arylalkynyl (N)-methanocarba derivatives demonstrated that the $A_3$AR is highly permissive of bulky aryl groups on the alkyne, e.g., $N^6$-(3-chlorobenzyl) derivatives **53** and **54**, which was also confirmed in the case of $N^6$-methyl analogues, such as **62** (Tosh et al. 2014). A sulfonated agonist that would not diffuse across biological membranes was desired for in vivo studies; compound **58** was predicted computationally and proved to be highly potent and selective at both the $mA_3$AR and $hA_3$AR (Paoletta et al. 2013). An in vivo phenotypic screen allowed the comparison of C2-arylalkynyl (N)-methanocarba analogues based on efficacy and duration of action in a model of chronic neuropathic pain (Tosh et al. 2014; Janes et al. 2016). In this screen, a 5-chlorothienylethynyl group was particularly conducive to in vivo activity and therefore was incorporated in adenine derivatives MRS5980 **55**, MRS7154 **56**, and MRS5914 **57** and in 1-deazaadenine derivatives MRS7140 **60** and MRS7144 **61** and other analogues (Tosh et al. 2015, 2016).

A C2-triazole group, as in (N)-methanocarba analogue **59**, was found to be a suitable bioisosteric replacement for the diarylalkyne of MRS5980 and its congeners (Tosh et al. 2015). In the ribose series, C2-triazoles were similarly shown to promote $A_3$AR affinity in compounds **36** and **37** (Cosyn et al. 2006).

## 7.3 Nucleosides as $A_3$AR Antagonists and Partial Agonists

The conversion of selective $A_3$AR agonists into selective $A_3$AR antagonists was found to be relatively facile compared to comparable attempts at other AR subtypes. Modifications of the ribose moiety, particularly around the 5′-position, were found to be effective in reducing the relative efficacy of the nucleosides in functional assays, i.e., inhibition of the formation of cyclic AMP (cAMP, Gao et al. 2002a). Steric constraint, truncation, and reducing the H-bond donor ability of the ribose ring moiety all had the effect of reducing $A_3$AR efficacy resulting in partial agonists or antagonists (Gao et al. 2006).

Several issues in determining the $E_{max}$ (as % of a full agonist effect, typically at 10 μM) of a given nucleoside derivative are (1) the reference full agonist used for comparison and (2) the dependence of $E_{max}$ on the pathway measured. Both NECA **8**

and Cl-IB-MECA **15** are full agonists in inhibition of cAMP accumulation. However, the $E_{max}$ of Cl-IB-MECA is only ~50% of NECA in some signaling events, such as $A_3$AR-induced GTP-γ-S binding and mobilization of $Ca^{2+}$ (Gao et al. 2008; Gao et al. 2011). Therefore, even for the same readout, which reference compound is used is important in classifying the nucleoside as a low- or high-efficacy partial agonist.

Introduction of an 8-(hexyn-1-yl) group reduced the $A_3$AR efficacy of adenosine in antagonist **65** (Volpini et al. 2001, Fig. 7.3). However, most nucleoside-based antagonists reported are modified at other sites on the adenine or ribose moieties. Commonly used $A_1$AR agonist **67** proved to be an antagonist at the h$A_3$AR, while substitution of the $N^6$ group with a 3-iodobenzyl moiety in **71** produced a low-efficacy agonist (Gao et al. 2002a). Steric constraint of the 5′-amide in the form of a spirolactam reduces the efficacy such that compound **68** is a potent $A_3$AR antagonist ($K_i$ 29 nM) that likely retains binding selectivity, by analogy to an earlier acyclic 4′-methyl-5′-amide derivative (structure not shown, Gao et al. 2002a; Siddiqi et al. 1995). Furthermore, in a limited number of cases, modifications of the $N^6$ and C2 substituents also were found to reduce efficacy. For example, although the sterically bulky fluorenylmethyl derivative **28** is a full agonist at the h$A_3$AR, its more flexible analogue **69** is an $A_3$AR antagonist. Thus, introducing rigidity at various nucleoside positions may either reduce or increase $E_{max}$.

4′-Truncation of adenosine derivatives in both ribo, e.g., **74**–**76**, and (N)-methanocarba series, e.g., **77**, **78**, and **82**, were $A_3$AR antagonists or low-efficacy agonists, although truncation tends to lower their affinity at r and m$A_3$ARs. However, some truncated derivatives, e.g., **81**, were noted to bind appreciably at the m$A_3$AR, with moderate selectivity as an antagonist (Tosh et al. 2012b). 4′-Truncated 4′-thionucleoside **76** both activated the $A_{2A}$AR and antagonized $A_3$AR (Hou et al. 2012). *N,N*-Dimethyl oxo-nucleoside **79** and thionucleoside **80** were pure antagonists at the $A_3$AR, with selectivity in binding and $K_i$ values of 29 and 9 nM, respectively (Jeong et al. 2008). 4′-Ester derivatives of adenosine in the ribo, e.g., **70**, and (N)-methanocarba series, e.g., **83** ($K_i$, 5.4 nM, $E_{max}$ 12% of NECA in forskolin-stimulated cAMP production in CHO cells) and **84**, also tend to be partial h$A_3$AR agonists (Tosh et al. 2017). 4′-Tetrazole derivative **85** of adenosine was recently reported to potently activate $A_1$AR and antagonize the $A_3$AR, while other $N^6$ substitutions produced mixed $A_1$AR/$A_3$AR agonists (Petrelli et al. 2017).

2-Substituted adenosine analogues display a range of $A_3$AR efficacies (relative to NECA **8**, cAMP), e.g., 2-(2-(3-chlorophenyl)ethyl)-adenosine ($K_i$, 41 nM, $E_{max}$ 31%) and 2-(3-chlorobenzyl)-adenosine ($K_i$, 72 nM, $E_{max}$ 16%) (structures not shown, Gao et al. 2004).

## 7.4 Nonnucleoside Heterocycles as $A_3$AR Antagonists

In addition to the nucleoside antagonists of the $A_3$AR, diverse classes of heterocycles have been identified as scaffolds for h$A_3$AR antagonists. Broad screening of various heterocyclic libraries, including known pharmacological agents and

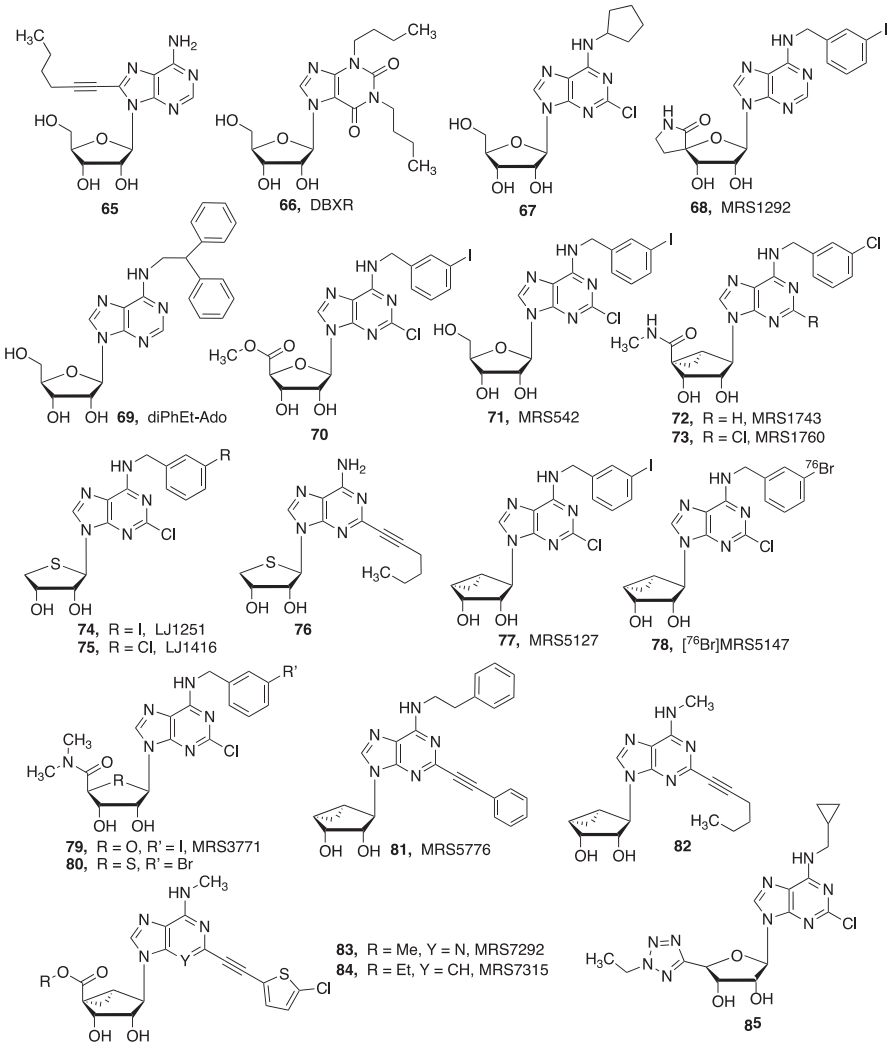


**Fig. 7.3** Nucleoside-derived $A_3$AR antagonists and partial agonists

phytochemicals, has been performed in order to obtain new leads for potent and highly selective $A_3$AR antagonists. Xanthine or purine analogues were examined first, but none of the tested compounds showed significant affinity or selectivity at $rA_3$AR (Jacobson et al. 2009). Inhibition of $rA_3$AR binding by diverse structures identified novel ligands, e.g., sulfonylpiperazines, a pyridazinone, imidazopyrimidines, pteridines, and a carbazolenine, as weak ligands (Siddiqi et al. 1996). Currently, virtual screening for AR antagonists is based on either antagonist-bound $A_{2A}$AR X-ray structures or homology models of the other AR subtypes. Often, new chemotypes are found for other ARs, including the $A_3$AR when docking chemical libraries to an $A_{2A}$AR structure (Rodriguez et al. 2015).

Subsequent to early broad library screening, a large number of compounds with high potency and selectivity as $hA_3AR$ antagonists were documented that are generally characterized as structurally diverse nitrogen-containing aromatic monocyclic/bicyclic/tricyclic systems. Nonnucleoside $A_3AR$ antagonists can be grouped into two broad categories: (1) xanthine analogues and (2) other aromatic monocyclic/bicyclic/tricyclic systems.

### *7.4.1 Xanthine Analogues (Table 7.2)*

The natural products 1,3-dimethylxanthine (theophylline) and 1,3,7-trimethylxanthine (caffeine) showed negligible affinity at the $rA_3AR$ (Müller 2001). Structural modifications at different positions of the xanthine core aimed at improving $A_3AR$ affinity led to a series of tricyclic analogues of xanthine such as 1-benzyl-3-propyl-1*H*,3*H*-pyrido[2,1-*f*]purine-2,4-dione **86** (Fig. 7.4), which showed good affinity ($K_i$ 4.0 nM) but low selectivity over the other ARs (Priego et al. 2002). Introduction of a cyclopropylmethyl group at the $N^3$-position in combination with a 4-methylbenzyl group at the 1-position led to compound **87**, which preserved affinity at the $A_3AR$ with a significant enhancement of selectivity (Priego et al. 2008). The strictly correlated imidazo[2,1-*i*]purinones were found to be potent and selective $A_3AR$ antagonists. The most important compound of this series is PSB-11 (**88**) that showed a $K_i$ value of 2.3 nM at the $hA_3AR$ and good selectivity versus the other AR subtypes (Müller et al. 2002b). The radiolabeled derivative of this compound exhibited a $K_d$ value of 4.9 nM (Müller et al. 2002a). Another similar compound KF-26777 (**89**, 2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one) offered high affinity and selectivity to the $hA_3AR$ ($K_i$ 0.20 nM) (Ozola 2003).

Subsequently, substitution of the 2-phenyl ring of **88** and congeners with five-membered heterocycles, in particular 1,5-disubstituted (not shown) and 1,3-disubstituted pyrazoles or 3-substituted isoxazoles, led to the tricyclic xanthine derivatives such as compounds **90** and **91**, respectively. These antagonists were endowed with high affinity and selectivity for $hA_3AR$. The hypothetical binding mode of these $A_3AR$ antagonists was determined in docking studies to an $A_3AR$ homology model (Baraldi et al. 2011).

In this class of compounds, triazolopurine derivatives in which a simple xanthine structure is elaborated with an additional pyrimidine-fused ring are also reported. One example is OT-7999 (**92**), which proved to be a potent and selective $hA_3AR$ ligand ($K_i$ 0.95 nM) and > 10,000-fold selectivity compared to other AR subtypes (Okamura et al. 2002).

**Table 7.2** Affinity of selected $A_3$AR antagonists

| Compound | $pK_i$ value or % inhibition at 10 μM | | | Ref. |
|---|---|---|---|---|
| | $A_1$AR | $A_{2A}$AR | $A_3$AR | |
| **86** | 7.30 (h) | 6.92 (h) | 8.40 (h) | Priego et al. (2002) |
| **87** | *24%* | *0%* | 8.66 (h) | Priego et al. (2008) |
| **88**, PSB-11 | 5.79 (h) | 5.89 (h) | 8.63 (h) | Müller et al. (2002b) |
| **89**, KF26777 | 5.74 (h) | 6.33 (h) | 9.70 (h) | Ozola (2003) |
| **90** | 5.60 (h) | <5.3 (h) | 8.84 (h) | Baraldi et al. (2011) |
| **91** | 5.52 (h) | 5.82 (h) | 8.71 (h) | Baraldi et al. (2011) |
| **92**, OT-7999 | *4%* (h) | *31%* (h) | 9.02 (h) | Hou et al. (2012) |
| **93**, MRS1523 | <5 (h) | 5.44 (h) | 7.72 (h) | Li et al. (1998) |
| | 4.81 (r) | 5.69 (r) | 6.95 (r) | Müller and Jacobson (2011) |
| **94**, MRS1097 | 5.23 (r) | 5.32 (r) | 6.97 (h) | Jiang et al. (1996) |
| **95**, MRS1191 | 3.40 (r) | *<10%* (r) | 7.50 (h) | Jiang et al. (1997) |
| **96**, ISVY130 | *1%* (h) | *10%* (h) | 8.44 (h) | Cosimelli et al. (2008) |
| **97** | <6.18 (h) | <6.08 (h) | 9.44 (h) | Jung et al. (2004) |
| **98** | *24%* (h) | *28%* (h) | 9.10 (h) | Huffman et al. (2005) |
| **99**, VUF5574 | *52%* (r) | *43%* (r) | 8.39 (h) | Van Muijlwijk-Koezen et al. (2000) |
| **100** | 6.37 (h) | 5.09 (h) | 8.22 (h) | Biagi et al. (2005) |
| **101**, MRS3777 | *26%* (h) | *16%* (h) | 7.33 (h) | Perreira et al. (2005) |
| **102**, MRS1067 | *36%* (r) | *19%* (r) | 6.25 (h) | Karton et al. (1996) |
| **103** | *0%* (h) | *19%* (h) | 10.11 (h) | Poli et al. (2011) |
| **104** | 5.98 (h) | 5.50 (h) | 10.74 (h) | Taliani et al. (2010) |
| **105** | >5.0 (h) | >5.0 (h) | 10.11 (h) | Taliani et al. (2010) |
| **106** | 8.92 (h) | *5%* (h) | *1%* (h) | Lenzi et al. (2009) |
| **107** | *1%* (h) | *1%* (h) | 11.57 (h) | Squarcialupi et al. (2016) |
| **108**, CGS15943 | 7.68 (r) | 8.49 (r) | 7.86 (h) | Kim et al. (1998) |
| **109**, MRS1220 | 7.28 (r) | 8.00 (r) | 9.19 (h) | Jacobson et al. (1997) |
| | 7.09 (m) | 8.04 (m) | ~4[a] (m) | Wan et al. (2004) |
| **110**, MRE3008-F20 | <5 (r) | 5.70 (r) | 9.54 (h) | Baraldi et al. (2000) |
| **111**, MRE3005-F20 | 6.60 (h) | 7.22 (h) | 10.40 (h) | Maconi et al. (2002) |
| **112** | 5.47 (h) | <5.3 (h) | 8.01 (h) | Baraldi et al. (2012) |
| **113** | *0%* (h) | *21%* (h) | 8.05 (h) | Colotta et al. (2007) |
| **114** | >6 (h) | >6 (h) | 8.05 (h) | Baraldi et al. (2005) |
| **115** | >5 (h) | >5 (h) | 10.10 (h) | Jacobson et al. (2009) |
| **116** | 5.57 (h) | >5 (h) | 8.80 (h) | Da Settimo et al. (2007) |

*h* human, *r* rat

[a]31% inhibition at 100 μM

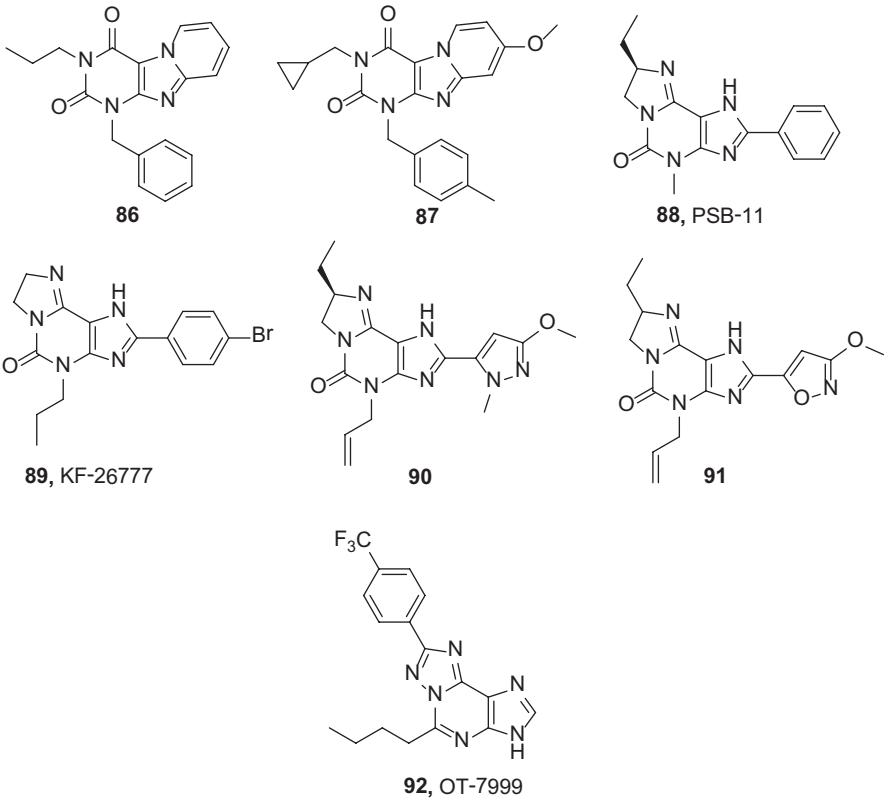


**Fig. 7.4** Xanthine analogues as $A_3$AR antagonists

### *7.4.2 Aromatic Monocyclic/Bicyclic/Tricyclic Systems (Table 7.2)*

Jacobson and coworkers investigated the SAR profile of the pyridine and the 1,4-dihydropyridine nucleus as $A_3$AR antagonists (van Rhee et al. 1996). Introduction of sterically bulky groups at the 6-position of pyridine led to one of the first heterocyclic, selective, and competitive $A_3$AR antagonist MRS1523 (**93**, Fig. 7.5). This compound showed good potency in both humans and rodents, with $K_i$ values of 18.9 nM for h$A_3$AR and 113 nM for r$A_3$AR. A later study comparing the species dependence of common AR antagonists showed MRS1523 **93** to be only moderately selective for the r$A_3$AR (Alnouri et al. 2015).

The $A_3$ antagonists related to the 1,4-dihyropyridine nucleus with sterically bulky groups at the 4-, 5-, and 6-positions, such as 2-methyl-6-phenyl-4-styryl-1,4-dihydro-pyridine-3,5-dicarboxylic acid diethyl ester and 2-methyl-6-phenyl-4-phenylethynyl-1,4-dihydropyridine-3,5-dicarboxylic acid 5-benzyl ester, named MRS1097 and MRS1191, respectively (**94** and **95**), were also reported (Jiang et al. 1996; Jiang et al. 1997).

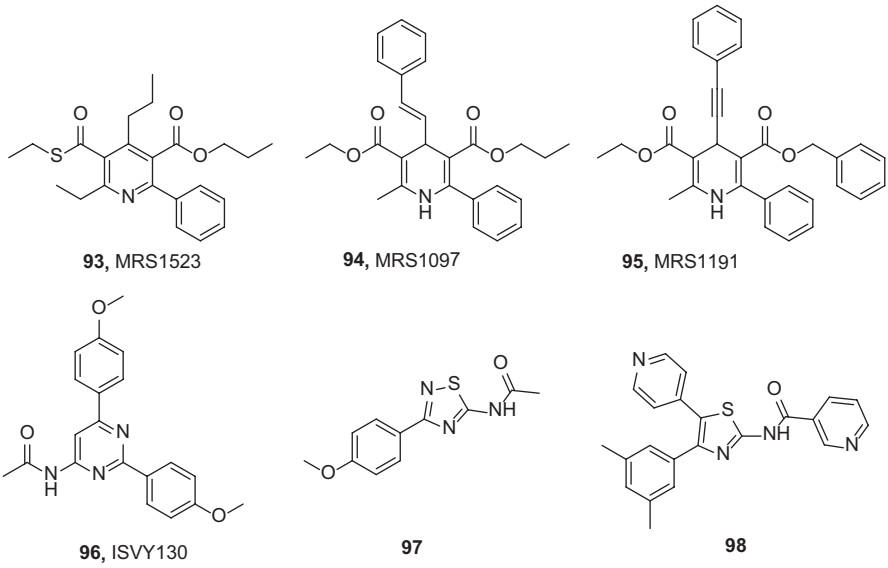


**Fig. 7.5** Aromatic monocyclic systems: pyridine, dihydropyridine, pyrimidine, thiazole, and thiadiazole derivatives as $A_3$AR antagonists

Among monocyclic compounds, the diaryl 2- or 4-amidopyrimidines have been reported as $A_3$AR antagonists. In particular, *N*-(2,6-bis(4-methoxyphenyl) pyrimidin-4-yl)acetamide derivative **96** named ISVY130 showed favorable affinity at the $hA_3$AR ($K_i$ 3.6 nM) (Cosimelli et al. 2008).

Thiazole and thiadiazole analogues were initially identified by simplifying the bicyclic ring system of isoquinolines and quinazolines with several monocyclic rings as a promising class of adenosine $A_3$AR antagonists (Jung et al. 2004). In this group, *N*-[3-(4-methoxyphenyl)-[1,2,4]thiadiazol-5-yl]acetamide (**97**) was reported as a potent $hA_3$AR antagonist with a $K_i$ value of 0.79 nM (Jung et al. 2004). Subsequently, a series of 4-phenyl-5-pyridyl-1,3-thiazole derivatives with $hA_3$AR affinity was identified (Miwatashi et al. 2008). As a result, the SAR study identified a potent $A_3$AR antagonist **98** with $K_i$ values of 0.36 nM for $hA_3$AR and 1.6 nM for $rA_3$AR, although no further studies have been published using this compound.

A class of $hA_3$AR antagonists, structurally related to the bicyclic isoquinoline and quinazoline urea derivatives, has been reported. The combination of the optimal substituents in the two series led to the potent $hA_3$AR antagonist *N*-(2-methoxyphenyl)-$N^9$-(2-(3-pyridyl)quinazolin-4-yl)urea **99** (VUF5574, Fig. 7.6) with a $K_i$ value of 4.0 nM and > 2400-fold selectivity versus $A_1$ and $A_{2A}$ARs (Van Muijlwijk-Koezen et al. 2000).

The first class of $A_3$AR antagonists with a bicyclic structure, rigorously related to the adenine nucleus, was described within a series of $N^6$-ureido-substituted 2-phenyl-9-benzyl-8-azaadenines. In this family, the adenine-like structure was responsible for the antagonist activity, while the phenylcarbamoyl group was

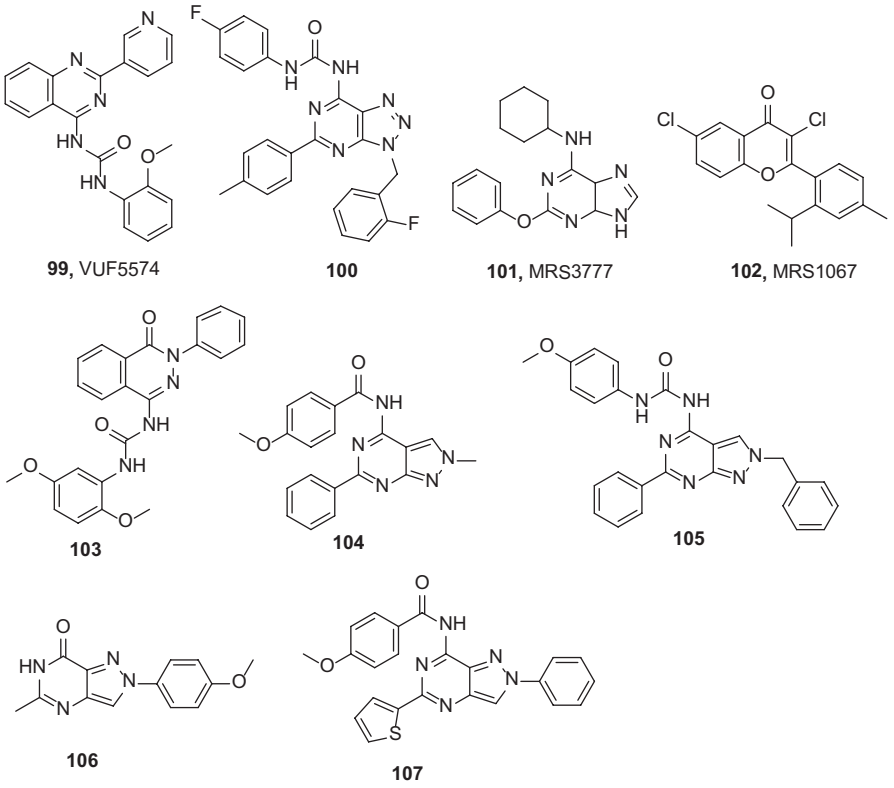


**Fig. 7.6** Aromatic bicyclic systems: quinazoline, (aza) adenine, flavone, 2-phenylphthalazine, pyrazolo[3,4-d]pyrimidine, and pyrazolo[4,3-d]pyrimidin derivatives as $A_3AR$ antagonists

important for selectivity at the $A_3AR$ (**100**) (Biagi et al. 2005). A series of adenine-based derivatives was also synthesized using "reversine" (2-(4-morpholinoanilino)-$N^6$-cyclohexyladenine) as a template. One of the most interesting compounds in terms of $hA_3AR$ affinity and selectivity was MRS3777 (**101**, $K_i$ $hA_3AR$ = 47 nM), which was derived from substitution of the $N^6$-cyclohexyl moiety of reversine with a 2-phenyloxy group. In $rA_3AR$ binding assays, these adenine derivatives reflected the species dependence of affinity that is typical of most known nonnucleoside $A_3AR$ antagonists, i.e., they were inactive at 10 μM (Jacobson et al. 2009).

The SAR optimization of the bicyclic flavone nucleus led to the MRS1067 (**102**) as the most potent and selective $hA_3AR$ compound of this series ($K_i$ $hA_3AR$ = 591 nM) (Jacobson et al. 1997). At the $rA_3AR$, MRS1067 (30 μM) completely antagonized agonist effects in RBL-2H3 rat basophilic cells (Shin et al. 1996).

Among bicyclic systems, the 2-phenylphthalazin-1(2*H*)-one scaffold was identified for the design of $hA_3AR$ antagonists. Introduction of different amide and ureido moieties led to the 2,5-dimethoxyphenylphthalazin-1(2*H*)-one **103** being the most potent and selective $A_3$ antagonist among this series ($K_i$ $hA_3AR$ = 0.77 nM) (Poli et al. 2011).

The pyrazolo[3,4-*d*]pyrimidine nucleus structurally related to the adenine nucleus has been also reported (Taliani et al. 2010). The SAR profile of this series highlighted the importance of amide or ureide functions at the 4-position along with a phenyl ring at the 6-position for $A_3$AR affinity and selectivity, such as in compounds **104** and **105**, respectively. In a related work, the 2-arylpyrazolo[4,3-*d*] pyrimidin-7-one derivatives were also examined, in which the new derivatives showed high affinity for the h$A_3$AR and increasing selectivity versus the other AR subtypes in comparison with the pyrazolo[3,4-*d*]pyrimidine isomers. Aryl/arylalkyl substitution at the 5-position of such derivatives was poorly tolerated for $A_3$AR binding affinity, while small groups at the same position were shown to increase ligand−receptor interaction. In addition, the introduction of a methoxy group on the 2-phenyl ring led to the most potent compound of the series (**106**) (Lenzi et al. 2009) Furthermore, a large number of 2-arylpyrazolo[4,3-*d*]pyrimidin-7-amine or 7-acylamine derivatives have been reported as potent $A_3$AR antagonists (Squarcialupi et al. 2013, 2016). In particular, the 2-phenyl-5-(2-thienyl)-pyrazolo[4,3-*d*] pyrimidin-7-(4-methoxybenzoyl)amine **107** was a potent h$A_3$AR antagonist in this series with a $K_i$ value of 0.02 nM (Squarcialupi et al. 2016).

The tricyclic triazoloquinazoline scaffold represented by compound CGS15943 (**108**, Fig. 7.7) was one of the first nonxanthine h$A_3$AR antagonists. CGS15943 displayed a $K_i$ value of 514 nM for h$A_3$AR and thus was a nonselective AR antagonist. This heterocycle proved to be a suitable starting template for the design of potent and selective h$A_3$AR antagonists (Kim et al. 1998). Acylation of the free amino group at the $N^5$-position of CGS15943 with aryl or arylalkyl moieties has enhanced both h$A_3$AR affinity and selectivity. This finding was exemplified by MRS1220 (**109**) that showed subnanomolar affinity at the h$A_3$AR with ~400- and ~40-fold selectivity vs. r$A_1$AR and r$A_{2A}$AR subtypes, respectively (Kim et al. 1996, 1998). However, the selectivity in human was not maintained in rat and mouse. In particular, MRS1220 is $A_{2A}$AR-selective in those species, with a $K_i$ values >10 μM at the r and m$A_3$ARs (Wan et al. 2004; Gao et al. 2009). The structurally related pyrazolo-triazolo-pyrimidines for the development of AR antagonists have been broadly reviewed (Baraldi et al. 2008; Cheong et al. 2013). Bioisosteric replacement of the phenyl ring of CGS15943 with a heterocyclic pyrazole ring led to the first example of an $A_{2A}$AR antagonist named 8FBPTP, featuring an 8-substituted pyrazolo-triazolo-pyrimidine core (Gatta et al. 1993; Dionisotti et al. 1994). Subsequently, a large number of tricyclic compounds (MRE series) were prepared during SAR optimization studies based on facile synthetic chemistry leading to substitutions at the $C^2$-, $C^5$-, $C^9$-, $N^7$-, and $N^8$-positions of the pyrazolo-triazolo-pyrimidine nucleus (Baraldi et al. 2008). Attention was focused on the $N^8$ substitution patterns, due to the complete inactivity of the $N^7$-substituted derivatives at the h$A_3$AR. The most potent and selective compounds at the h$A_3$AR subtype emerged from the combination of a small alkyl chain at the $N^8$-pyrazole position with a (substituted)phenylcarbamoyl residue at the $N^5$-position (Baraldi et al. 2000). Compound **110** is one of the most favorable examples representing this class, with high affinity ($K_i$ h$A_3$AR = 0.29 nM) and selectivity over both rat and h$A_1$ARs and $A_{2A}$ARs (Varani et al. 2000). Another important compound of this series is the 4-pyridyl-carbamoyl derivative **111** that showed high affinity with a $K_i$ value of 10 pM at h$A_3$AR (Maconi et al. 2002).

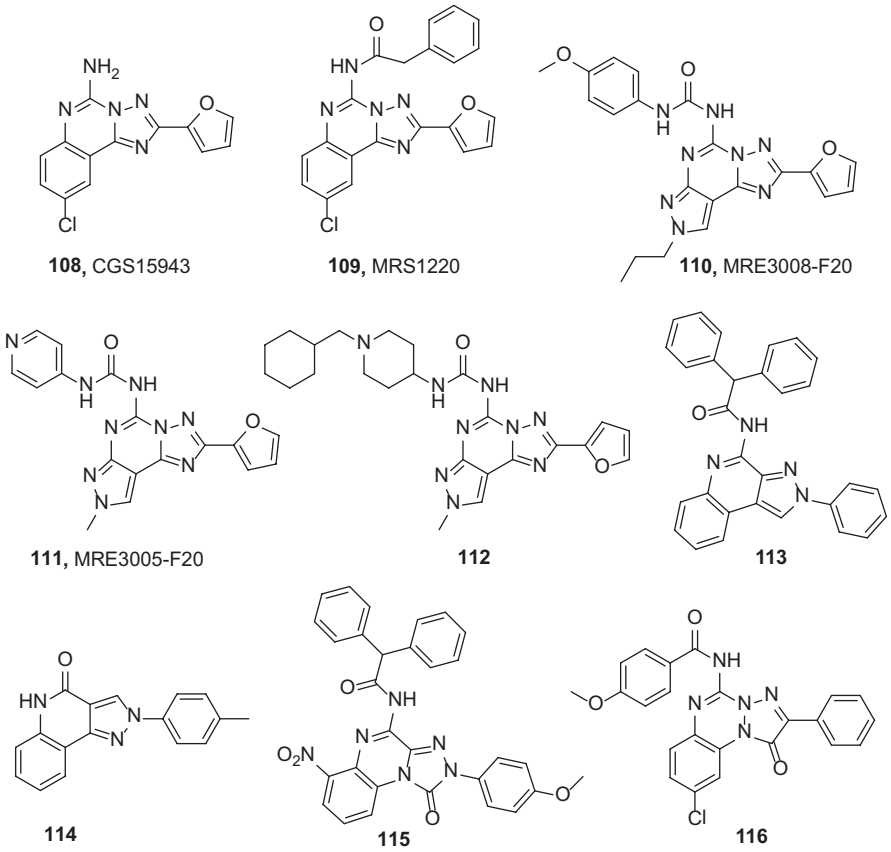


**Fig. 7.7** Aromatic tricyclic systems: triazoloquinazoline, pyrazolo-triazolo-pyrimidine, pyrazoloquinolines, triazoloquinoxaline, and aminophenyltriazolobenzotriazinone derivatives as $A_3AR$ antagonists

Consequently, replacement of pyridin-4-yl moiety of MRE3005-F20 **111** with a substituted piperidine ring led to the hydrochloride salt of 1-(1-(cyclohexylmethyl) piperidin-4-yl)-3-(2-(furan-2-yl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)urea **112**. This compound was the most active of the series showing high $hA_3AR$ affinity and selectivity against the other subtypes, with aqueous solubility of 8 mg/mL at physiological pH (Baraldi et al. 2012).

The tricyclic pyrazolo[3,4-*c*]/[4,3-*c*]quinolines have been reported as $A_3AR$ antagonists. Several 4-benzoylamido derivatives were prepared by introduction of bulky and lipophilic (hetero)aroylamino groups or a benzylcarbamoyl residue at the 4-position of pyrazolo[3,4-*c*]quinoline. An example of these derivatives is compound **113,** shown in Fig. 7.7, that exhibited a $K_i$ value of 8.9 nM in binding experiments (Colotta et al. 2007). In a related effort, further pyrazoloquinolines as structural isomers of the parent 2-arylpyrazolo[3,4-*c*]quinoline derivatives have also

been reported. Among them, the 2-(*p*-tolyl)-2*H*-pyrazolo[4,3-*c*]quinolin-4(5*H*)-one derivative **114** showed high affinity and selectivity ($K_i$ $hA_3AR$ = 9 nM) as evaluated in radioligand binding assays (Baraldi et al. 2005).

Triazolo[4,3-*a*]quinoxaline was also identified as a suitable scaffold for $A_3AR$ antagonists (Colotta et al. 2004; Lenzi et al. 2006). Efficient substitution of the 2-, 4-, and 6-positions of the tricyclic template, with molecular modeling investigations, led to the identification of optimal structural requirements for $A_3AR$ affinity and selectivity. In particular, sterically hindered and lipophilic acylamino moieties at the 4-position enhanced $A_3AR$ affinity and selectivity (**115**, $K_i$ $hA_3AR$ = 0.8 nM, Fig. 7.7) (Jacobson et al. 2009).

The aminophenyltriazolobenzotriazinone $A_3AR$ antagonists have been reported. In this series, the structural modifications by introduction of appropriate moieties on the 5-amino function and in the 4′-and/or 9-positions led to compound **116** (Fig. 7.7) which showed a $K_i$ value of 1.6 nM at the $A_3AR$ and no significant affinity at the other ARs (Da Settimo et al. 2007).

## 7.5 Allosteric Modulators of the $A_3AR$

The SAR of three major heterocyclic classes of positive allosteric modulators (PAMs) have been explored: 3-(2-pyridinyl)isoquinolines (e.g., **117**, Fig. 7.8), 1*H*-imidazo-[4,5-*c*]quinolin-4-amines, and 2,4-disubstituted quinolines (Göblyös et al. 2006; Kim et al. 2009; Heitman et al. 2009). The imidazo-[4,5-*c*]quinolin-4-amines (**118**–**121**) have been most extensively explored, and a key PAM in this series is LUF6000 **119**. The closely related series of 2,4-disubstituted quinolines is represented by amide derivative LUF6096 **122**, which was shown to be a potent PAM, but with a short half-life in vivo (Du et al. 2012). Species differences are evident in the $A_3AR$ PAMs, and a potent PAM at the r or $mA_3ARs$ is still lacking (Du et al. 2018). However, **119** was reported to alleviate erectile dysfunction in rats treated with streptozotocin to induce diabetes (Cohen and Fishman 2016).

A functional bias in the allosteric actions of imidazo-[4,5-*c*]quinolin-4-amines has been characterized (Gao et al. 2011). LUF6000 was found to be more efficacious in enhancing agonist $E_{max}$ of low-efficacy partial agonists than high-efficacy agonists, suggesting flexibility in modulating $E_{max}$.

## 7.6 Modeling and Structural Probing of the $A_3AR$

The facility of having a consistent model of ligand recognition at the $A_3AR$ has guided the design of novel orthosteric ligands. Extensive site-directed mutagenesis (SDM) of the $hA_3AR$ has been performed to locate the residues involved in ligand recognition (Gao et al. 2002b; Duong et al. 2005). Constitutively active mutations

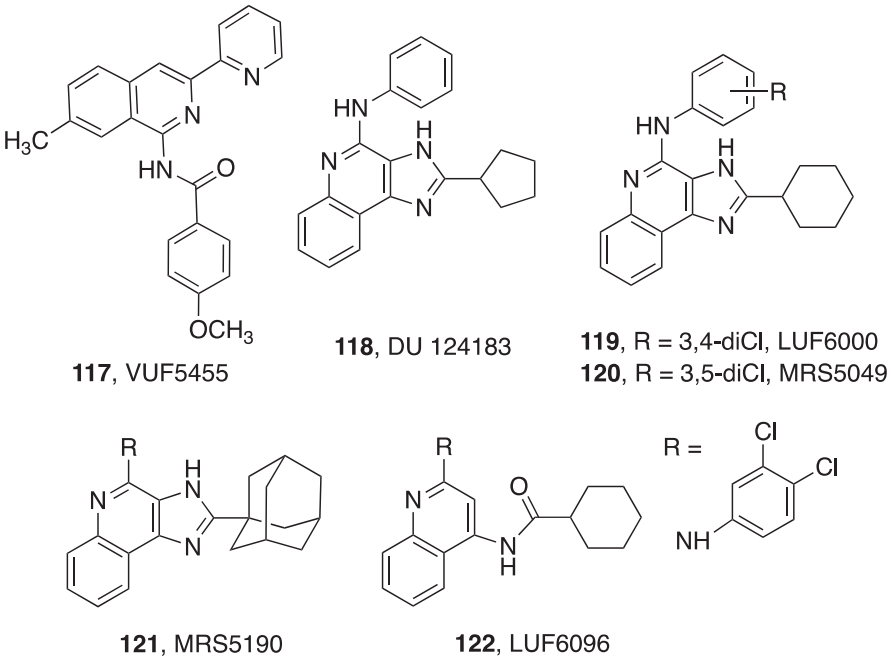


**Fig. 7.8** Representative positive allosteric modulators (PAMs) of the $A_3AR$

of the $A_3AR$ were reported (Chen et al. 2001). Homology modeling of the $hA_3AR$ based on several successive templates (rhodopsin and the $hA_{2A}AR$) has identified conserved residues in the putative binding site that recognize the ribose moiety and the adenine moiety (Cheong et al. 2013; Ciancetta and Jacobson 2017; Dal Ben et al. 2014). Both docking and molecular dynamics simulations have been performed to predict ligand complexes of the $A_3AR$. In addition, a neoceptor approach to identifying complementarity between the receptor protein and a bound agonist analogue has been applied to the $A_3AR$ (Jespers et al. 2018), and its prediction of proximity of the ribose moiety to hydrophilic side chains in TM3 and TM7 has been supported by experimental and computational methods. A hybrid model of the agonist-bound $hA_3AR$ has been proposed in order to accommodate the bulky C2-arylethynyl groups when combined with the (N)-methanocarba modification. An outward movement of TM2 (second transmembrane helix), similar to its position in active states of opsin and the $\alpha_2$-adrenergic receptor, is needed to prevent steric clash of the receptor protein with the C2 substituent. A functional bias in the efficacy of orthosteric agonists to favor the cAMP pathway has been found to correlate with the length of the rigid C2-substituent (Baltos et al. 2016); compound **54** was the most elongated analogue tested.

The amino acid residues that are associated with the allosteric action of 3-(2-pyridinyl)isoquinolines and imidazo-[4,5-*c*]quinolin-4-amines have been probed through mutagenesis (Gao et al. 2003b) and molecular modeling (Deganutti et al. 2015). However, the precise binding site of the $A_3AR$ PAMs has not been established.

## 7.7 Conclusions

The clinical studies with two $A_3$AR agonists (**14** and **15**) for treating autoimmune inflammatory disorders and liver diseases are continuing and appear encouraging. The interest in both agonists and antagonists of the $A_3$AR for therapeutic application has motivated numerous SAR studies of selective agonists, antagonists, partial agonists, and allosteric modulators. Subnanomolar affinity and selectivity of >10,000-fold have been achieved for various compound classes. The issue of species dependence of the $A_3$AR affinity has to be addressed in each medicinal chemistry study, especially considering that most antagonist classes greatly favor the $hA_3$AR over the rat and mouse homologues. The design of $A_3$AR orthosteric ligands is now largely guided by computational approaches. We have amassed a large body of data concerning the relationship of receptor structure and function and have supported many of the hypotheses generated with experimental data.

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