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Original article

Synthesis of a series of 8-(substituted-phenyl)xanthines and a study on the effects of substitution pattern of phenyl substituents on affinity for adenosine A_1 and A_{2A} receptors

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ABSTRACT

A new series of 8-(substituted-phenyl)xanthines have been synthesized and compounds were evaluated for their affinity for A_1 and A_2 adenosine receptors (AR) using radioligand binding assays. The effects of varying the positions of 8-phenyl substituents on affinity and selectivity at A_1 and A_{2A} adenosine receptors have been studied. Isovanilloid 1,3-dimethyl-8-[4-methoxy-3-(2-morpholin-4-ylethoxy)phenylxanthine (**9d**) displayed the highest affinity and selectivity towards A_{2A} AR subtypes with $K_i = 100$ nM over A_1 receptors (Ki > 100 mM). It has been observed that substitution pattern on 8-phenyl group greatly affects the affinity and selectivity at adenosine receptors, with A_{2A} tolerating bulkier substituents than did A_1 receptors.

1. Introduction

In recent years, considerable efforts have been dedicated to the development of potent and selective adenosine receptor antagonists as therapeutic agents [1–3]. Substituted xanthines represent the most potent class of adenosine receptors antagonists reported to date [4–7]. A variety of xanthine analogues have already been synthesized and assessed for their potency and selectivity at A_1 and A_2 adenosine receptors [5,6]. Structure–activity studies have established that structural modifications at 1- and 3-positions of the xanthine nucleus do not greatly affect the binding ability of the compounds for adenosine receptors. However the most dramatic alterations in potencies of the xanthines as antagonists of adenosine receptors result from substitution in the 8-position of this heterocyclic system [5,8]. Introduction of alkyl, cycloalkyl or a phenyl ring in the 8-position of 1,3-dipropylxanthines generates a variety of potent and selective adenosine receptor antagonists [8,9].

8-Phenyltheophylline is the parent member of a variety of potent adenosine receptor antagonists. It has been reported that appropriate substituents on the 8-phenyl ring not only affects the potency and selectivity towards adenosine receptors but also the solubility properties [9]. On one side, monosubstituted 8-(*p*-hydroxyphenyl)xanthine has been chosen as a suitable lead compound to develop MRS-1754 (**1**) as a potent and selective A_{2B} receptor antagonist [10], and on other disubstitution as in the case of 8-(2-hydroxy-4-methoxyphenyl)xanthine (**2**) (Fig. 1) is reported to increase 90 fold selectivity towards A_1 versus A_2 receptors [9]. The incorporation of polar substituents has been shown to improve the otherwise extremely limited water solubility of 8-phenylxanthines and increase their usefulness as potential therapeutic agents [9,10].

In view of the above observations, it was decided to study the impact of substituting polar dialkylaminoethoxy substituents at *para* or *meta* positions of the 8-phenyl ring along with an *ortho* methoxy group on the adenosine receptors binding affinity and selectivity. Herein we report the synthesis of a new series of 8-(substituted-phenyl)xanthines and the effects of the substitution pattern of phenyl substituents on binding affinity at A_1 and A_{2A} adenosine receptors.

2. Chemistry

The synthetic routes to various 8-(substituted-phenyl)xanthines have been depicted in Schemes 1–4. The synthesis of 5,6-diamino-1,3-dimethyluracil (**3**), a key compound for the synthesis of all the 8-substituted derivatives, was performed according to the general

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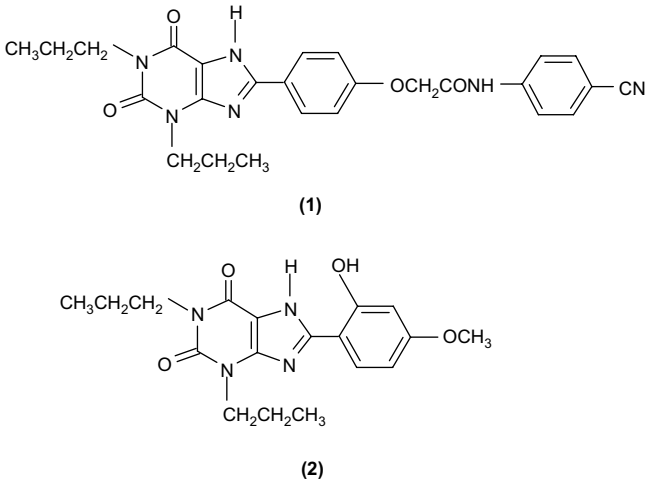
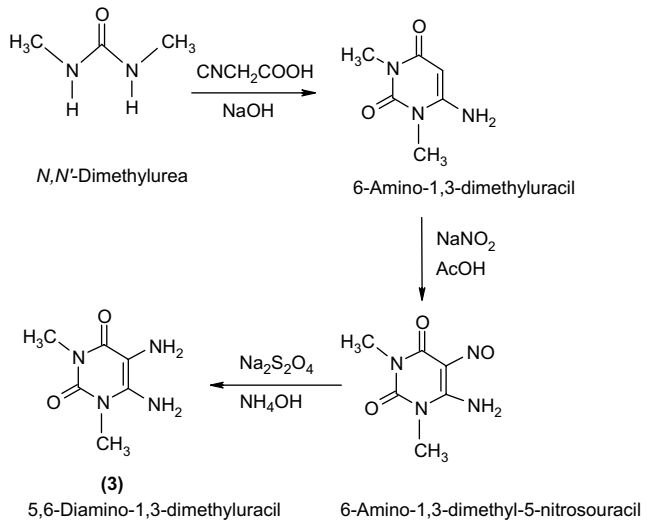


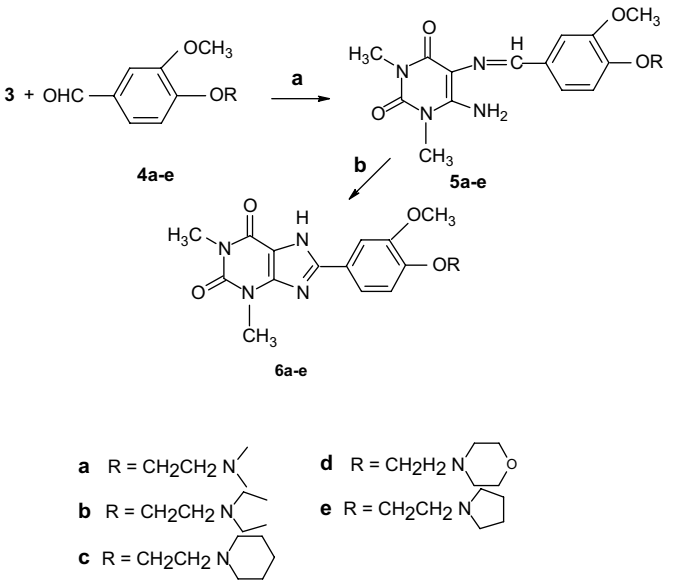
Fig. 1. Structures of xanthine based adenosine receptor antagonists MRS-1754 (**1**) and 8-(2-hydroxy-4-methoxyphenyl)xanthine (**2**).

method [11,12] summarized in Scheme 1. 1,3-Dimethyl-5-nitrosouracil was prepared by condensing *N*,*N*′-dimethylurea and cyanoacetic acid in the presence of acetic anhydride to obtain 6-aminouracil and subsequent nitrosation with sodium nitrite. Reduction of nitrosouracil with sodium dithionite in concentrated ammonium hydroxide afforded quite an unstable diaminouracil **3**, which was then reacted with appropriately substituted aldehydes **4a**–**e**, **7a**–**e** and **10a**–**e** to afford corresponding benzylidene derivatives **5a**–**e**, **8a**–**e** and **11a**–**e**. Subsequent cyclization of these compounds by refluxing in thionyl chloride for 1 h yielded the desired 8-substituted 1,3-dimethylxanthines **6a**–**e**, **9a**–**e** and **12a**–**e**.

Substituted-aldehydes **4a**–**e**, **7a**–**e** and **10a**–**e** were prepared by treating vanillin, isovanillin and 3-hydroxybenzaldehyde, respectively, with hydrochloride of the requisite dialkylaminoethyl chloride such as β-dimethylaminoethyl chloride, β-diethylaminoethyl chloride, 1-(2-chloroethyl)piperidine, 4-(2-chloroethyl)morpholine and 1-(2-chloroethyl)-pyrrolidine in refluxing ethyl methyl ketone in the presence of anhydrous potassium carbonate. The completion of the reaction was monitored by thin layer chromatography (TLC). The oily residues obtained after processing the reaction mixture were used as such for further reaction. Treatment of these substituted aldehydes with 5,6-diamino-1,3-dimethyluracil (**3**) in MeOH–AcOH (4:1) at room temperature resulted in the formation



Scheme 1. Synthesis of starting compound 5,6-diamino-1,3-dimethyluracil (**3**).

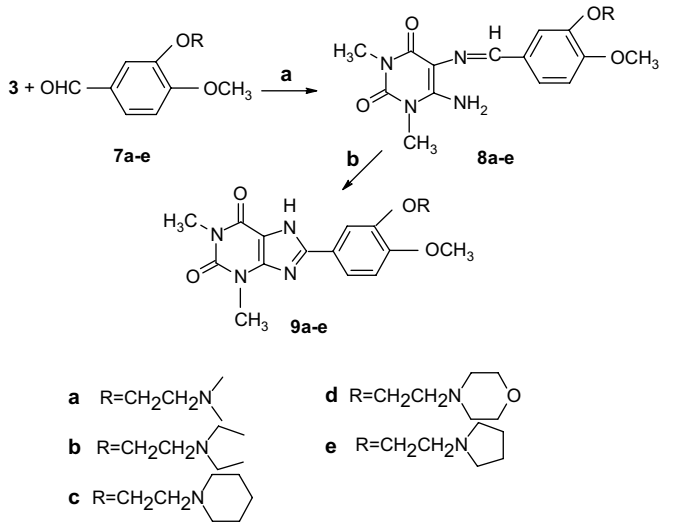


Scheme 2. Synthetic route to compounds **6a**–**e**. Reagents and conditions. (a) MeOH/CH_3COOH, room temperature, 18 h; (b) $SOCl_2$, reflux, 30–40 min; NH_4OH.

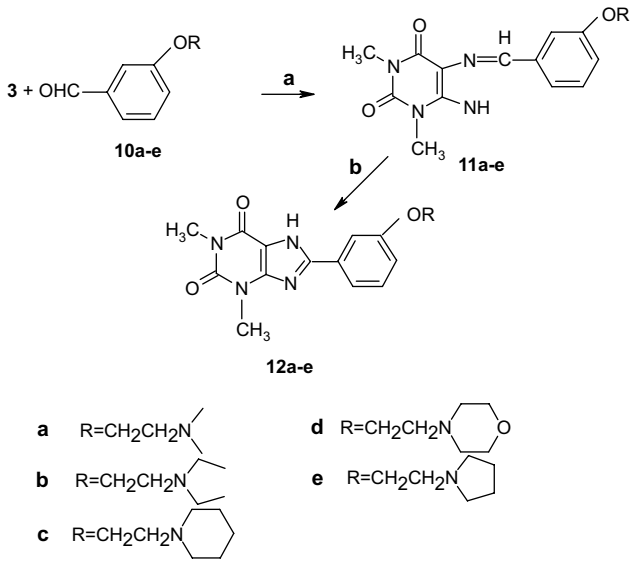
of corresponding benzylidene adducts **5a**–**e**, **8a**–**e** and **11a**–**e**. The structures of the compounds were characterized using various spectral analyses. ^{1}H NMR spectra of these benzylidenes exhibited a characteristic one proton singlet at ∼δ 9.75 for N═C*H* and a slightly broad singlet, which disappeared on deuterium exchange, for two exchangeable protons of –NH_2 group at δ 6 ppm. Protons of phenyl ring and its substituents resonated at their required positions. Subsequent ring closure of these intermediates by refluxing in thionyl chloride for 30–40 min afforded the target 8-(substituted-phenyl)xanthines **6a**–**e**, **9a**–**e** and **12a**–**e**. Singlets for N═C*H* at ∼δ 9.75 and of –NH_2 at ∼δ 6 were found missing in the ^{1}H NMR spectra of these cyclized products, while peaks for other protons were present at their expected values.

3. Biological evaluation

The newly synthesized compounds were evaluated in radioligand binding studies at cloned human A_1 and A_{2A} adenosine



Scheme 3. Synthetic route to compounds **9a**–**e**. Reagents and conditions. (a) MeOH/CH_3COOH, room temperature, 18 h; (b) $SOCl_2$, reflux, 30–40 min; NH_4OH.



Scheme 4. Synthetic route to compounds **12a–e**. Reagents and conditions. (a) MeOH/CH_3COOH, room temperature, 18 h; (b) $SOCl_2$, reflux, 30–40 min; NH_4OH.

receptors. [^{3}H]DPCPX and [^{3}H]ZM-241385 were used as radioligands for A_1 and A_{2A} adenosine receptors, respectively [10,13].

4. Results and discussion

Table 1 summarizes the observed affinities of various newly synthesized 8-phenylxanthine derivatives in radioligand binding assays at human A_1 and A_{2A} receptors. Data for the standard A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and the A_{2A} receptor antagonist 4-[2-[[7-amino-2-(furyl)1,2,4-triazolo[2,3-*a*]1,3,5-triazin-5-yl]-amino]ethyl]phenol (ZM-241385) has also been provided for comparison.

The three series of xanthine derivatives **6a–e**, **9a–e** and **12a–e** exhibited varying degrees of affinity and selectivity towards A_1 and A_{2A} receptor subtypes. Disubstituted vanilloid based xanthine derivatives (**6a–c**) with a methoxy group *ortho* to polar substituents such as dimethylaminoethoxy, diethylaminoethoxy and piperidinoethoxy on 4-position of phenyl ring were found to possess ~100 times more binding affinity for adenosine A_{2A} receptors ($K_i = \sim 1$ μM) in comparison to A_1 receptors ($K_i > 100$ μM). Pyrrolidinyl derivative **6e** was found to be only 10 times more selective for A_{2A} over A_1 receptors. However, the results with morpholinoethoxy substituted compound **6d** seem anomalous as this compound showed little binding affinity for either receptor (Table 1). In general, these compounds substituted with a polar *para* substituent along with an *ortho* methoxy group on the phenyl group seems to be more selective towards A_{2A} over A_1 receptors. Similarly, for the second series of isovanilloid based xanthine derivatives **9a–d** also, the overall selectivity and potency of compounds was observed at A_{2A} over A_1 receptors. The rearrangement of two side substituents of the 8-phenyl ring so that the methoxy is at 4-position and polar substituents at 3-position on the phenyl group resulted in moderate decrease in the selectivity of open ring analogues **9a** (30 times), **9b** (70 times) and piperidino substituted derivative **9c** (38 times) for A_{2A} receptors versus A_1 receptors. However the data obtained with morpholinyl substituted product **9d** was surprising in comparison to its vanilloid based analogue **6d**. 1,3-Dimethyl-8-[4-methoxy-3-(2-morpholin-4-ylethoxy)phenylxanthine **(9d)** emerged as the most active and selective compound of the series with a $K_i = 100$ nM at A_{2A} receptors but showing little displacement of binding at A_1 receptors at concentrations up to 100 μM. It can be said that interchange of position of alkylaminoalkoxy side chain and methoxy group on 8-phenyl ring brings about changes in binding properties of xanthine derivatives for adenosine receptor subtypes significantly. This also depends on the type of attached substituent.

Table 1
Adenosine A_1 and A_{2A} binding affinities of compounds **6a–e**, **9a–e** and **12a–e** and reference compounds.

S. No.	Compd. No.	Code	A_1K_i (μM)	$A_{2A}K_i$ (μM)
1	**6a**	(RG-DPJ-23)	>100	0.6 (0.4–0.9)
2	**6b**	(RG-DPJ-24)	>100	1.1 (0.8–1.6)
3	**6c**	(RG-DPJ-26)	>100	0.7 (0.3–1.4)
4	**6d**	(RG-DPJ-33)	>100	~100
5	**6e**	(RG-DPJ-35)	11.2 (2.1–59)	1.0 (0.3–2.6)
6	**9a**	(RG-DPJ-66)	13.5 (2.7–64)	0.4 (0.2–2.0)
7	**9b**	(RG-DPJ-76)	43 (10–181)	0.6 (0.4–1.2)
8	**9c**	(RG-DPJ-92)	>100	2.7 (1.3–55)
9	**9d**	(RG-DPJ-79)	>100	0.1 (0.02–0.4)
10	**12a**	(RG-DPJ-174)	0.8 (0.5–1.3)	0.4 (0.2–0.7)
11	**12b**	(RG-DPJ-99)	14 (1.3–145)	0.1 (0.03–0.4)
12	**12c**	(RG-DPJ-172)	2.1 (0.9–4.9)	0.8 (0.3–7.2)
13	**12d**	(RG-DPJ-168)	2.1 (0.7–17)	1.4 (0.3–2.8)
14	**12e**	(RG-DPJ-170)	1.6 (0.8–3.3)	0.4 (0.2–0.6)
	DPCPX		0.095 (0.06–0.15)	0.13[a]
	ZM 24,1385		0.54[a]	0.064 (0.03–0.14)

K_i values are given with 95% confidence limits.
[a] Ref. [14].

Monosubstituted xanthine derivatives (**12a–e**), in which only polar side chain is present at the 3-position of 8-phenyl group, exhibited potent affinity for both adenosine receptor subtypes. The binding affinity was a little more pronounced at A_{2A} than at A_1 receptors, except for diethylaminoethoxy substituted compound **12b**, which was 140 times more potent at A_{2A} than at A_1 receptors. Introduction of polar substituents at *meta* position of 8-phenyl ring without an *ortho* methoxy group in this series results in decreased selectivity for A_2 receptors over A_1 receptors.

In all, it has been observed that substitution pattern and type of substituents on 8-phenyl group greatly affects the affinity and selectivity of xanthine derivatives at adenosine receptors. Previous studies on substituted phenyl [9] or cyclohexyl xanthines [8] had revealed compounds that were generally selective for A_1 over A_2 receptors. We achieved the opposite selectivity with several compounds (notably **6a–c**, **9a–d**, **12b**). In order to investigate the structure activity relationship, we can compare the affinity of the new xanthine derivatives with the parent analogue, 8-(2-hydroxy-4-methoxyphenyl)xanthine (**2**), which is reported to be 90 times more selective for A_1 ($K_i = 0.01$ μM) over A_2 ($K_i = 0.9$ μM) receptors. In the new compounds, the presence of a methoxy substituent *ortho* to a polar side chain at 3- or 4-position of phenyl ring results in increased selectivity for A_2 over A_1 receptors. A polar side chain at 3-position of 8-phenyl ring without a methoxy group results in almost equal selectivity for both subtypes. It can be concluded that suitable selection and positioning of aryl substituents may lead to the development of potent and selective xanthine based adenosine receptor antagonists.

5. Experimental

5.1. Chemistry

The melting points reported are uncorrected, ^{1}H NMR spectra were recorded on Brucker AC-300F, 300 MHz instrument using Me_4Si (TMS) as an internal standard (chemical shifts in δ, ppm). The IR spectra were recorded on Perkin–Elmer 882 spectrophotometer. The purity of the compounds was established by thin layer chromatography and elemental analyses (C, H, N). Elemental analyses were carried out on a Perkin–Elmer 2400 model. IR spectra were

obtained with potassium bromide discs (ν_{max} in cm^{-1}). Plates for TLC were prepared according to Stahl (E. Merck) using EtOAc as solvent (activated at 110 °C for 30 min) and were visualized by exposure to iodine vapours. Anhydrous sodium sulphate was used as a drying agent. All solvents were dried and freshly distilled prior to use according to standard procedures.

*5.1.1. General procedure for the synthesis of various aldehydes **4a–e**, **7a–e** and **10a–e***

Requisite alkylaminoethyl chloride hydrochloride (6.0 mmol) was added to a stirred and refluxing slurry of vanillin (1.0 g, 6.57 mmol), isovanillin (1.0 g, 6.57 mmol) or 3-hydroxybenzaldehyde (1.0 g, 8.19 mmol) in ethyl methyl ketone (C_4H_8O) (40 mL) in the presence of anhydrous potassium carbonate (2.0 g, 14.47 mmol). The reaction mixture was further refluxed for 6 h with continuous stirring. The completion of the reaction was monitored by TLC. On completion, the reaction mixture was cooled, filtered and the solvent was removed under reduced pressure to obtain oily residue of corresponding aldehyde **4a–e**, **7a–e** and **10a–e**, which were used as such for further reaction.

*5.1.2. General procedure for the synthesis of various benzylidene derivatives **5a–e**, **8a–e** and **11a–e***

To a stirred solution of 5,6-diamino-1,3-dimethyluracil (**3**) (1.0 g, 5.87 mmol) in MeOH–AcOH (4:1, 40 mL) was slowly added the solution of above obtained oily residue of respective aldehyde **4a–e**, **7a–e** and **10a–e** in methanol (24 ml). The reaction mixture was further stirred overnight at room temperature. The residue obtained after removal of solvent under reduced pressure was dissolved in ice-cold water and alkalized with sodium hydroxide. The resultant turbid solution was cooled in ice for complete precipitation. The precipitate obtained was filtered off, washed with ice cold water and dried to obtain corresponding benzylidene derivatives **5a–e**, **8a–e** and **11a–e**.

*5.1.2.1. 6-Amino-5-[{4-(2-dimethylaminoethoxy)-3-methoxybenzylidene}amino]-1,3-dimethyluracil (**5a**).* Yield: 20.52%; m.p. 178–186 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.34 (s, 6H, –N(CH_3)$_2$), 2.80 (t, 2H, –CH_2N<), 3.38 (s, 3H, N–CH_3), 3.52 (s, 3H, N–CH_3), 3.90 (s, 3H, –OCH_3), 4.14 (t, 2H, –OCH_2–), 5.76 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.89 (d, 1H, C*H*, arom, J_o = 8.29 Hz), 7.28 (dd, 1H, C*H*, arom, J_o = 8.86, J_m = 1.80 Hz), 7.35 (d, 1H, C*H*, arom, J_m = 1.55 Hz), 9.72 (s, 1H, N=C*H*).

*5.1.2.2. 6-Amino-5-[{4-(2-diethylaminoethoxy)-3-methoxybenzylidene}amino]-1,3-dimethyluracil (**5b**).* Oily residue; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.04 (t, 6H, –N(CH_2CH_3)$_2$), 2.54 (m, 4H, –N(CH_2CH_3)$_2$), 2.86 (t, 2H, –CH_2N<), 3.38 (s, 3H, N–CH_3), 3.61 (s, 3H, N–CH_3), 3.90 (s, 3H, –OCH_3), 4.08 (t, 2H, –OCH_2–), 5.76 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.97 (s, 1H, C*H*, arom), 7.35 (s, 1H, C*H*, arom), 7.46 (s, 1H, C*H*, arom), 9.72 (s, 1H, N=C*H*).

*5.1.2.3. 6-Amino-5-[{3-methoxy-4-(2-piperidin-1-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**5c**).* Yield: 26.8%; m.p. 110–114 °C. ^{1}H NMR ($CDCl_3$): δ 1.46 (m, 2H, CH_2, piperidine), 1.60 (pent, 4H, 2 × CH_2, piperidine), 2.50 (br s, 4H, –N(CH_2)$_2$, piperidine), 2.81 (t, 2H, –CH_2N<), 3.35 (s, 3H, N–CH_3), 3.49 (s, 3H, N–CH_3), 3.88 (s, 3H, –OCH_3), 4.14 (t, 2H, –OCH_2–), 5.98 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.86 (d, 1H, C*H*, arom, J_o = 8.34 Hz), 7.27 (d, 1H, C*H*, arom, J_o = 8.32 Hz), 7.34 (s, 1H, C*H*, arom), 9.69 (s, 1H, N=C*H*).

*5.1.2.4. 6-Amino-5-[{3-methoxy-4-(2-morpholin-4-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**5d**).* Yield: 38.10%; m.p. 180–184°C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.59 (t, 4H, –N(CH_2)$_2$, morpholine), 2.85 (t, 2H, –CH_2N<), 3.37 (s, 3H, N–CH_3), 3.50 (s, 3H, N–CH_3), 3.75 (t, 4H, O(CH_2)$_2$, morpholine), 3.90 (s, 3H, –OCH_3), 4.17 (t, 2H, –OCH_2–), 5.86 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.88 (d, 1H, C*H*, arom, J_o = 8.20 Hz), 7.28 (dd, 1H, C*H*, arom, J_o = 8.26, J_m = 1.70 Hz), 7.35 (d, 1H, C*H*, arom, J_m = 1.80 Hz), 9.71 (s, 1H, N=C*H*).

*5.1.2.5. 6-Amino-5-[{3-methoxy-4-(2-pyrrolidin-1-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**5e**).* Yield: 53.58%; m.p. 112–114 °C. ^{1}H NMR ($CDCl_3$): δ 1.82 (br s, 4H, 2 × CH_2, pyrrolidine), 2.62 (br s, 4H, –N(CH_2)$_2$, pyrrolidine), 2.95 (t, 2H, –CH_2N<), 3.35 (s, 3H, N–CH_3), 3.49 (s, 3H, N–CH_3), 3.88 (s, 3H, –OCH_3), 4.14 (t, 2H, –OCH_2–), 6.09 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.85 (d, 1H, C*H*, arom, J_o = 8.22 Hz), 7.26 (dd, 1H, C*H*, arom, J_o = 8.21, J_m = 1.53 Hz), 7.34 (d, 1H, C*H*, arom, J_m = 1.45 Hz), 9.68 (s, 1H, N=C*H*).

*5.1.2.6. 6-Amino-5-[{3-(2-dimethylaminoethoxy-4-methoxybenzylidene}amino]-1,3-dimethyluracil (**8a**).* Yield: 15.36%; m.p. 208–210 °C. ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 6H, –N(CH_3)$_2$), 2.75 (t, 2H, –CH_2N<), 3.30 (s, 3H, N–CH_3), 3.50 (s, 3H, N–CH_3), 3.83 (s, 3H, –OCH_3), 4.15 (t, 2H, –OCH_2–), 5.65 (br s, 2H, –NH_2, disappeared on D_2O exchange), 6.80 (m, 1H, C*H*, arom), 7.39 (m, 2H, C*H*, arom), 9.65 (s, 1H, N=C*H*).

*5.1.2.7. 6-Amino-5-[{3-(2-diethylaminoethoxy)-4-methoxybenzylidene}amino]-1,3-dimethyluracil (**8b**).* Yield: 34.5%; m.p. 186–188 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.09 (t, 6H, –N(CH_2CH_3)$_2$), 2.72 (m, 6H, –CH_2N(CH_2CH_3)$_2$), 3.38 (s, 3H, N–CH_3), 3.50 (s, 3H, N–CH_3), 3.90 (s, 3H, –OCH_3), 4.20 (t, 2H, –OCH_2), 5.79 (br s, 2H, –NH_2, disappeared on D_2O exchange), 7.50 (m, 3H, C*H*, arom), 9.75 (s, 1H, N=C*H*).

*5.1.2.8. 6-Amino-5-[{4-methoxy-3-(2-piperidin-1-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**8c**).* Yield: 63.45%; m.p. 179–180 °C. ^{1}H NMR ($CDCl_3$): δ 1.80 (s, 6H, CH_2, piperidine), 2.50 (m, 4H, –N(CH_2)$_2$, piperidine), 2.80 (t, 2H, –CH_2N<), 3.40 (s, 3H, N–CH_3), 3.56 (s, 3H, N–CH_3), 3.90 (s, 3H, –OCH_3), 4.10 (t, 2H, –OCH_2–), 5.60 (br s, 2H, –NH_2, disappeared on D_2O exchange), 6.90 (m, 1H, C*H*, arom), 7.30 (m, 2H, C*H*, arom), 9.60 (s, 1H, N=C*H*).

*5.1.2.9. 6-Amino-5-[{4-methoxy-3-(2-morpholin-4-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**8d**).* Yield: 29.93%; m.p. 218–220 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.68 (m, 6H, –CH_2N(CH_2)$_2$, morpholine), 3.30 (s, 3H, N–CH_3), 3.50 (s, 3H, N–CH_3), 3.70 (t, 4H, O(CH_2)$_2$, morpholine), 3.90 (s, 3H, –OCH_3), 4.20 (t, 2H, –OCH_2), 5.86 (s, 2H, –NH_2, disappeared on D_2O exchange), 6.90 (m, 1H, C*H*, arom), 7.36 (m, 2H, C*H*, arom), 9.70 (s, 1H, N=C*H*).

*5.1.2.10. 6-Amino-5-[{4-methoxy-3-(2-pyrrolidin-1-ylethoxy)benzylidene}amino]-1,3-dimethyluracil (**8e**).* Yield: 31.05%; m.p. 196–198 °C. ^{1}H NMR ($CDCl_3$): δ 1.80 (m, 4H, 2 × CH_2, pyrrolidine), 2.60 (m, 6H, –CH_2N(CH_2)$_2$), 3.30 (s, 3H, N–CH_3), 3.50 (s, 3H, N–CH_3), 3.80 (s, 3H, –OCH_3), 4.20 (t, 2H, –OCH_2–), 5.70 (br s, 2H, –NH_2, disappeared on D_2O exchange), 6.90 (s, 1H, C*H*, arom), 7.30 (m, 2H, C*H*, arom), 9.60 (s, 1H, N=C*H*).

*5.1.2.11. 6-Amino-5-[{3-2-dimethylaminoethoxybenzylidene}amino]-1,3-dimethyluracil (**11a**).* Yield: 71.2%; m.p. 160–164 °C. ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 6H, –N(CH_3)$_2$), 2.78 (t, 2H, –CH_2N<), 3.31 (s, 3H, N–CH_3), 3.55 (s, 3H, N–CH_3), 4.16 (t, 2H, –OCH_2–), 5.90 (br s, 2H, –NH_2, disappeared on D_2O exchange), 6.95 (d, 1H, C*H*, arom, J_o = 8.29 Hz), 7.31 (t, 1H, C*H*, arom, J_o = 7.81 Hz), 7.72 (m, 2H, C*H*, arom), 9.80 (s, 1H, N=C*H*).

*5.1.2.12. 6-Amino-5-[{3-(2-diethylaminoethoxy)benzylidene}amino]-1,3-dimethyluracil (**11b**).* Yield: 37.6%; m.p. 150–152 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.10 (t, 6H, –N(CH_2CH_3)$_2$), 2.60 (m, 6H,

–C*H*$_2$N(C*H*$_2$CH$_3$)$_2$), 3.30 (s, 3H, N–C*H*$_3$), 3.40 (s, 3H, N–C*H*$_3$), 4.10 (t, 2H, –OC*H*$_2$–), 5.90 (br s, 2H, –N*H*$_2$, disappeared on D_2O exchange), 7.00 (m, 1H, C*H*, arom), 7.30 (m, 3H, C*H*, arom), 9.80 (s, 1H, N=C*H*).

*5.1.2.13. 6-Amino-5-[{3-(2-piperidin-1-yl-ethoxy)-benzylidene}amino]-1,3-dimethyluracil (**11c**).* Yield: 56.5%; m.p. 198–202 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (m, 2H, –C*H*$_2$, piperidine), 1.59 (p, 4H, 2 × C*H*$_2$, piperidine), 2.50 (br s, 4H, –N(C*H*$_2$)$_2$, piperidine), 2.81 (t, 2H, –C*H*$_2$N<), 3.35 (s, 3H, N–C*H*$_3$), 3.55 (s, 3H, N–C*H*$_3$), 4.14 (t, 2H, –OC*H*$_2$–), 5.98 (s, 2H, –N*H*$_2$, disappeared on D_2O exchange), 6.86 (dd, 1H, C*H*, arom, $J_o = 8.34$, $J_m = 1.75$ Hz), 7.23 (t, 1H, C*H*, arom, $J_o = 8.10$ Hz), 7.74 (m, 2H, C*H*, arom), 9.69 (s, 1H, N=C*H*).

*5.1.2.14. 6-Amino-5-[{3-(2-morpholin-4-yl-ethoxy)-benzylidene}amino]-1,3-dimethyluracil (**11d**).* Yield: 51.7%; m.p. 196–200 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.58 (t, 4H, –N(C*H*$_2$)$_2$, morpholine), 2.81 (t, 2H, –C*H*$_2$N<), 3.37 (s, 3H, N–C*H*$_3$), 3.60 (s, 3H, N–C*H*$_3$), 3.70 (t, 4H, O(C*H*$_2$)$_2$, morpholine), 4.17 (t, 2H, –OC*H*$_2$–), 5.86 (s, 2H, –N*H*$_2$, disappeared on D_2O exchange), 6.95 (dd, 1H, C*H*, arom, $J_o = 8.31$, $J_m = 1.92$ Hz), 7.32 (m, 1H, C*H*, arom), 7.72 (m, 2H, C*H*, arom), 9.71 (s, 1H, N=C*H*).

*5.1.2.15. 6-Amino-5-[{3-(2-pyrrolidin-1-ylethoxy)-benzylidene}amino]-1,3-dimethyluracil (**11e**).* Yield: 29.7%; m.p. 192–196 °C. ^{1}H NMR ($CDCl_3$): δ 1.86 (br s, 4H, 2 × C*H*$_2$, pyrrolidine), 3.08 (m, 6H, –N(C*H*$_2$)$_2$, pyrrolidine and –C*H*$_2$N<), 3.36 (s, 3H, N–C*H*$_3$), 3.61 (s, 3H, N–C*H*$_3$), 4.26 (t, 2H, –OC*H*$_2$–), 6.09 (s, 2H, –N*H*$_2$, disappeared on D_2O exchange), 6.92 (dd, 1H, C*H*, arom, $J_o = 8.05$, $J_m = 1.98$ Hz), 7.32 (m, 2H, C*H*, arom), 7.72 (m, 1H, C*H*, arom), 9.68 (s, 1H, N=C*H*).

*5.1.3. General procedure for the synthesis of various 8-(substituted-phenyl)xanthine derivatives **6a–e**, **9a–e** and **12a–e***

Benzylidene derivatives **5a–e**, **8a–e** and **11a–e** (1.0 g, 2.5 mmol) thus obtained were refluxed separately in thionyl chloride (20 mL) for 30–40 min to affect cyclization. The excess thionyl chloride was removed under reduced pressure to obtain a solid product. Ice cold water was added to it and resultant suspension was neutralized with ammonium hydroxide solution. The precipitate obtained was collected by filtration, dried and recrystallized from a mixture of DMF and methanol to afford the desired products **6a–e**, **9a–e** and **12a–e**, respectively.

*5.1.3.1. 8-[4-(2-Dimethylaminoethoxy)-3-methoxyphenyl]-1,3-dimethylxanthine (**6a**).* Yield: 48.28%; m.p. 246–250 °C. IR: 3290, 1690, 1650, 1495, 1215; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.23 (s, 6H, –N(C*H*$_3$)$_2$), 2.65 (t, 2H, –C*H*$_2$N<), 3.24 (s, 3H, N–C*H*$_3$), 3.48 (s, 3H, N–C*H*$_3$), 3.84 (s, 3H, –OC*H*$_3$), 4.09 (t, 2H, –OC*H*$_2$–), 7.07 (d, 1H, C*H*, arom, $J_o = 8.47$ Hz), 7.68 (m, 2H, C*H*, arom); Anal. Calc. for $C_{18}H_{23}N_5O_4$: C, 57.89; H, 6.20; N, 18.75. Found: C, 57.53; H, 6.05; N, 18.67%.

*5.1.3.2. 8-[4-(2-Diethylaminoethoxy)-3-methoxyphenyl]-1,3-dimethylxanthine (**6b**).* Yield: 19.11%; m.p. 176–180 °C. IR: 3300, 1690, 1650, 1490, 1210; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.08 (t, 6H, –N(CH$_2$C*H*$_3$)$_2$), 2.65 (m, 4H, –N(C*H*$_2$CH$_3$)$_2$), 2.94 (t, 2H, –C*H*$_2$N<), 3.44 (s, 3H, N–C*H*$_3$), 3.67 (s, 3H, N–C*H*$_3$), 3.94 (s, 3H, –OC*H*$_3$), 4.12 (t, 2H, –OC*H*$_2$–), 6.98 (s, 1H, C*H*, arom), 7.37 (s, 1H, C*H*, arom), 7.51 (s, 1H, C*H*, arom); Anal. Calc. for $C_{20}H_{27}N_5O_4$: C, 59.83; H, 6.78; N, 17.40. Found: C, 59.41; H, 6.29; N, 17.12%.

*5.1.3.3. 1,3-Dimethyl-8-[3-methoxy-4-(2-piperidin-1-ylethoxy)-phenyl]xanthine (**6c**).* Yield: 20.12%; m.p. 204–206 °C. IR: 3350, 2940, 1695, 1640, 1480, 1225; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.45 (m, 2H, C*H*$_2$, piperidine), 1.57 (p, 4H, 2 × C*H*$_2$, piperidine), 2.52 (br s, 4H, –N(C*H*$_2$)$_2$, piperidine), 2.80 (t, 2H, –C*H*$_2$N<), 3.38 (s, 3H, N–C*H*$_3$), 3.61 (s, 3H, N–C*H*$_3$), 3.89 (s, 3H, –OC*H*$_3$), 4.17 (t, 2H, –OC*H*$_2$–), 7.03 (s, 1H, C*H*, arom), 7.31 (s, 1H, C*H*, arom), 7.83 (s, 1H, C*H*, arom); Anal. Calc. for $C_{21}H_{27}N_5O_4$: C, 61.00; H, 6.58; N, 16.93%. Found: C, 60.90; H, 6.29; N, 16.57%.

*5.1.3.4. 1,3-Dimethyl-8-[3-methoxy-4-(2-morpholin-4-ylethoxy)-phenylxanthine (**6d**).* Yield: 35.75%; m.p. 264–268 °C. IR: 3300, 2940, 1695, 1645, 1490, 1220; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.54 (br s, 4H, –N(C*H*$_2$)$_2$, morpholine), 2.78 (t, 2H, –C*H*$_2$N<), 3.31 (s, 3H, N–C*H*$_3$), 3.54 (s, 3H, N–C*H*$_3$), 3.62 (t, 4H, O(C*H*$_2$)$_2$, morpholine), 3.88 (s, 3H, –OC*H*$_3$), 4.18 (t, 2H, –OC*H*$_2$–), 7.02 (d, 1H, C*H*, arom, $J_o = 8.28$ Hz), 7.29 (s, 1H, C*H*, arom), 7.73 (m, 1H, C*H*, arom), 13.50 (br s, 1H, N–*H*); Anal. Calc. for $C_{20}H_{25}N_5O_5$: C, 57.82; H, 6.06; N, 16.85. Found: C, 57.38; H, 5.96; N, 16.52%.

*5.1.3.5. 1,3-Dimethyl-8-[3-methoxy-4-(2-pyrrolidin-4-ylethoxy)-phenyl]xanthine (**6e**).* Yield: 22.27%; m.p. 232–234 °C. IR: 3300, 2925, 1695, 1640, 1470, 1260; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.71 (br s, 4H, 2 × C*H*$_2$, pyrrolidine), 2.57 (br s, 4H, –N(C*H*$_2$)$_2$, pyrrolidine), 2.85 (t, 2H, –C*H*$_2$N<), 3.28 (s, 3H, N–C*H*$_3$), 3.52 (s, 3H, N–C*H*$_3$), 3.86 (s, 3H, –OC*H*$_3$), 4.12 (t, 2H, –OC*H*$_2$–), 7.04 (d, 1H, C*H*, arom, $J_o = 7.25$ Hz), 7.72 (m, 2H, C*H*, arom); Anal. Calc. for $C_{20}H_{25}N_5O_4$: C, 60.13; H, 6.30; N, 17.53. Found: C, 60.08; H, 6.08; N, 17.24%.

*5.1.3.6. 8-[3-(2-Dimethylaminoethoxy)-4-methoxyphenyl]-1,3-dimethylxanthine (**9a**).* Yield: 49.29%; m.p. 230–232 °C. IR: 3220, 1680, 1640, 1470, 1270; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.34 (s, 6H, –N(C*H*$_3$)$_2$), 2.77 (t, 2H, –C*H*$_2$N<), 3.36 (s, 3H, N–C*H*$_3$), 3.61 (s, 3H, N–C*H*$_3$), 3.90 (s, 3H, –OC*H*$_3$), 4.13 (t, 2H, –OC*H*$_2$-), 6.87 (m, 1H, C*H*, arom), 7.69 (m, 2H, C*H*, arom); Anal. Calc. for $C_{18}H_{23}N_5O_4$: C, 57.89; H, 6.20; N, 18.75. Found: C, 57.71; H, 6.25; N, 18.59%.

*5.1.3.7. 8-[3-(2-Diethylaminoethoxy)-4-methoxyphenyl]-1,3-dimethylxanthine (**9b**).* Yield: 36.21%; m.p. 168–170 °C. IR: 3350, 1690, 1650, 1490, 1210; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.09 (t, 6H, –N(CH$_2$C*H*$_3$)$_2$), 2.65 (m, 4H, –N(C*H*$_2$CH$_3$)$_2$), 2.95 (t, 2H, –C*H*$_2$N<), 3.44 (s, 3H, N–C*H*$_3$), 3.67 (s, 3H, N–C*H*$_3$), 3.91 (s, 3H, –OC*H*$_3$), 4.19 (t, 2H, –OC*H*$_2$–), 6.90 (s, 1H, C*H*, arom), 7.30 (s, 1H, C*H*, arom), 7.80 (s, 1H, C*H*, arom); Anal. Calc. for $C_{20}H_{27}N_5O_4$: C, 59.83; H, 6.78; N, 17.40. Found: C, 59.44; H, 6.51; N, 17.33%.

*5.1.3.8. 1,3-Dimethyl-8-[4-methoxy-3-(2-piperidin-1-ylethoxy)-phenyl]xanthine (**9c**).* Yield: 60.36%; m.p. 230–232 °C. IR: 3380, 1680, 1640, 1480, 1210; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.45 (m, 2H, C*H*$_2$, piperidine), 1.57 (m, 4H, 2 × C*H*$_2$, piperidine), 2.51 (br s, 4H, –N(C*H*$_2$)$_2$, piperidine), 2.78 (t, 2H, –C*H*$_2$N<), 3.36 (s, 3H, N–C*H*$_3$), 3.61 (s, 3H, N–C*H*$_3$), 3.89 (s, 3H, –OC*H*$_3$), 4.16 (t, 2H, –OC*H*$_2$–), 6.93 (d, 1H, C*H*, arom, $J_m = 2.21$ Hz), 7.35 (s, 1H, C*H*, arom), 7.83 (s, 1H, C*H*, arom); Anal. Calc. for $C_{21}H_{27}N_5O_4$: C, 61.00; H, 6.58; N, 16.93%. Found: C, 60.90; H, 6.31; N, 16.74%.

*5.1.3.9. 1,3-Dimethyl-8-[4-methoxy-3-(2-morpholin-4-ylethoxy)-phenylxanthine (**9d**).* Yield: 53.32%; m.p. 250–252 °C. IR: 3350, 1690, 1650, 1480, 1250; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.62 (t, 4H, –N(C*H*$_2$)$_2$, morpholine), 2.88 (t, 2H, –C*H*$_2$N<), 3.41 (s, 3H, N–C*H*$_3$), 3.64 (s, 3H, N–C*H*$_3$), 3.73 (t, 4H, O(C*H*$_2$)$_2$, morpholine), 3.90 (s, 3H, –OC*H*$_3$), 4.26 (t, 2H, –OC*H*$_2$-), 6.96 (d, 1H, C*H*, arom, $J_o = 8.28$ Hz), 7.76 (m, 2H, C*H*, arom) and 13.20 (br s, 1H, N–*H*); Anal. Calc. for $C_{20}H_{25}N_5O_5$: C, 57.82; H, 6.06; N, 16.85. Found: C, 57.57; H, 6.03; N, 16.65%.

*5.1.3.10. 1,3-Dimethyl-8-[4-methoxy-3-(2-pyrrolidin-4-ylethoxy)-phenyl]xanthine (**9e**).* Yield: 80.48%; m.p. 234–236 °C. IR: 3200, 2925, 1690, 1670, 1490, 1210; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.67 (br s, 4H, 2 × C*H*$_2$, pyrrolidine), 2.86 (br s, 4H, –N(C*H*$_2$)$_2$, pyrrolidine), 3.12 (t, 2H, –C*H*$_2$N<), 3.12 (s, 3H, N–C*H*$_3$), 3.37 (s, 3H, N–C*H*$_3$), 3.88

(s, 3H, –OCH_3), 4.29 (t, 2H, –OCH_2–), 6.90 (d, 1H, C*H*, arom, $J_o = 8.27$ Hz) and 7.71 (m, 2H, C*H*, arom); Anal. Calc. for $C_{20}H_{25}N_5O_4$: C, 60.13; H, 6.30; N, 17.53. Found: C, 60.08; H, 6.19; N, 17.35%.

*5.1.3.11. 8-[3-(2-Dimethylaminoethoxy)phenyl]-1,3-dimethylxanthine (**12a**).* Yield: 30.30%; m.p. 260–264 °C. IR: 3177, 1689, 1647, 1524, 1480, 1231, 1058; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.37 (s, 6H, –N(CH_3)$_2$), 2.80 (t, 2H, –CH_2N<), 3.33 (s, 3H, N–CH_3), 3.55 (s, 3H, N–CH_3), 4.16 (t, 2H, –OCH_2–), 6.98 (d, 1H, C*H*, arom, $J_o = 8.19$ Hz), 7.35 (t, 1H, C*H*, arom, $J_o = 7.84$ Hz) and 7.74 (m, 2H, C*H*, arom). Anal. Calc. for $C_{17}H_{21}N_5O_3$: C, 59.45; H, 6.16; N, 20.39. Found: C, 59.25; H, 6.13; N, 19.91%.

*5.1.3.12. 8-[3-(2-Diethylaminoethoxy)phenyl]-1,3-dimethylxanthine (**12b**).* Yield: 43.26%; m.p. 268–270 °C. IR: 3190, 1690, 1650, 1480, 1210; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.08 (t, 6H, -N(CH$_2$CH_3)$_2$), 2.65 (q, 4H, –N(CH_2CH$_3$)$_2$), 2.91 (t, 2H, –CH_2N<), 3.44 (s, 3H, N–CH_3), 3.66 (s, 3H, N–CH_3), 4.14 (t, 2H, –OCH_2–), 6.96 (d, 1H, C*H*, arom, $J_o = 7.31$ Hz), 7.33 (t, 1H, C*H*, arom, $J_o = 7.78$ Hz) and 7.75 (m, 2H, C*H*, arom). Anal. Calc. for $C_{19}H_{25}N_5O_3$: C, 61.43; H, 6.78; N, 18.85. Found: C, 61.23; H, 6.38; N, 18.45%.

*5.1.3.13. 1,3-Dimethyl-8-[3-(2-piperidin-4-ylethoxy)phenyl]xanthine (**12c**).* Yield: 62.82%; m.p. 264–266 °C. IR: 3153, 2932, 1696, 1651, 1523, 1479, 1449, 1225, 1059; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.46 (m, 2H, –CH_2, piperidine), 1.61 (p, 4H, 2 × CH_2, piperidine), 2.57 (br s, 4H, N–(CH_2)$_2$), 2.83 (t, 2H, –CH_2N<), 3.37 (s, 3H, N–CH_3), 3.60 (s, 3H, N–CH_3), 4.19 (t, 2H, –OCH_2-), 6.97 (dd, 1H, C*H,* arom, $J_o = 7.50$, $J_m = 1.75$ Hz), 7.35 (t, 1H, C*H*, arom, $J_o = 8.10$ Hz), and 7.74 (m, 2H, C*H*, arom). Anal. Calc. for $C_{20}H_{25}N_5O_3$: C, 62.65; H, 6.57; N, 18.26. Found: C, 62.38; H, 6.22; N, 18.15%.

*5.1.3.14. 1,3-Dimethyl-8-[3-(2-morpholin-4-ylethoxy)-phenyl]xanthine (**12d**).* Yield: 27.27%; m.p. 256–260 °C. IR: 3150, 2910, 170, 1650, 1290, 1230, 1120; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 2.60 (t, 4H, –N(CH_2)$_2$, morpholine), 2.83 (t, 2H, –CH_2N<), 3.42 (s, 3H, N–CH_3), 3.64 (s, 3H, N–CH_3), 3.72 (t, 4H, O(CH_2)$_2$, morpholine), 4.20 (t, 2H, –OCH_2-), 6.98 (dd, 1H, C*H*, arom, $J_o = 8.31$, $J_m = 1.92$ Hz), 7.34 (t, 1H, C*H*, arom, $J_o = 8.40$ Hz), 7.76 (m, 2H, C*H*, arom) and 13.49 (br s, 1H, N-*H*). Anal. Calc. for $C_{19}H_{23}N_5O_4$: C, 59.21; H, 6.01; N, 18.17. Found: C, 58.91; H, 5.87; N, 17.92%.

*5.1.3.15. 1,3-Dimethyl-8-[3-(2-pyrrolidin-1-ylethoxy)-phenyl]xanthine (**12e**).* Yield: 14.37%; m.p. >250 °C. IR: 3166, 1694, 1649, 1525, 1478, 1228, 1058; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 1.90 (br s, 4H, 2 × CH_2, pyrrolidine), 3.10 (m, 6H, –N(CH_2)$_2$, pyrrolidine and –CH_2N<), 3.38 (s, 3H, N–CH_3), 3.64 (s, 3H, N–CH_3), 4.30 (t, 2H, –OCH_2-), 6.97 (dd, 1H, 5-C*H*, arom, $J_o = 8.05$, $J_m = 2.23$ Hz), 7.34 (t, 2H, C*H*, arom, $J_o = 7.99$ Hz) and 7.75 (m, 1H, C*H*, arom). Anal. Calc. for $C_{19}H_{23}N_5O_3$: C, 61.77; H, 6.27; N, 18.96. Found: C, 61.38; H, 6.22; N, 18.42 %.

5.2. Adenosine binding assays

Radioligand binding assays of xanthine analogues were carried out using cloned human adenosine A_1 and A_{2A} receptors and [^{3}H]DPCPX and [^{3}H]ZM241385 as radioligands, respectively. [^{3}H]DPCPX (Tocris Cookson) (specific activity 103 Ci/mmol, concentration 1 mCi/ml) was used in assays at 20 nM. The receptor membrane preparation (Human recombinant Adenosine A_1 receptor – ES-010-M) was from Euroscreen. The recombinant adenosine A_1 receptor was stably expressed in CHO–K1 cells; the membrane suspensions (received as frozen aliquots in 7.5 mM Tris–HCl pH 7.5; 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose) were diluted in assay buffer on thawing. [^{3}H]ZM241385 (Tocris Cookson) (specific activity 0.777 TBq/mmol, concentration 37 MBq/ml) was used in assays at 20 nM. Receptor membrane preparation (Human A_{2A} receptor membrane-RBHA2AM) was from Perkin Elmer. The human recombinant A_{2A} receptor was expressed in HEK-293 cells. The membrane suspensions were received as frozen aliquots in 50 mM Tris–HCl (pH 7.4) and 10% sucrose and were diluted in assay buffer on thawing.

The assays were performed using a similar method as previously described [10,13]. Binding assays were performed using Milipore Multiscreen MHAF B3H60 filter plates presoaked in 0.3% polyethyleneimine (PEI). 10 μg of membrane protein was used in the final assay volume of 0.2 ml. Tris–HCl buffer [50 mM Tris–HCl, 0.5 mM EDTA and 10 mM $MgCl_2$, pH 7.4] supplemented with 1U/ml adenosine deaminase for A_{2A} binding assays and Hepes buffer [20 mM Hepes, 10 mM NaCl, 10 mM $MgCl_2$, pH 7.4] for A_1 binding assays were used. Stock solutions of the compounds were prepared in dimethylsulfoxide (DMSO), the final concentration of DMSO in assays was ≤1%. Nonspecific binding was measured in the presence of 100 μM known non-radioactive ligands and accounted for less than 5% of total binding.

The incubation time was 1 h at 25 °C. Termination of the incubation was performed by rapid filtration using a Millipore manifold at a pressure of 700 mbr. Filters were washed three times with 200 μl of the relevant assay buffer. Scintillation fluid was added (100 μl/well) and bound radioactivity was counted in a scintillation counter (Wallac Microbeta). Testing was done in three stages: first, all the compounds were tested at 100 μM; second, those causing greater than 60% displacement of binding were retested at three different concentrations from 1 to 100 μM; third, those that were consistently active in a concentration-dependent manner were tested over a full concentration range to determine the IC_{50} values and K_i. Data was analysed using GraphPad Prism, Version 2.0 (GraphPad, San Diego, CA). For nonlinear regression analysis, the Cheng–Prusoff equation and K_D values of 1.6 nM (human A_1) for [^{3}H]DPCPX and 1 nM (human A_{2A}) for [^{3}H]ZM-241385 were used to calculate K_i values from IC_{50} values.

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