Non-ribose ligands for the human adenosine A1 receptor

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CHAPTER 5

Structure-activity relationships of 2-amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles reveal full agonists with picomolar affinity for the human adenosine A_1 receptor

Recently, we reported on selective agonists for human adenosine A_1 receptors with an unusual structure lacking the ribose moiety of traditional adenosine-like agonists. The synthesis, affinity and activity of twelve such non-ribose ligands, all 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles, are described in this study. The substitution pattern of the phenyl ring determined the affinity of the compounds for the adenosine A_1 receptor, whereas efficacy was determined by the substituents on both the phenyl ring and the sulfanyl chain.
The di-methoxyphenyl substituted compounds had very little affinity. The monosubstituted compounds recognized one binding state/site with higher nanomolar affinity. cAMP second-messenger studies revealed an activity profile ranging from partial agonism to partial inverse agonism.
3,4-Methylenedioxyphenyl substituted compounds recognized two binding states/sites with a picomolar affinity for the high affinity site. These compounds were full agonists that largely superseded the prototypic agonist N^6-cyclopentyladenosine (CPA) in affinity and potency.

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Introduction

Traditionally agonists for adenosine receptors are structurally related to the endogenous ligand adenosine, consisting of an adenine base coupled to a ribose moiety. In the search for selective, high affinity agonists for the human adenosine A_1 receptor, adenosine has been substituted at different positions. Especially the introduction of a hydrophobic substituent such as cyclopentyl at the N^6-position resulted in potent adenosine A_1 agonists[1].

We recently learned from patent literature about a new class of adenosine ligands, the 2-amino-4-(3,4-disubstituted-phenyl)-6-(2-hydroxyethylsulfanyl)-pyridine-3,5-dicarbonitriles, structurally unrelated to adenosine itself[2,3]. We synthesized a number of such compounds ourselves and observed that several compounds displayed a significant affinity and efficacy towards different adenosine receptor subtypes.[4,5]. In our hands it turned out that the substitution pattern at the phenyl ring R^1 greatly affected the efficacy of the compounds (for structures see Table 5.1). But what would happen to the affinity and efficacy of the compounds for the human adenosine A_1 receptor if the ethanol substituent R^2 would be modified? To address this issue, ethanol was replaced with methylethanol or ethylpropionate for four classes of phenyl-substituted compounds.

This synthetic program yielded a number of compounds with unprecedented high affinity and potency, some of which were significantly more potent (10 to 100-fold) than the decades-long reference agonist, *N*^{6}-cyclopentyladenosine (CPA).

Experimental section

Materials and Methods

N^6-cyclopentyladenosine (CPA) and *N*^{6}-cyclopentyl-9-methyladenine (N0840) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Bovine serum albumin (BSA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), forskolin, DEAE dextran and chloroquine were from Sigma (St. Louis MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Biochemicals (Mannheim, Germany) and Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX with a specific activity of 124 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^{3}H] cyclic adenosine monophosphate ([^{3}H] cAMP with a specific activity of 32.1 Ci/mmol) was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). Protein kinase A (PKA) was isolated from bovine adrenal glands according to Leurs et al.[6]. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[7]. HEK293 cells expressing the human adenosine A_{2A} or the A_3 receptor

were kindly provided by Dr. J. Wang, Biogen, USA and Dr. K.-N. Klotz, University of Würzburg, Germany, respectively.

All other reagents were obtained from commercial sources and all solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (^{1}H NMR, 200 MHz; ^{13}C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ(ppm) and the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Melting points were determined on a Büchi melting point apparatus and are uncorrected. Elemental analyses on all end products (**13-24**) were performed by the Leiden Institute of Chemistry and are within 0.4% of the theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F_{254} plates.

Synthesis

General Procedure for functionalized malononitriles (1-4)

To malononitrile (1.32 mL, 20.8 mmol) dissolved in EtOH (14 mL) was added an aldehyde (20 mmol) followed by 3 drops of piperidine. This reaction mixture was then refluxed for 1 hour, and then allowed to cool to room temperature, upon which a precipitate was formed. The crude product was collected by filtration and used without any further purification.

2-(3,4-Dimethoxy-benzylidene)-malononitrile (1)

Off white solid, 53% yield. ^{1}H-NMR δ(DMSO): 8.39 (s, 1H, C*H*), 7.66-7.60 (m, 2H, phenyl-*H*), 7.27-7.22 (m, 1H, phenyl-*H*), 3.91 (s, 3H, CH_3), 3.83 (s, 3H, CH_3).

2-Benzo[1,3]dioxol-5-ylmethylene-malononitrile (2)

Off white solid, 46% yield. ^{1}H-NMR δ(DMSO): 8.39 (s, 1H, C*H*), 7.57-7.54 (m, 2H, phenyl-*H*), 7.24-7.19 (m, 1H, phenyl-*H*), 6.25 (s, 2H, -OCH_2O-).

2-(4-Dimethylamino-benzylidene)-malononitrile (3)

Off white solid, 45% yield. ^{1}H-NMR δ(DMSO): 8.08 (s, 1H, C*H*), 7.87 (d, 2H, J = 9.5 Hz phenyl-*H*), 6.89 (m, 2H, J = 9.5 Hz, phenyl-*H*), 3.13 (s, 6H, 2 × CH_3).

2-(3-Fluoro-benzylidene)-malononitrile (4)

Off white solid, 56% yield. ^{1}H NMR δ(DMSO): 8.59 (s, 1H, C*H*), 7.84-7.60 (m, 4H, phenyl-*H*).

General Procedure for 2-Amino-4-(substituted)-phenyl-6-phenylsulfanyl-pyridine-3,5-dicarbonitriles (5-8)

To a solution of the functionalized malononitrile (**1-4**) (10 mmol) in EtOH (10 mL), was added malononitrile (0.64 mL, 10 mmol), thiophenol (1.02 mL, 10 mmol) and triethylamine (50 µL), and the mixture heated at reflux for approximately 4 h. The reaction mixture was then allowed to cool to room temperature. The crude product precipitated upon cooling and was collected by filtration.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (5)

Yellow solid, 28% yield. ^{1}H-NMR δ(DMSO): 7.77 (br s, 2H, NH_2), 7.65-7.50 (m, 7H, phenyl-*H*), 7.15 (d, 2H, J = 8.8 Hz, phenyl-*H*), 3.67 (s, 3H, CH_3), 3.63 (s, 3H, CH_3).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (6)

Yellow solid, 34% yield. ^{1}H-NMR δ(DMSO): 7.80 (br s, 2H, NH_2), 7.64-7.60 (m, 2H, S-phenyl-*H*), 7.53-7.50 (m, 3H, S-phenyl-*H*), 7.20-7.04 (m, 3H, phenyl-*H*), 6.18 (s, 2H, -OCH_2O-).

2-Amino-4-(4-dimethylamino-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (7)

Yellow solid, 32% yield. ^{1}H-NMR δ(DMSO): 7.68 (br s, 2H, N*H*$_2$), 7.64-7.60 (m, 2H, phenyl-*H*), 7.54-7.48 (m, 2H, phenyl-*H*), 7.44 (d, 2H, J = 8.8 Hz, 4-NMe$_2$-phenyl-*H*), 6.86 (d, 2H, J = 8.8 Hz, 4-NMe$_2$-phenyl-*H*), 3.03 (s, 6H, 2 × C*H*$_3$).

2-Amino-4-(3-fluoro-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (8)

Yellow solid, 24% yield. ^{1}H-NMR δ(DMSO): 7.90 (br s, 2H, N*H*$_2$), 7.71-7.40 (m, 9H, phenyl-*H*).

General Procedure for 2-Amino-4-(substituted)-phenyl-6-mercapto-pyridine-3,5-dicarbonitriles (9-12)

The pyridine (**5-8**) (3 mmol) was dissolved in DMF (10 mL) and to this was added sodium sulfide (0.78 g, 10 mmol) and the mixture stirred at 80 °C for 2 h. Upon cooling to room temperature, 1 M HCl (20 mL) was added, resulting in the formation of a yellow precipitate. The crude product was collected by filtration.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (9)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.17-7.13 (m, 3H, phenyl-*H*), 3.85 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-mercapto-pyridine-3,5-dicarbonitrile (10)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.13-7.00 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-).

2-Amino-4-(4-dimethylamino-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (11)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.41 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.83 (d, 2H, J = 8.8 Hz, phenyl-*H*), 3.03 (s, 6H, 2 × C*H*$_3$).

2-Amino-4-(3-fluoro-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (12)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.65-7.59 (m, 1H, phenyl-*H*), 7.48-7.35 (m, 3H, phenyl-*H*).

General Procedure for 2-Amino-4-[(substituted)phenyl]-6-[(substituted)sulfanyl)]-pyridine-3,5-dicarbonitriles (13-24)

The free thiol (**9-12**) (1 mmol) was stirred with the bromo compounds (1 mmol), $NaHCO_3$ (0.34 g, 1 mmol) in DMF (2 mL) at room temperature for 2 h. Water (10 mL) was added to precipitate the crude product, which was collected by filtration. Purification by column chromatography on SiO_2, eluting with dichloromethane-methanol mixtures or ethylacetate-petroleum ether mixtures and/or ethylacetate, followed by recrystallisation gave the desired products (**13-24**) in approximately 40% yield.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (13)

White Solid. mp: 209 ºC. ^{1}H-NMR δ(DMSO): 7.96 (br s, 2H, N*H*$_2$) 7.19-7.13 (m, 3H, phenyl-*H*), 5.02 (m, 1H, O*H*), 3.86 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.69-3.66 (m, 2H, OC*H*$_2$), 3.32-3.19 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 167.0, 159.7, 158.0, 150.4, 148.3, 126.0, 121.5, 115.7, 115.6, 112.3, 111.5, 92.7, 85.9, 59.5, 55.7, 55.6, 32.8. MS (ESI): 356.9. Anal. ($C_{17}H_{16}N_4O_3S$) C, H, N, S.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (14)

White Solid. mp: 198 ºC. ^{1}H-NMR δ(DMSO): 7.97 (br s, 2H, N*H*$_2$) 7.19-7.13 (m, 3H, phenyl-*H*), 4.99 (d, 1H, J = 5.1 Hz, O*H*), 3.86 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.88-3.80 (m, 1H, C*H*), 3.42-3.17 (m, 2H, C*H*$_2$), 1.20 (d, 3H, J = 6.6 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 159.7, 158.0, 150.4, 148.3, 125.9, 121.6, 118.6, 112.3, 111.5, 93.6, 88.6, 68.1, 55.7, 55.6, 22.6. MS (ESI): 370.8. Anal. ($C_{18}H_{18}N_4O_3S$) C, H, N, S.

3-[2-amino-3,5-dicyano-4-(3,4-dimethoxy-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (15)

White Solid. mp: 172 ºC. ^{1}H-NMR δ(DMSO): 8.04 (br s, 2H, N*H*$_2$), 7.19-7.13 (m, 3H, phenyl-*H*), 4.12 (q, 2H, J = 7.3 Hz, OC*H*$_2$), 3.86 (s, 3H, OC*H*$_2$), 3.81 (s, 3H, OC*H*$_3$), 3.46-3.39 (m, 2H, C*H*$_2$CO), 2.82 (t, 2H, J = 6.9 Hz, C*H*$_2$S), 1.22 (t, 3H, J = 6.9 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 171.4, 166.6, 159.8, 158.1, 150.4, 148.3, 125.8, 121.5, 115.6, 112.3, 111.5, 91.9, 88.8, 60.2, 55.7, 55.6, 33.4, 24.9, 14.1. MS (ESI): 412.7. Anal. ($C_{20}H_{20}N_4O_4S$) C, H, N, S.

2-Amino-4-benzo[1,3]dioxol-5-yl-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (16)

White Solid. mp: 192 ºC. ^{1}H-NMR δ(DMSO): 7.99 (br s, 2H, N*H*$_2$), 7.16-7.01 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 5.02 (t, 1H, J = 5.5 Hz, O*H*), 3.67 (m, 2H, OC*H*$_2$), 3.38-3.32 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 197.7, 167.0, 159.7, 157.9, 148.9, 147.4, 127.4, 123.0, 115.6, 115.4, 108.9, 108.5, 101.8, 93.8, 59.5, 32.6. MS (ESI): 341.1. Anal. ($C_{16}H_{12}N_4O_3S$) C, H, N, S.

2-Amino-4-benzo[1,3]dioxol-5-yl-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (17)

White Solid. ^{1}H-NMR δ(DMSO): 7.98 (br s, 2H, N*H*$_2$), 7.12-7.00 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 4.99 (d, 1H, J = 4.7 Hz, O*H*), 3.94-3.83 (m, 1H, C*H*), 3.41-3.16 (m, 2H, C*H*$_2$), 1.20 (d, 3H, J = 6.2 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 159.6, 157.9, 148.9, 147.4, 127.5, 123.0, 115.6, 115.4, 108.9, 108.6, 101.9, 93.8, 85.8, 65.1, 38.3, 22.7. Anal. ($C_{17}H_{14}N_4O_3S$) C, H, N.

3-(2-Amino-4-benzo[1,3]dioxol-5-yl-3,5-dicyano-pyridin-6-ylsulfanyl)-propionic acid ethyl ester (18)

White Solid. mp: 162 ºC. ^{1}H-NMR δ(DMSO): 8.05 (br s, 2H, N*H*$_2$), 7.16-7.01 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 4.12 (q, 2H, J = 6.6 Hz, OC*H*$_2$), 3.44-3.35 (m, 2H, C*H*$_2$CO), 2.85-2.78 (m, 2H, C*H*$_2$S), 1.22 (t, 3H, J = 7.3 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 171.4, 166.5, 159.8, 157.9, 148.9, 147.4, 127.4, 123.0, 115.3, 108.9, 108.6, 101.8, 93.7, 86.0, 60.2, 33.3, 24.9, 14.1. MS (ESI): 396.7. Anal. ($C_{19}H_{16}N_4O_4S$) C, H, N, S.

2-Amino-4-(4-dimethylamino-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (19)

White Solid. mp: 268 ºC dec. ^{1}H-NMR δ(DMSO): 7.87 (br s, 2H, N*H*$_2$) 7.39 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.82 (d, 2H, J = 8.8 Hz, phenyl-*H*), 4.99 (t, 1H, J = 5.5 Hz, O*H*), 3.69-3.60 (m, 2H, OC*H*$_2$), 3.38-3.32 (m, 2H, C*H*$_2$S), 3.00 (s, 6H, 2 × C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.1, 160.0, 158.3, 151.5, 129.9, 120.0, 116.1, 116.0, 111.3, 93.2, 85.0, 59.6, 39.5, 32.6. MS (ESI): 339.8. Anal. ($C_{17}H_{17}N_5OS \cdot 0.2CH_2Cl_2$) C, H, N, S.

2-Amino-4-(4-dimethylamino-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (20)

White Solid. mp: 245 ºC. ^{1}H-NMR δ(DMSO): 7.86 (br s, 2H, N*H*$_2$) 7.39 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.83 (d, 2H, J = 8.8 Hz, phenyl-*H*), 4.98 (d, 1H, J = 5.1 Hz, O*H*), 3.94-3.83 (m, 1H, C*H*), 3.40-3.10 (m, 2H, C*H*$_2$S), 3.02 (s, 6H, 2 × C*H*$_3$), 1.20 (d, 3H, J = 5.8 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.4, 160.0, 158.3, 151.6, 129.9, 120.1, 116.1, 111.4, 93.2, 84.9, 65.2, 39.6, 38.3, 22.7. MS (ESI): 353.9. Anal. ($C_{18}H_{19}N_5OS \cdot 0.2$ MeOH) C, H, N, S.

3-[2-Amino-3,5-dicyano-4-(4-dimethylamino-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (21)

White Solid. mp: 186 ºC. ^{1}H-NMR δ($CDCl_3$): 7.47 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.76 (d, 2H, J = 8.8 Hz, phenyl-*H*), 5.71 (br s, 2H, N*H*$_2$), 4.18 (q, 2H, J = 7.3 Hz, OC*H*$_2$), 3.43 (t, 2H, J = 6.6 Hz, C*H*$_2$CO), 3.03 (s, 6H, 2 × C*H*$_3$), 2.76 (t, 2H, J = 6.6 Hz, C*H*$_2$S), 1.27 (t, 3H, J = 7.3 Hz, C*H*$_3$). ^{13}C-NMR δ($CDCl_3$): 171.6, 168.2, 159.6, 158.2, 151.9, 130.0, 119.5, 116.3, 115.7, 111.4, 85.8, 60.9, 39.9, 34.0, 25.4, 14.1. MS (ESI): 396.0. Anal. ($C_{20}H_{21}N_5O_2S$) C, H, N, S.

2-Amino-4-(3-fluoro-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (22)

White Solid. mp: 189 ºC. ^{1}H-NMR δ(DMSO): 8.09 (br s, 2H, N*H*$_2$), 7.70-7.59 (m, 1H, phenyl-*H*), 7.52-7.37 (m, 3H, phenyl-*H*), 5.05 (t, 1H, J= 5.5 Hz, O*H*), 3.72-3.64 (m, 2H, OC*H*$_2$), 3.40-3.33 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 167.8, 159.5, 130.3, 130.2, 123.7, 117.1, 116.7, 115.2, 114.8, 114.3, 105.6, 83.3, 59.8, 31.5. MS (ESI): 314.8 Anal. ($C_{15}H_{11}FN_4OS \cdot 0.3$MeOH) C, H, N, S.

2-Amino-4-(3-fluoro-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (23)

White Solid. mp: 185 ºC. ^{1}H-NMR δ(DMSO): 8.09 (br s, 2H, N*H*$_2$), 7.61-7.57 (m, 1H, phenyl-*H*), 7.52-7.36 (m, 3H, phenyl-*H*), 5.00 (d, 1H, J= 5.1 Hz, O*H*), 3.91-3.82 (m, 1H, C*H*), 3.21 (d, 2H, J = 6.6 Hz, C*H*$_2$S), 1.18 (d, 3H, J = 5.8 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 164.2, 159.5, 156.9, 136.3, 136.1,

131.2, 131.0, 124.8, 117.5, 117.1, 115.9, 115.5, 115.3, 115.1, 93.6, 85.7, 65.1, 22.7. MS (ESI): 328.7 Anal. ($C_{16}H_{13}FN_4OS \cdot 0.1CH_2Cl_2$) C, H, N, S.

3-[2-Amino-3,5-dicyano-4-(3-fluoro-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (24)

White Solid. mp: 131 ºC. ^{1}H-NMR δ(MeOD): 6.86-6.75 (m, 1H, phenyl-*H*), 6.57-6.50 (m, 3H, phenyl-*H*), 3.39 (q, 2H, J = 7.3 Hz, O*CH*$_2$), 2.71 (t, 2H, J = 6.6 Hz, *CH*$_2$CO), 2.03 (t, 2H, J = 6.6 Hz, *CH*$_2$S), 0.49 (t, 3H, J = 7.3 Hz, *CH*$_3$). ^{13}C-NMR δ(MeOD): 173.3, 168.9, 166.4, 161.3, 158.5, 137.6, 132.1, 131.9, 125.7, 118.5, 118.1, 117.0, 116.5, 115.9, 95.5, 89.8, 61.9, 35.0, 26.4, 14.5. MS (ESI): 370.9. Anal. ($C_{18}H_{15}FN_4O_2S$) C, H, N, S.

Cell culture

CHO cells stably expressing the human adenosine A_1 receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/mL), penicillin (50 IU/mL) and 0.2 or 0.8 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly.

Preparation of cell membranes

Membranes of CHO cells stably expressing the human adenosine A_1 receptor and of HEK293 cells stably expressing the human adenosine A_{2A} receptor or human adenosine A_3 receptor were prepared as previously described[8,9]. The protein concentration in the membrane preparation was determined using the BCA method with BSA as a standard[10].

Radioligand displacement assay

Radioligand binding studies were carried out as previously described[11]. In short, membrane aliquots containing 10 µg of protein were incubated in 400 µL of 50 mM Tris-HCl buffer, pH 7.4 at 25 ºC for 60 min in the presence of ADA (1 U/mL) and approximately 1.6 nM [^{3}H]DPCPX. Increasing concentrations of cold ligand were added to determine the affinity of the ligands. Non-specific binding was measured in the presence of 10 µM CPA. Incubations were stopped by rapid dilution with 1 mL ice-cold 50 mM Tris-HCl, pH 7.4 and bound radioligand was separated from free radioligand by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with 2 mL ice-cold buffer. Filter-bound radioactivity was measured by liquid scintillation counting (Tri-Carb 2900TR, Perkin Elmer) after the addition of 3.5 mL of Packard Emulsifier Safe. Experiments were performed in triplicate. Radioligand binding to membranes of HEK293 cells expressing the adenosine A_{2A} and A_3 receptors was performed in duplicate with [^{3}H]ZM241385 (K_D = 1.0 nM) and [^{125}I]I-ABMECA (K_D = 5.0 nM) as radioligands, respectively. Details of these experiments have been previously described[8].

cAMP assay

cAMP assays were performed according to Kourounakis et al. (2001)[12]. In short, cells stably expressing the human adenosine A_1 receptor were harvested using a trypsin solution (0.25% in PBS/EDTA), resuspended in medium and plated in 24-well plates at 500 µl or 2 × 10^5 cells/well. After 24 hr, the cells were washed two times with 500 µl DMEM containing 50 mM HEPES, pH 7.4. Subsequently, cells were incubated at 37 ºC in 250 µL DMEM + HEPES supplemented with ADA (2 IU/mL), rolipram (50 µM) and cilostamide (50 µM). After 30 min of preincubation, ligands (50 µL) were added, giving a final concentration between 1 to 20 µM based on the K_i value of the ligand. For the same reason, the reference compounds CPA, DPCPX and N0840 were tested at 1, 1 and 100 µM, respectively. The concentration was at least 100 × K_i, to achieve maximal receptor activation as reported e.g. by Beukers et al. (2004)[4]. In addition, concentration-effect curves were generated for CPA, LUF5853 and LUF6037 with concentrations ranging from 1 pM to 1 µM. After 10 min of incubation (37 ºC), 100 µL forskolin was added (final concentration 10 µM) and cells were incubated for an additional 15 min at 37 ºC. The assay was terminated by quick aspiration of the medium and cells were lysed by adding 200 µL of 0.1 M ice-cold HCl. Plates were stored at -20 ºC until further use. To determine the amount of cAMP produced, 100 µL PKA in buffer (150 mM K_2HPO_4; 10 mM EDTA; 0.2% BSA, pH 7.5), 50 µL [^{3}H]cAMP (20,000 dpm) in K_2HPO_4/EDTA buffer (150 mM K_2HPO_4; 10 mM EDTA, pH 7.7) and 50 µL of the cell lysate or cAMP solution for generation of a standard curve (0-16 pmol/200 µL 0.1 M HCl) were incubated on ice for 2.5 hr. Separation of bound and free radioligand was performed by rapid filtration through Whatman GF/C filters that were subsequently washed once with ice-cold buffer. Scintillation fluid (Emulsifier Safe, Packard Bioscience), 3.5 mL, was added and after 2 hr extraction, radioactivity was counted using a Tri-Carb 2900TR, Perkin Elmer scintillation counter.

Data analysis

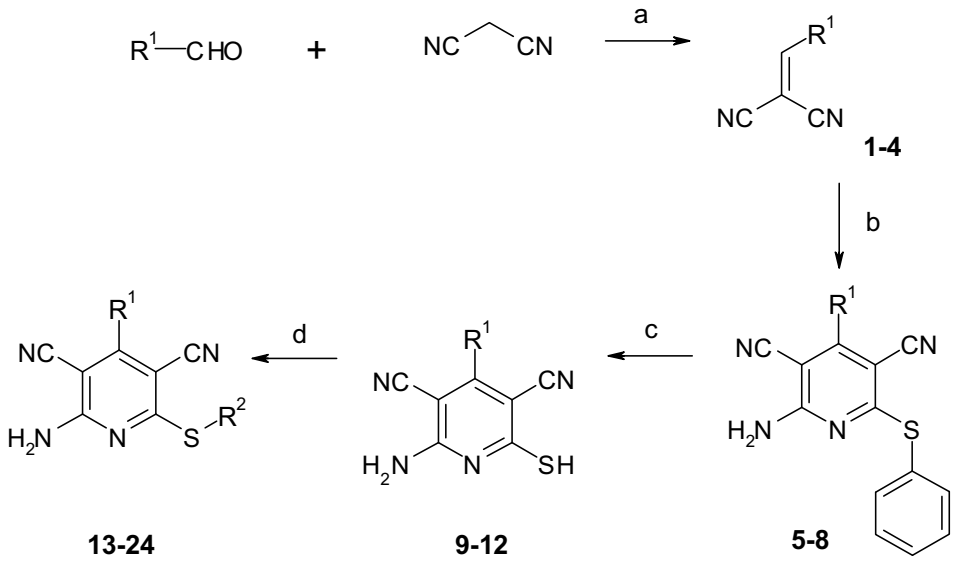
Data of radioligand binding and cAMP experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). Radioligand displacement curves were fitted to one and two site binding curves. For LUF5853 (**16**), LUF6037 (**17**), LUF5854 (**18**) and CPA the data were best fitted to a two site binding model. K_i values were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i = IC_{50}/(1+[L^*]/K_d)$, where [L*] is the radioligand concentration, and K_d its dissociation constant[13].

Results

Chemical synthesis

Compounds **13-24** were synthesized according to Scheme 5.1 essentially as previously described[2,3,5]. Intermediates (**1-4**) were obtained in moderate to good yields (46-56%) via a straightforward Knoevenagel condensation of the respective aldehyde with malononitrile in the presence of a few drops of piperidine. The formation of the pyridine occurred according to the preparation previously described by Kambe et al. (1981) resulting in the phenyl protected sulfide at the 6-position of the ring[14]. The functionalized malononitrile was refluxed with another equivalent of malononitrile and an equivalent of thiophenol in ethanol and triethylamine, resulting in compounds **5-8** (24-34% yields). The free thiol in the 6-position of the pyridine ring was obtained by adding 3.3 equivalents of sodium sulfide in DMF at 80 °C for 2-3 hours and resulted in quantitative yields of compounds **9-12**. The yields stated correspond to the crude material, and it was this crude substance that was used in subsequent reactions. Purification of the crude product was not performed throughout the synthesis, nor were the reactions optimized. The final step was the reaction of the free thiol with the bromo compounds in the presence of $NaHCO_3$ in DMF at room temperature. The final compounds **13-24** were obtained in 35-43% yields. They were purified by chromatography and subsequent recrystallisation gave clean, fully characterized compounds.

Scheme 5.1. Synthetic route to 2-Amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles **13-24**.[a]



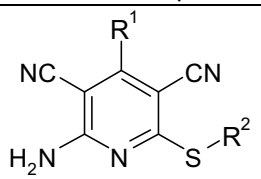
(a) piperidine, EtOH, 1 h reflux; (b) malononitrile, thiophenol, triethylamine, EtOH, 4 h reflux; (c) (i) Na_2S, DMF, (ii) 1M HCl; (d) bromo compound, $NaHCO_3$, DMF.

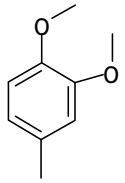
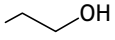
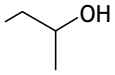
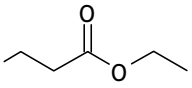
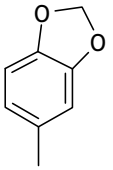
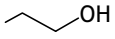
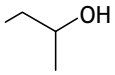
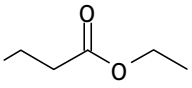
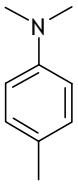
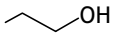
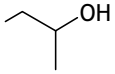
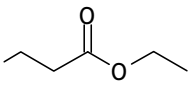
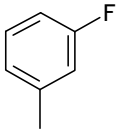
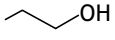
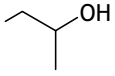
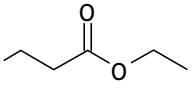
Affinities of the novel, non-ribose ligands for the human adenosine A_1 receptor

The affinity of the newly synthesized non-ribose ligands was assessed with help of radioligand binding studies on membranes of CHO cells stably expressing the human adenosine A_1 receptor. First of all, single point measurements at a concentration of 1 µM were performed for each ligand to determine the percentage inhibition of radioligand binding. For those compounds that gave more than 50% displacement at a concentration of 1 µM, whole displacement curves were generated, to determine the K_i value of the ligand. In Table 5.1, the percentages displacement at 1 µM or the K_i values in nM for each non-ribose ligand are presented. From this table it appears that within the different subsets – based on the R^1 group - a change in R^2 group from ethanol, to methylethanol or ethylpropionate did not affect the affinity of the ligand for the human adenosine A_1 receptor to a great extent.

On the other hand, modification of the R^1 substituent had striking effects on the affinity. LUF5850 – 5852 (**13-15**), containing a 3,4-dimethoxyphenyl-group as R^1 group, showed a displacement between 22% and 37% at a concentration of 1 µM, whereas introduction of a (4)-*N,N*-dimethylphenyl group as in LUF5874 (**19**), LUF5875 (**20**) and LUF5876 (**21**) resulted in affinities of 245, 340 and 231 nM, respectively. A 3-fluoro-phenyl group at the R^1 position resulted in ligands (**22-24**) with a rather high affinity for the human adenosine A_1 receptor (K_i of 40 – 69 nM).

Table 5.1. Reported K_I values or % displacement of compounds 13-24 for human adenosine A_1 receptors. The double figures for compounds 16-18 represent the $K_{i, high}$ and $K_{i, low}$ values.



R^1	R^2	Compound	K_i [nM][b] or Displacement at 1 μM [%]	
		LUF5850 (**13**)[a]	25 ± 1%	
		LUF5851 (**14**)	22 ± 6%	
		LUF5852 (**15**)	37 ± 9%	
		LUF5853 (**16**)	0.026 ± 0.020	12 ± 3.9
		LUF6037 (**17**)	0.021 ± 0.008	7.9 ± 2.8
		LUF5854 (**18**)	0.28 ± 0.18	45 ± 6.6
		LUF5874 **(19)**[a]	245 ± 103	
		LUF5875 (**20**)	340 ± 111	
		LUF5876 (**21**)	231 ± 93	
		LUF5877 (**22**)[a]	40 ± 10	
		LUF5878 (**23**)	43 ± 24	
		LUF5879 (**24**)	69 ± 17	

[a] Data from Chang et al. (2005) *J Med Chem* **48**:2045-2053[5].
[b] Mean of three experiments ± SEM

Much higher affinities were obtained with LUF5853 (**16**), LUF6037 (**17**) and LUF5854 (**18**), containing a 3,4-methylenedioxyphenyl as R^1 group. Interestingly, the three compounds recognized two binding states/sites on the receptor, whereas all other non-ribose compounds recognized just one binding site (see Figure 5.1). Moreover, the affinity of LUF5854 (**18**) for the high affinity site was 10 fold higher than CPA, 0.28 ± 0.18 nM and 2.2 ± 0.9 nM, respectively[15]. The affinities of LUF5853 (**16**) and LUF6037 (**17**) for the high affinity site were even higher, 100-fold, compared with CPA, 26 pM and 21 pM, respectively.

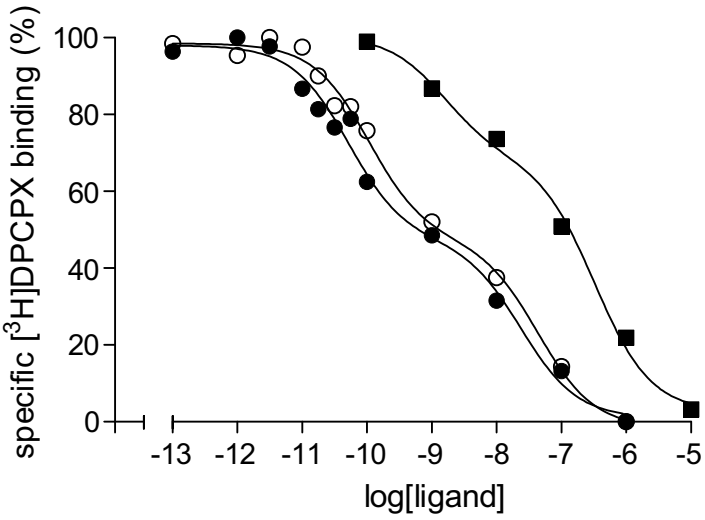


Figure 5.1. Displacement of ^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF5853 (**16**) (○), LUF6037 (**17**) (●) or CPA (■). A representative curve from one experiment performed in duplicate is shown.

Selectivity of the novel, non-ribose ligands for the human adenosine A_1 receptor

To determine the selectivity of the non-ribose ligands for the adenosine A_1 receptor the interaction of the compounds with the adenosine A_{2A} and adenosine A_3 receptors expressed on HEK293 cells was investigated. None of the compounds (**13-24**) was able to displace to a considerable extent either [^{3}H]ZM241385 or [^{125}I]I-ABMECA from the adenosine A_{2A} or adenosine A_3 receptors, respectively (Table 5.2).

Efficacies of the novel, non-ribose ligands on the human adenosine A_1 receptor

Subsequently, the functional activity of the novel, non-ribose ligands was tested in a cAMP assay. The final concentration varied between 1-20 µM, to obtain a concentration of at least 100 × K_i to ensure full receptor occupancy. LUF5850 – LUF5852 (**13-15**) were not considered in the activity assay, because of their low affinity. The basal cAMP concentration in the CHO cells was set at 0%, and the cAMP concentration in forskolin-stimulated cells was set at 100%. For comparison, three reference ligands, CPA (1 µM), DPCPX (1,3-dipropyl-8-cyclopentylxanthine, 1 µM) and N0840 (N^6-cyclopentyl-9-methyladenine, 100 µM) were also included. The agonist CPA inhibited the forskolin-stimulated cAMP production from 100% to 41 ± 9%. DPCPX, an inverse agonist, increased the cAMP production from 100% to 297 ± 37%. A somewhat smaller increase was observed for N0840 (210 ± 41%). The set of non-ribose ligands represented the whole range of full agonists to inverse agonists (Figure 5.2). LUF6037 (**17**) (29 ± 7%), LUF5853 (**16**) (31 ± 20%), LUF5854 (**18**) (50 ± 14%), and LUF5877 (**22**) (40 ± 14%) performed better than or equally well as CPA (41 ± 9%) in inhibiting the cAMP production, and are thus considered full agonists. LUF5874 (**19**) (68 ± 19%) and LUF5878 (**23**) (56 ± 13%) rather behaved as partial agonists in this test system. LUF5875 (**20**) and LUF5879 (**24**) can be regarded as neutral antagonists, given their slight influence on forskolin-stimulated cAMP

production, 107 ± 19% and 118 ± 9%, respectively. Finally, LUF5876 (**21**) appeared to be a (partial) inverse agonist, given its increase in cAMP production to 187 ± 31%.

Table 5.2. Reported% displacement of compounds **13-24** for human adenosine A_{2A} and A_3 receptors.

Compound	hA_{2A} Displacement at 1 μM	hA_3 Displacement at 1 μM
LUF5850 (**13**)[a]	25%	0%
LUF5851 (**14**)	19%	1%
LUF5852 (**15**)	26%	4%
LUF5853 (**16**)[a]	33%	13%
LUF6037 (**17**)	22%	1%
LUF5854 (**18**)	28%	2%
LUF5874 (19)[a]	0%	7%
LUF5875 (**20**)	3%	9%
LUF5876 (**21**)	5%	11%
LUF5877 (**22**)[a]	19%	8%
LUF5878 (**23**)	19%	1%
LUF5879 (**24**)	24%	16%

[a] Data from Chang et al. (2005) *J Med Chem* **48**:2045-2053[5].

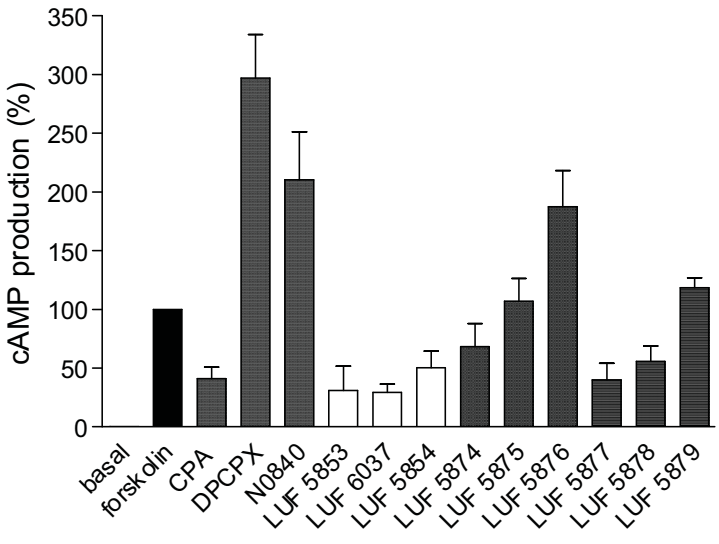


Figure 5.2. Modulation of forskolin-induced cAMP production in CHO cells stably expressing the human adenosine A_1 receptor, after exposure to reference ligands (CPA, DPCPX, N0840) and non-ribose ligands (LUF5853-5879, **16-24**) at a concentration between 1 to 100 μM to obtain full receptor occupancy (n = 3). Each subset of non-ribose ligands with identical R^1 group but different R^2 groups is labeled with the same bar pattern.

Full cAMP concentration-effect curves were generated for the high, picomolar, affinity non-ribose agonists LUF6037 (**17**) and LUF5853 (**16**). Representative curves for CPA, LUF6037 (**17**) and LUF5853 (**16**) are shown in Figure 5.3. The EC_{50} values were 2.1 ± 0.7 nM, 0.06 ± 0.05 nM and 0.1 ± 0.03 nM, respectively.

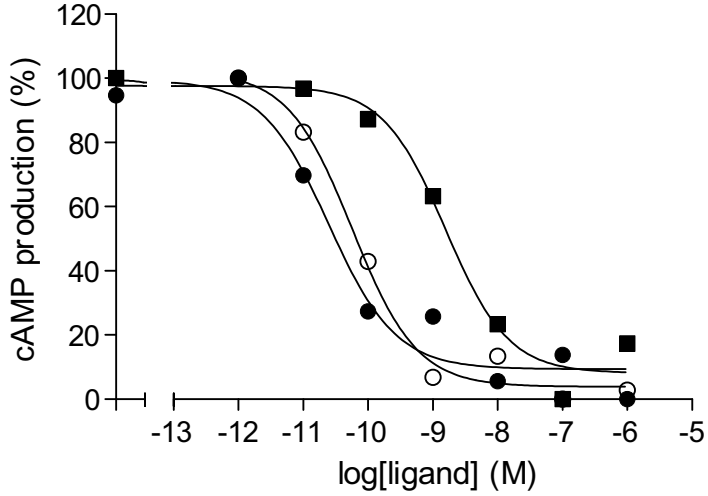


Figure 5.3. Representative curves of the inhibition of forskolin-stimulated cAMP production by CPA (■), LUF5853 (**16**) (○) and LUF6037 (**17**) (●) in CHO cells stably expressing the human adenosine A_1 receptor (n = 5, except for LUF5853 (**16**), n = 4).

Discussion

Structural analogues of adenosine, especially when substituted at the N^6 position are known to, often selectively, activate the human adenosine A_1 receptor[1]. Moreover the presence of a ribose moiety has always been considered essential for receptor activation[16,17]. The publication of several patents[2,3] describing a series of compounds as agonists for the adenosine receptors lacking a ribose moiety firmly challenged that hypothesis. These findings prompted us to synthesize a number of such ligands, all substituted 3,5-dicyanopyridine derivatives, that indeed showed a high affinity, activity and selectivity for the human adenosine A_1 receptor[5]. In the present study, we extended our research program by synthesizing and evaluating compounds **13-24**.

Affinity and selectivity of the novel, non-ribose ligands for the human adenosine A_1 receptor

The compounds with an ethanol substituent at the R^2 position were part of a larger series of non-ribose ligands, with more variation in the 3,4-substituents at the phenyl ring at the R^1 position[5]. It was found that increasing the electronegativity by introducing e.g. a fluorine atom or a hydroxyl group at especially the 3-position is important to increase the affinity of the ligands[5]. These findings were confirmed and extended in our current experiments, demonstrating that the R^2 group itself is important for affinity, but does not affect the rank order of potency that is dictated by the R^1 group. Moreover, three highly potent ligands, LUF5853 (**16**) and LUF6037 (**17**) and LUF5854 (**18**), were identified, all with unprecedented picomolar affinity. In fact

we had missed the very high affinity of LUF5853 (**16**) in our previous publication[5]. but due to our experimental observations with **17** and **18** we were prompted to reexamine these earlier findings. These compounds share a 3,4-methylenedioxy group at R^1 with an alcohol or ester function at R^2. Whereas these agonists recognized two binding states of the receptor, all other non-ribose compounds recognized just one binding state/site. This is in line with previous data on a similar compound, LUF5831, a partial adenosine A_1 receptor agonist that recognized a single binding state/site only[15]. Strikingly, the rigidity and somewhat smaller size provided by the closed ring structure of these three compounds is strongly preferred over the low affinity compounds **13-15**. The latter have a minor change in substitution pattern, namely two adjacent methoxy groups, which may cause too much steric hindrance in the receptor binding site.

To determine the selectivity of these compounds for the adenosine A_1 receptor the interaction with the adenosine A_{2A} and A_3 receptors was determined. In agreement with the previously reported data for a series of related analogues, none of the compounds (**13-24**) was able to interact with these receptors to a considerable extent[5].

Efficacy of the novel, non-ribose ligands for the human adenosine A_1 receptor

Whereas the affinity was mainly determined by the substitution pattern on the phenyl ring, different results were obtained with respect to efficacy. In this series of compounds (**13-24**) the efficacy was affected both by the substitution pattern on the phenyl ring, and by the substitution pattern on the sulfanyl chain. The effects of the substitution pattern on the phenyl ring were in line with previously reported data[5]. Interestingly, however, the substitution pattern on the sulfanyl side-chain was also important. The efficacy to activate the receptor decreased with increasing chain length. Thus a change from ethanol to methylethanol and ethylpropionate resulted in a decrease in efficacy from an agonist such as LUF5874 (**19**), a neutral antagonist such as LUF5875 (**20**), to an inverse agonist such as LUF5876 (**21**). In our hands and in this cAMP assay N0840, which is generally claimed to be a neutral antagonist and behaved as such in [^{35}S]GTPγS binding studies[18,19], was an inverse agonist (210 ± 41%), quite similar to LUF5876 (**21**).

In conclusion, structure-activity relationship studies documented very potent full agonists for the adenosine A_1 receptor in a series of 3,5-dicyanopyridines substituted at both the phenyl and sulfanyl position. These compounds lack any obvious similarity with the traditional ribose, adenosine-like compounds. Substitution at the phenyl ring mostly dictated affinity, whereas the substitution pattern at the sulfanyl position appeared to rather govern intrinsic activity. Compounds such as LUF5853 (**16**) and LUF6037 (**17**) largely superseded CPA in its affinity and potency.

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