Structure—Activity Relationship of Adenosine 5′-diphosphoribose at the Transient Receptor Potential Melastatin 2 (TRPM2) Channel: Rational Design of Antagonists

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ABSTRACT: Adenosine 5′-diphosphoribose (ADPR) activates TRPM2, a Ca2+, Na+, and K+ permeable cation channel. Activation is induced by ADPR binding to the cytosolic C-terminal NudT9-homology domain. To generate the first structure—activity relationship, systematically modified ADPR analogues were designed, synthesized, and evaluated as antagonists using patch-clamp experiments in HEK293 cells overexpressing human TRPM2. Compounds with a purine C8 substituent show antagonist activity, and an 8-phenyl substitution (8-Ph-ADPR, $S$) is very effective. Modification of the terminal ribose results in a weak antagonist, whereas its removal abolishes activity. An antagonist based upon a hybrid structure, 8-phenyl-2′-deoxy-ADPR (86, $IC_{50} = 3 \mu M$), is more potent than 8-Ph-ADPR ($S$). Initial biosisosteric replacement of the pyrophosphate linkage abolishes activity, but replacement of the pyrophosphate and the terminal ribose by a sulamate-based group leads to a weak antagonist, a lead to more drug-like analogues. 8-Ph-ADPR ($S$) inhibits Ca2+ signalling and chemotaxis in human neutrophils, illustrating the potential for pharmacological intervention at TRPM2.

INTRODUCTION

Transient receptor potential (TRP) channels are six-transmembrane polypeptide subunits that assemble as tetramers to form cation-permeable pores.1 TRP subfamily melastatin, type 2 (TRPM2), is a Ca2+ permeant channel which is also permeant to Na+, K+, and Cs+ ions.2 TRPM2 is unique among the known ion channels as it contains a C-terminal domain which is homologous to NUDT9 ADPR-hydrolase, and this has led to considerable interest. The NUDT9-homology (NUDT9H) domain of human TRPM2 extends from residue 1236 to the C-terminus. NUDT9 was identified in an EST database screen for proteins with homology to the C-terminus of TRPM2. It is an enzyme of the Nudix family of pyrophosphatases, with proteins with homology to the C-terminus of TRPM2. It is an enzyme of the Nudix family of pyrophosphatases, with adenosine 5′-diphosphoribose (ADPR, 1, Figure 1) as sole substrate. A NUDT9 crystal structure illustrated that this is a two-domain enzyme with a C-terminal ADPRase and N-terminal domain which enhances affinity for ADPR.3 Sequence alignment suggests that NUDT9H has the same features in TRPM2 channels and revealed that TRPM2 is gated by binding of ADPR,2,4 controlling cation entry through the plasma membrane channel. Interestingly, TRPM2 is not a very effective hydrolase.5

The TRPM2 channel is gated by micromolar cytosolic concentrations of ADPR ($EC_{50} \sim 100 \mu M$). In contrast to other nicotinamide adenosine 5′-diphosphoribose (cdADPR, 3,

Figure 1) and nicotinic acid adenine dinucleotide phosphate (NAADP), free ADPR has only recently been considered a second messenger.6 Cellular ADPR derives from NAD+ glycohydrolases such as CD38 that predominantly produce ADPR but also hydrolyze cADPR to ADPR (Figure 1) and other NAD+ metabolizing processes, including activation of poly-ADPribose polymerase (PARP) and poly-ADPribose glycohydrolase (PARG) pathways.7,8

TRPM2 is expressed in a variety of tissues, with highest transcript levels being detected in the brain, bone marrow, and cells of the immune system.9 Because the free cytosolic Ca2+ concentration ([Ca2+]i) sensitizes TRPM2 for activation by ADPR,10 resulting in a positive feedback loop and massive Ca2+ entry, it has been related to cell death from early on.11–13 Its activation downstream of reactive oxygen species suggested that it might be involved in the pathogenesis of neurodegenerative diseases,14 myocardial infarction,15 and type I and type II diabetes.16,17

More recent data point to an important physiological role for TRPM2 in cells of the innate immune system (reviewed in ref 18). In macrophages/monocytes, TRPM2 is required for the production of the pro-inflammatory chemokine CXCL2 in response to reactive oxygen species19 and in the production of...
different cytokines in response to lipopolysaccharides. In neutrophils, TRPM2 is involved in Ca2+ signalling and the chemotactic response to fMLP. TRPM2 has also been shown to be involved in dendritic cell maturation and their chemotaxis to chemokines. Because TRPM2 is upregulated in T-lymphocytes after activation and appears to play a role in cytokine secretion and proliferation, it might also be important for the adaptive immune response.

In pancreatic β-cells, TRPM2 has been shown to be involved in modulation of insulin secretion. In neurons, it is much less clear whether TRPM2 also performs a physiological function besides its potential role in the pathophysiology of neurodegeneration. However, there have been initial reports of a contribution of TRPM2 in synaptic transmission.

Research in these areas and identification of additional physiological processes involving ADPR/TRPM2 critically depend on the availability of specific small molecule tools. Over the years, some compounds that inhibit the TRPM2 channel have been described. The first TRPM2 inhibitor was flufenamic acid (IC50 70 μM), belonging to the fenamate group of nonsteroidal anti-inflammatory drugs. Other fenamates like mefenamic acid and niflumic acid also inhibit the channel although with lower potency (IC50 124 and 149 μM, respectively). A fenamate with reduced nonspecific effects, 2-(3-methylphenyl)aminobenzoic acid (3-MFA, IC50 76 μM), has recently been developed by Chen et al. Other TRPM2 inhibitors include the PLA2 inhibitor N-(p-amlycinnamoyl)anthranilic acid (ACA, IC50 1.7 μM) that shares the anthranilic acid motif with the fenamates, the antifungal imidazoles miconazole, econazole, and clotrimazole (IC50 <3 μM for both), and copper ions (IC50 2.6 μM). All of these pharmacological compounds apparently act as channel blockers and inhibit a variety of other ion channels (see ref 30 for citations). ACA affects other signal transduction pathways, thereby severely limiting its usefulness.

In 2007, some of us developed 8-Br-ADPR (4, Figure 1) as the first specific NUDT9H-TRPM2 antagonist. Both Ca2+ signalling and chemotaxis of murine neutrophils were very sensitive to 4. The introduction of an 8-bromo substituent turned the natural agonist ADPR into an antagonist, highlighting the sensitivity of ADPR to small structural modifications.

To better understand the ADPR/NUDT9H-TRPM2 system, it was necessary to develop further this early lead and begin to build a structure—activity relationship. Here, for the first time, we report a chemo-enzymatic approach involving chemical synthesis, coupled with use of Neurospora crassa NADase, to interrogate each major motif of the ADPR structure and

Figure 1. Formation of ADPR by CD38.

Scheme 1. Synthesis of 8-Substituted ADPR Analogues

Reagents and conditions: (i) NADase, Tris buffer (0.1 M, pH 7.2), rt, 92%; (ii) Br2, NaOAc buffer (0.5 M, pH 4), rt, 25%; (iii) Cs2CO3, Pd(OAc)2, TPPTS, X(BOH)3, MeCN–H2O, 125 °C, 5 min, 12–25%.

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subsequently evaluate the effect of these modifications on antagonist activity at the NUDT9 domain of the TRPM2 channel.

**RESULTS AND DISCUSSION**

To explore the SAR of ADPR at TRPM2, we undertook a systematic approach, splitting ADPR into four major motifs: adenine, the adenosine ribose, the pyrophosphate, and the terminal ribose (Figure 1). We synthesized analogues with modifications in each of the four motifs to evaluate the resultant effect on antagonist activity and provide a comprehensive early SAR.

**Adenine Modified Analogues: 8-Modified.** The introduction of substituents at the 8-position of a nucleoside can directly affect the syn/anti orientation of the base residue. Following on from the discovery that 8-Br-ADPR (4) is an antagonist,21 we wanted to further explore the effect of other substituents in this area. We prepared three analogues with hydrophobic substitutions by Suzuki coupling (compounds 5–7, Scheme 1) and one more polar substitution in the form of 8-NH$_2$-ADPR (12, Scheme 3).

8-Br-ADPR (4) was prepared by treatment of commercially available NAD$^+$ with NADase followed by bromination with Br$_2$ in NaOAc buffer (pH 4). The Suzuki reaction of 4 with phenylboronic acid was not straightforward. The conditions that had been successfully applied to 8-Br-cIDPR34,35 did not result in any reaction with 4; possibly the less rigid phosphate conformation was able to bind with the palladium source, resulting in any reaction with 8-Br-ADPR.

Unfortunately, this was not directly transferable to 8-NH$_2$-ADPR because in this instance the lack of N7 for cyclization results in the linear analogue on treatment with cyclase rather than a cyclic product.41

6-O-Methyl-IDPR (6-O-Me-IDPR, 24) was prepared from 6-chloropurine (17) and tetracyctyl-d-ribose (18) that were coupled together at N9 using Vorbrüggen conditions,42 followed by simultaneous deprotection of the three hydroxyls and conversion of the 6-chloro to a 6-methoxy substituent using sodium methoxide. Phosphorylation of the 5'-OH to generate 6-O-Me-AMP (21) was followed by activation of the phosphate, subsequent coupling to β-NMN$^+$, and hydrolysis of the 6-O-Me-NHD$^+$ (23) using NADase to generate the desired analogue 24 (Scheme 4).

**Adenosine Ribose Modified Analogues.** Three modifications were prepared at the adenosine ribose: individual deletion of either the 2'- or 3'-hydroxyl group or complete removal of the ribose by replacing it with a more flexible and hydrophobic butyl chain. An acyclic analogue (acyclic-ADPR, 30) was prepared by introduction of an n-butyl chain at N9, via alkylation of 17 with chlorobutyacetate (25).43 Subsequent treatment with MeOH/NH$_3$ simultaneously removed the acetate protecting group and substituted the 6-chloro substituent with a 6-amino group.44 Nucleoside analogue 27 was then phosphorylated under the standard POCl$_3$/TEP conditions.41 Activation of the phosphate, followed by coupling with β-NMN$^+$ and MnCl$_2$ in formamide, delivered the desired linear acyclic-NAD$^+$ (29). Treatment with NADase hydrolyzed the nicotinamide to afford 30 in very good yield (Scheme 5).

By removing an individual hydroxyl group, we hoped to uncover key interactions, i.e., the 2'-deoxy group in some cADPR analogues is critical for their antagonist activity.55 The preparation of 2'-deoxy-NAD$^+$ (32) has previously been reported,45 and treating this with NADase generated 2'-deoxy-ADPR (33, Scheme 6).

The synthesis of 3'-deoxy-ADPR (43) was more challenging as 3'-deoxy-AMP (41) is not commercially available. Using a published synthesis,46 we prepared 3'-deoxyadenosine (37). However, in this instance, our usual phosphorylation conditions (POCl$_3$/ TEP) were not selective for the primary hydroxyl group, and a mixture of products was obtained. We therefore had to adopt a longer route, in which both hydroxyl groups were protected as the TBDMS ether, followed by selective cleavage of the primary ether and phosphorylation with a protected phosphoramidite, followed by oxidation under basic conditions. Reagents and conditions: (i) TPPTS, Pd(OAc)$_2$, Cs$_2$CO$_3$, MeCN–H$_2$O, 18%.

![Scheme 2. Synthesis of 8-(4-Ph-ala)-AMP](image-url)
conditions using \( \text{H}_2\text{O}_2 \) and triethylamine. Simultaneous deprotection of the phosphate esters and 2'-hydroxyl group afforded 41 (Scheme 7).

3'-Deoxy-AMP (41) was activated and coupled to \( \beta\)-NMN\(^+\) to generate 3'-deoxy-NAD\(^+\) (42), and the nicotinamide cleaved with NADase to generate 43.

**Pyrophosphate-Modified Analogues.** The diphosphate linkage is in most cases essential for biological activity. We prepared an analogue with increased negative charge, length, and flexibility in the form of three phosphate groups (ATPR,

Scheme 3. Synthesis of 8-NH\(_2\)−ADPR\(^a\)

\[ \text{Scheme 3. Synthesis of 8-NH}_2\text{−ADPR}^a \]

\[ \text{Reagents and conditions: (i) morpholine, dipyridyl disulfide, PPh}_3\text{, DMSO, rt, 1 h then } \beta\text{-NMN}^+, \text{MgSO}_4, 0.2 \text{ M MnCl}_2, \text{13%; (ii) NADase, Tris buffer (0.1M, pH 7.3), rt, 40%.} \]

Figure 2. Further purine modified ADPR analogues.

Scheme 4. Synthesis of 6-O-Me-IDPR\(^a\)

\[ \text{Scheme 4. Synthesis of 6-O-Me-IDPR}^a \]

\[ \text{Reagents and conditions: (i) DBU, DMF, 60 °C, 16 h, 48%; (ii) MeOH/NH}_3\text{, 80 °C, 16 h, 88%; (iii) POCl}_3\text{, TEP, H}_2\text{O, 0 °C, 1 h, 72%; (iv) morpholine, dipyridyl disulfide, PPh}_3\text{, DMSO, rt, 1 h then } \beta\text{-NMN}^+, \text{MgSO}_4, 0.2 \text{ M MnCl}_2; (v) NADase, Tris buffer (0.1M, pH 7.3), rt, 8% for steps iv−v.} \]

Scheme 5. Synthesis of Acyclic-ADPR\(^a\)

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\[ \text{Reagents and conditions: (i) DBU, DMF, 60 °C, 16 h, 48%; (ii) MeOH/NH}_3\text{, 80 °C, 16 h, 88%; (iii) POCl}_3\text{, TEP, H}_2\text{O, 0 °C, 1 h, 72%; (iv) morpholine, dipyridyl disulfide, PPh}_3\text{, DMSO, rt, 1 h then } \beta\text{-NMN}^+, \text{MgSO}_4, 0.2 \text{ M MnCl}_2; (v) NADase, Tris buffer (0.1M, pH 7.3), rt, 81% for steps iv−v.} \]
ATPR (47) was prepared by degradation of cyclic adenosine triphosphoribose (cATPR,47 Scheme 8). The cyclic precursor 46 was synthesized in two steps from the coupling of ADP (44) and β-NMN+ with EDC to afford the linear nicotinamide adenine trinucleotide (NAT,45) that was cyclized by incubation with Aplysia cyclase as previously reported47 (Scheme 8).

In contrast, we developed analogues in which the pyrophosphate was replaced entirely by a phosphate or pyrophosphate bioisostere. The limited bioavailability of pyrophosphate-bearing compounds, their instability in a physiological environment, low membrane permeability, and difficulties in synthesis and scale up have led to considerable effort toward the development of isosteres. These include phosphate-containing, sulfur-containing, and carboxylate linkages as well as more unique cyclic mimics.48 However, no generally applicable bioisostere has so far been identified and with so many to choose from, our choices were based on ease of synthesis in the first instance. We therefore chose to utilize squarate, triazole, or sulfamate groups.

A small library of squarate compounds was prepared by coupling protected 5′-aminoadenosine 59 with a suitable squarate (Scheme 10). The analogue in which only the pyrophosphate is altered required 5′-aminoribofuranose (52) that was synthesized according to Scheme 9: One-step protection of the 1-, 2-, and 3-OH under acidic conditions 49.49 The 5-OH was then converted to the tosylate (50) that was displaced using sodium azide to generate 51 and the product reduced to the desired amine 52.

Four amines were coupled to diethylsquarate 53 in the presence of DIPEA and EtOH; 52 gave 54, cyclopentylamine gave 55, butylamine gave 56, and hexylamine gave 57. The squarate compounds 54−57 were then coupled to protected 5′-aminoadenosine 59 that was synthesized according to Scheme 9: One-step protection of the 1-, 2-, and 3-OH under acidic conditions 49.59 The 5-OH was then converted to the tosylate (50) that was displaced using sodium azide to generate 51 and the product reduced to the desired amine 52.

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An analogue was prepared in which the pyrophosphate was replaced by a 1,2,3-triazole. Suitable precursors were prepared by generating an azide at the 5′-position of adenosine and introducing a propargyl ether on the terminal ribofuranose for copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition ("click" cyclization). 2′,3′-O-Isopropylidene adenosine (58) was treated with diphenylphosphoryl azide followed by sodium azide to generate 5′-azidoadenosine 68 that was then brominated in the 8-position. The propargyl counterpart was prepared by deprotonating 49 with sodium hydride, followed by addition of propargyl chloride to prepare the protected 5-O-propargyl ether 70. The two precursors 69 and 70 underwent a 1,3-dipolar cycloaddition promoted by Cu(I), generated in situ from copper sulfate and sodium ascorbate, to afford 71. Triazole 71 underwent Suzuki coupling with phenyl boronic acid at the preinstalled 8-bromo substituent to afford 72. This was deprotected under acidic conditions to generate 8-phenyladenosine-1,4-triazole ribose (8-Ph-ATrR, 73, Scheme 11). Despite multiple attempts, it was not possible to introduce the propargyl ether on the 5′-position of adenosine as the nucleoside was degraded during deprotonation with sodium hydride.

The third pyrophosphate replacement explored was a sulfonamide. The sulfonamide was introduced to the 5′-hydroxyl group of a protected adenosine by treatment of 58 with sulfamoyl chloride. However, introduction of a terminal ribose proved difficult, both in the preparation of a suitable amide or carboxylic acid and in the condensation reaction. Condensation of 74 with salicylic acid afforded an opportunity to introduce a terminal ribose surrogate and generate a known phosphate bioisostere. The protected sulfonamide analogue 75 was then deprotected under acidic conditions to afford salicyladenosine monosulfamide (Sal-AMS, 76, Scheme 12).

**Terminal Ribose-Modified Analogues.** A cyclopentyl group was introduced in place of the terminal ribose to assess its importance in binding to TRPM2. This has none of the polar hydroxyl groups but a similar overall shape. Analogues modified at the terminal ribose have the advantage that they do not require coupling using expensive β-NMN+. Cyclopentanol (77) was phosphorylated and deprotected to give the

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**Scheme 8. Synthesis of ATPR**

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\[
\text{ADP. 44} \xrightarrow{\text{i}} \text{ATPR. 47}
\]
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"Reagents and conditions: (i) β-NMN⁺, MgCl₂ (1M), HEPES (2M), EDC, 24 h, rt, 38%; (ii) Aplysia cyclase, HEPES (25 mM, pH 7.5), 3 days, rt, 39%; (iii) HEPES (25 mM, pH 7.5), reflux, 1 h, 54%.

**Scheme 9. Preparation of 5′-Aminoribofuranose**

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\[
\text{d-ribose, 48} \xrightarrow{\text{i}} \text{RO} \xrightarrow{\text{ii}} \text{R} \xrightarrow{\text{MeO}} \xrightarrow{\text{iii}} \text{R} \xrightarrow{\text{MeO}} \xrightarrow{\text{iv}} \text{R}
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"Reagents and conditions: (i) H₂SO₄ acetone, MeOH, 48 h, 98%; (ii) pTsCl, DMAP, pyridine, 5 h, 81%; (iii) NaN₃, DMP, 120 °C, 16 h, 91%; (iv) PPh₃, THF, 16 h, 85%.

**Scheme 10. Synthesis of the Squarate Series: ASqR, Cyclopentyl-ASq, Butyl-ASq, and Hexyl-ASq**

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\[
\text{EtO}_2 \xrightarrow{\text{i}} \text{N} \xrightarrow{\text{ii}} \text{N} \xrightarrow{\text{iii}} \text{N} \xrightarrow{\text{iv}} \text{N} \xrightarrow{\text{v}} \text{N}
\]
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"Reagents and conditions: (i) R-NH₂, DIPEA, EtOH, rt, 1 h; (ii) diphenylphosphoryl azide, DBU, dioxane, rt, 3 h then TBAI, 15-crown-5, Na₂S₂O₅, reflux, 4 h, 88%; (iii) 10% Pd/C, EtOH, H₂, rt, 16 h, 95%; (iv) DIPEA, EtOH, rt, 16 h; (v) 75% aq TFA, rt, 1 h.

An analogue was prepared in which the pyrophosphate was replaced by a 1,2,3-triazole. Suitable precursors were prepared by generating an azide at the 5′-position of adenosine and introducing a propargyl ether on the terminal ribofuranose for copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition ("click" cyclization). 2′,3′-O-Isopropylidene adenosine (58) was treated with diphenylphosphoryl azide followed by sodium azide to generate 5′-azidoadenosine 68 that was then brominated in the 8-position. The propargyl counterpart was prepared by deprotonating 49 with sodium hydride, followed by addition of propargyl chloride to prepare the protected 5-O-propargyl ether 70. The two precursors 69 and 70 underwent a 1,3-dipolar cycloaddition promoted by Cu(I), generated in situ from copper sulfate and sodium ascorbate, to afford 71. Triazole 71 underwent Suzuki coupling with phenyl boronic acid at the preinstalled 8-bromo substituent to afford 72. This was deprotected under acidic conditions to generate 8-phenyladenosine-1,4-triazole ribose (8-Ph-ATrR, 73, Scheme 11). Despite multiple attempts, it was not possible to introduce the propargyl ether on the 5′-position of adenosine as the nucleoside was degraded during deprotonation with sodium hydride.

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corresponding free-phosphate 79 that was coupled to AMP-morpholidate to afford cyclopentyl-ADP (Scheme 13).

**Combined Modifications.** After initial positive results, we designed analogues combining substitutions that appeared to improve antagonist properties. Cyclopentyl-8-Ph-ADP (Scheme 14) was prepared by introduction of an 8-bromo substituent to 58, followed by Suzuki cross coupling with phenyl boronic acid to introduce an 8-phenyl substituent in 82. The free S'-OH was phosphorylated to afford 8-Ph-AMP (83) that was activated and coupled to 79 to afford 84 (Scheme 14).

8-Phenyl-2′-deoxyadenosine-5′-diphosphoribose (8-Ph-2′-deoxy-ADPR, 86) was prepared by degradation of 8-phenyl-2′-deoxy-cADPR (Scheme 15).

### PHARMACOLOGY

To evaluate ADPR analogues for antagonist activity, HEK293 clones with stable expression of human TRPM2 were established. Clones derived from HEK293 cells transfected with an expression vector for human TRPM2 were selected according to their increase in [Ca2+]i in response to hydrogen peroxide (Figure 3A). Further, expression of TRPM2 at the protein level was confirmed in responsive clones by Western blot analysis (Figure 3B). The specificity of the antibody was checked by preincubation with a blocking peptide (Figure 3B, right). For different clones, maximum [Ca2+]i response to hydrogen peroxide increased with magnitude of TRPM2 expression (Figure 3).

Whole-cell patch-clamp experiments were performed to evaluate ADPR analogues using clone #24 showing intermediate expression of TRPM2. To avoid excessive inward currents, the sodium chloride within the bath solution for the patch clamp experiments was replaced by N-methyl-D-glucamine (NMDG) hydrochloride that does not pass through the TRPM2 channel. Under these conditions, activation of TRPM2, after break-in with 100 μM ADPR (1) in the pipet solution, resulted in a small but measurable inward current of Ca2+ and a more pronounced outward current mainly carried by K+ ions (Figure 4A, gray line) similar to previous observations.50 In contrast, no such currents were observed when a control cell line stably expressing EGFP (EGFP #8, Figure 4A, dashed gray line) was used instead of TRPM2 #24 or the pipet was filled with a solution devoid of 1 (Figure 4A, dashed black line). As a read-out for TRPM2 activation the maximum net outward current at +15 mV obtained from repetitive voltage ramps between −85 and +20 mV was chosen.

The first specific ADPR antagonist 8-Br-ADPR (4)21 significantly inhibited TRPM2 activation when applied at a 9-fold excess over ADPR (1). Thus, alongside the positive control (100 μM of 1) in most experiments, a combination of 100 μM 1 and 900 μM 4 was included as an inhibitor control (Figure 5). Most ADPR analogues were tested for antagonism to 100...
μM of 1 at a concentration of 900 μM. Because some compounds were poorly soluble in water, they were resolubilized in DMSO. To avoid high concentrations of DMSO in the pipet solution, these compounds were tested for antagonist activity at a concentration of 100 μM in the presence of 100 μM of 1.

**Purine-Base Modifications.** The ADPR analogues with modifications at C2 of the purine ring [2-F-ADPR (16)] or C6 [IDPR (13) and 6-O-Me-IDPR (24)] did not antagonize activation of TRPM2 by 1 (Figure 5). ADPR analogues with modifications at N7 of the purine ring [7-deaza-IDPR (14) and 7-deaza-8-Br-ADPR (15)] were also without significant effect. In contrast, introduction of a bulky, hydrophobic substitution at C8 as in 8-thiophenyl-ADPR (6), 8-(3-acetylphenyl)-ADPR (7), or 8-phenyl-ADPR (8) resulted in ADPR analogues that effectively antagonized TRPM2 activation at 900 μM (Figure 5), while a compound with a small polar substitution at C8, e.g., 8-NH2-ADPR (12), was inactive as an antagonist. This stark

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Scheme 14. Synthesis of Cyclopentyl-8-Ph-ADP

**Reagents and conditions:** (i) Br2, NaOAc buffer, 89%; (ii) Na2ClPd, PhB(OH)2, TPPTS, MeCN–H2O, 80 °C, 1 h, 81%; (iii) 75% aq TFA, rt; (iv) POCl3, TEP, 0 °C, 1 h then cyclopentylphosphate 79, MnCl2 in formamide, MgSO4, rt, 16 h, 12%.

Scheme 15. Synthesis of 8-Ph-2′-deoxy-ADPR

**Reagents and conditions:** (i) KH2PO4 buffer, 70 °C, 2.5 h, 39%.

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Figure 3. Characterization of HEK 293 cell clones stably expressing TRPM2. (A) Cell lines from limiting dilution cloning of transfected HEK293 cells were selected by measuring their [Ca2+]i response to H2O2 (300 μM, time of addition indicated by arrow). Characteristic tracings of different HEK293 clones (TRPM2#23, TRPM2#24) transfected with a vector for coupled expression of TRPM2 and EGFP and a HEK293 clone (EGFP#8) transfected with the empty vector (expressing EGFP) are shown (n = 2–4, mean). (B) TRMP2 protein expression of clones TRPM2#23, TRPM2#24, EGFP#8, and wild-type HEK293 cells was determined by Western blot analysis. Specificity of TRPM2 detection was tested by preincubation of the primary antibody with a corresponding blocking peptide (right part). Specific bands of full length TRPM2 (black arrow) and degradation products (gray arrows) are marked. None of these bands were seen in wild-type HEK293 cells, in EGFP#8 control cell line, or in the presence of blocking peptide.
Contrast in activity may be due to the alteration of the purine base conformation from anti to syn upon substitution with large groups in the 8-position. In contrast to an 8-bromo or 8-amino substituent, the 8-aryl group introduces a new conformational parameter, as not only will such groups tend to a predominantly syn configuration around the glycosidic bond, but they also introduce the possibility of torsional conformational effects of, as yet, unknown significance.

**Adenosine Ribose Modifications.** The adenosine ribose was replaced by an alkane chain, resulting in the weak antagonist acyclic-ADPR \(30\) that showed nearly complete inhibition at 900 \(\mu\)M, whereas at 100 \(\mu\)M, no inhibition of the ADPR induced current was observed, indicating a steep dose response relationship with an IC\(_{50}\) value in the upper micromolar range (Figure 6). It was not possible to measure the antagonist activity of 2′-deoxy- or 3′-deoxy-ADPR \(33, 34\). In contrast, 2′-deoxy-AMP \(31\) was active as a weak antagonist, whereas AMP \(88\) showed no antagonist activity in

Figure 4. Stimulation of clone TRPM2#24 by infusion of 100 \(\mu\)M ADPR. Membrane currents of TRPM2#24 and EGFP#8 were measured in the whole-cell configuration using repetitive voltage ramps from −85 to +20 mV. Experiments were carried out with 1 mM Ca\(^{2+}\) and 140 mM NMDG instead of sodium in the extracellular solution. The free [Ca\(^{2+}\)]\(_{\text{free}}\), in the pipet solution was buffered to 200 nM by EGTA. (A) Characteristic tracings of membrane current at +15 mV and at −80 mV over time from representative experiments of TRPM2#24 (gray and black broken line) and EGFP#8 (gray broken line) stimulated with 100 \(\mu\)M ADPR (1) or vehicle (buffer) are shown. (B) Current–voltage relationship of the representative experiments shown in (A) at ramp 30 (corresponding to maximum ADPR induced current of TRPM2#24). (C) Maximum potassium outward current at +15 mV induced by infusion of buffer or 100 \(\mu\)M ADPR in TRPM2#24 or EGFP#8 indicated as mean ± SEM \((n = 7–11),**, P < 0.01 (t test).

Figure 5. Effect of purine-modified ADPR analogues on activation of TRPM2 by 1. Maximum outward currents were recorded as outlined in the Experimental Section. Pipet solutions contained either no nucleotide or 100 \(\mu\)M ADPR (1) alone or in combination with the indicated ADPR analogue. 8-Br-ADPR (4) was included as inhibitor control. Bars are grouped to indicate the position of purine modification (label on top). A horizontal dashed line marks the mean of the control (100 \(\mu\)M of 1). Data are shown as mean ± SEM (number of experiments denoted in or on top of the bar) and were analyzed by a nonparametric one-way ANOVA (Kruskal–Wallis test) followed by comparison against control (100 \(\mu\)M of 1) using Dunn’s correction for multiple testing. Results significantly different from control \((p < 0.05)\) are indicated by asterisks.

Figure 6. Effect of adenosine ribose-modified ADPR analogues on TRPM2 activation by ADPR (1). Maximum outward currents were recorded as outlined in the Experimental Section. Pipet solutions contained either no nucleotide or 100 \(\mu\)M of 1 alone or in combination with the indicated ADPR analogue. Data are shown as mean ± SEM (number of experiments denoted in or on top of the bar) and were analyzed by a one-way ANOVA followed by comparison against control (100 \(\mu\)M of 1) using Dunn’s correction for multiple testing. Results significantly different from control \((p < 0.05)\) are indicated by asterisks.
our hands (Figure 7A). There are conflicting reports on the antagonistic effect of 88; while it was previously described to inhibit TRPM2 activation completely with an IC50 value of 70 μM in whole cell patch clamp experiments,51 other studies have not shown any inhibition of ADPR-mediated currents in cell free patches from Xenopus oocytes expressing TRPM2, even at 60-fold excess of 88 over 1.52 Interestingly, the combination of this 2′-deoxy-modification and the 8-phenyl substitution at C8 in 8-Ph-2′-deoxy-ADPR 86 also completely inhibited activation of TRPM2 when applied at a concentration of 900 μM. Full concentration–response relationships for 4 and 86 are displayed in Figure 10. The adenosine ribose, particularly the C2′ position, seems to be important for binding of analogues to NUDT9H; possibly the absence of the hydroxyl group results in lower steric hindrance or lower repulsion between ligand and channel ultimately resulting in stronger binding.

**Pyrophosphate Modifications.** Introduction of an additional phosphate in ATPR (47) generated an analogue that appears to have some antagonist effect at 900 μM, although this was not statistically significant (Figure 7a). It remains unclear whether this is a result of increased length or the increased charge, the latter unattractive from a drug discovery point of view. Squarate analogues 64–67 and triazole analogue 73 could only be tested at 100 μM because the usual method of solubilizing the compounds in HEPES buffer was not successful, so DMSO was used. However, only as much as 0.1% (v/v) DMSO was tolerated in the pipet solution, allowing a maximum concentration of 100 μM to be reached. However, at this concentration, none of these compounds showed antagonist activity (Figure 7B). The acyl sulfamate analogue 76 was soluble in aqueous media and showed noticeable antagonist activity at 900 μM. Although this was not statistically significant, this analogue (in particular, because it combines both a neutral pyrophosphate bioisostere and simple substitution of the terminal ribose) represents a tractable lead toward future, more drug-like ADPR antagonists. Interestingly, Sal-AMS has previously been prepared as an antibiotic lead for the inhibition of siderophore biosynthesis in tuberculosis and plague.53,54 However, in ADPR, the pyrophosphate moiety is more than simply a “spacer” between the two riboses; to date, no analogues with a pyrophosphate substitution have shown a significant antagonist effect. These results indicate that the negative charge at the pyrophosphate is important for interaction with the NUDT9H domain. This finding is of great significance for all applications of such an antagonist in cell and animal models because the pyrophosphate moiety potentially may be attacked by pyrophosphatase enzyme activity. Thus, analogues containing the pyrophosphate moiety may suffer from a short half-life. Metabolically stable phosphate-type analogues or alternative pyrophosphate replacement groups might be a future solution for this problem.55

**Terminal Ribose Modifications.** A number of analogues were prepared or are commercially available, including completely truncated “AMP-like” compounds. The latter are attractive analogues due to the presence of only one phosphate group; they are therefore likely to be more membrane permeant and are more easily synthetically accessible as they do not require the difficult coupling to expensive reagent β-NMN. In contrast to AMP (88) that was ineffective, 2′-deoxy-AMP (31) and 8-(4-phenylalanine)-AMP (9) suppressed activation of TRPM2 by 1 when applied at 900 μM (Figure 8). However, when equimolar to 1 (100 μM each), the antagonist effect was lost, indicating a steep concentration–response relationship with an IC50 value considerably higher than 100 μM. Interestingly, 8-Cl-AMP (87) showed no antagonist effect at 900 μM (Figure 8). The observed variation among AMP analogues suggests that 8-substitution plays a role in introducing antagonist properties, but these are much reduced in magnitude compared to the full-length analogues.

Replacing only the terminal ribose, while maintaining the complete pyrophosphate, generated the antagonist cyclopentyl-ADP (80) that showed partial inhibition at 900 μM (Figure 9). This suggested that the hydroxyl groups on the terminal ribose may not be critical for antagonist activity. In contrast, the parent ADP (44) was inactive, suggesting that the five-membered ring does play a critical role in filling the binding site. Combining the terminal cyclopentyl substituent with the 8-phenyl substituent afforded cyclopentyl-8-phenyl-ADPR (84), which showed significant antagonist activity when applied in an

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**Figure 7.** Effect of pyrophosphate-modified ADPR analogues on TRPM2 activation by ADPR (1) Maximum outward currents were recorded as outlined in the Experimental Section. Pipet solutions contained either no nucleotide or 100 μM of 1 alone or in combination with the indicated ADPR analogue. (A) Analogues that were soluble in buffer. (B) Analogues that were only soluble on addition of DMSO. Because squarate and triazole compounds (64–67, 73) were resolubilized in DMSO, the pipet solution contained 0.1% DMSO. For the control conditions, DMSO was included in the pipet solution at the same final concentration. Data are shown as mean ± SEM (number of experiments denoted in or on top of the bar) and were analyzed by a nonparametric one-way ANOVA (Kruskal–Wallis test) followed by comparison against the respective control (100 μM of 1) using Dunn’s correction for multiple testing. Results significantly different from control (p < 0.05) are indicated by asterisks.

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**Figure 8.** Effect of pyrophosphate-modified ADPR analogues on TRPM2 activation by ADPR (1) Maximum outward currents were recorded as outlined in the Experimental Section. Pipet solutions contained either no nucleotide or 100 μM of 1 alone or in combination with the indicated ADPR analogue. (A) Analogues that were soluble in buffer. (B) Analogues that were only soluble on addition of DMSO. Because squarate and triazole compounds (64–67, 73) were resolubilized in DMSO, the pipet solution contained 0.1% DMSO. For the control conditions, DMSO was included in the pipet solution at the same final concentration. Data are shown as mean ± SEM (number of experiments denoted in or on top of the bar) and were analyzed by a nonparametric one-way ANOVA (Kruskal–Wallis test) followed by comparison against the respective control (100 μM of 1) using Dunn’s correction for multiple testing. Results significantly different from control (p < 0.05) are indicated by asterisks.
equimolar concentration to 1 (100 μM each, Figure 9). Replacing the terminal ribose with a cyclopentyl group is attractive because, as well as removing any complication of both anomeric center stereochemistry and equilibrium with a ring-opened ribose form, it negates the possibility of intramolecular attack on the pyrophosphate by a ribose hydroxyl group, which may lead to instability. Thus, analogue 84 was analyzed in more detail (see Figure 10).

The compounds that showed enhanced antagonist activity were assessed in a concentration–response manner using patch-clamp experiments (Figure 10). The IC50 value of the previously reported 8-Br-ADPR (4) was around 300 μM. 8-Thiophenyl-ADPR (6) and 8-(3-Ac-Ph)-ADPR (7) showed a 6-fold increase in potency (IC50 values of 51 and 49 μM, respectively). This was further improved to 15 μM in cyclopentyl-8-phenyl-ADP (84) and to 11 μM in 8-phenyl-ADPR (5). Combining the 8-phenyl substitution on the adenine ring with removal of the hydroxyl group on C2′ of the adenosine ribose further enhanced the antagonist effect, resulting in an IC50 value of 3 μM for 8-Ph-2′-deoxy-ADPR (86), the best evaluated TRPM2 antagonist to date.

The five most potent inhibitors are all substituted with an aromatic ring at the 8-position of adenine. Comparison of the three 8-aromatic analogues 5–7 suggest that appended groups (e.g., 3-acetylphenyl-) or alternative ring sizes (e.g., thiophene) do not improve antagonist effects, although this has not been fully exploited due to the difficulties encountered with Suzuki chemistry to prepare these analogues. The small improvement in activity observed for cyclopentyl-8-phenyl-ADP (84) suggests that the terminal ribose is not critical.

Importantly, we also studied the biological activity of one of the best antagonist compounds with a low IC50 value, in neutrophil Ca2+ signalling and chemotaxis; a system where we have already established the physiologically important role of TRPM2.8-Phenyl-ADPR (5) was accessed via 4, as opposed to 8-phenyl-2′-deoxy-ADPR (86), which was prepared by degradation of its valuable cyclic counterpart. Thus, we assessed the effect of 5 on Ca2+ signalling and chemotaxis in human neutrophils stimulated with the chemotactic peptides A5 or fMLP (Figures 11 and 12).

To investigate the effect of NUDT9H-TRPM2 antagonism on Ca2+ signalling, freshly isolated primary human neutrophils from healthy donors and loaded with Fura2 were stimulated either by 1 μM fMLP (high affinity ligand for human FPR1) or 10 μM of the FPR2 specific A5 peptide.56,57 When the
neutrophils were preincubated with 100 μM of S before application of the chemoattractant, the increase in [Ca²⁺]i upon stimulation was significantly reduced for both ligands (31.8%–34.7% for fMLP and 31.4% for A5 peptide, Figure 11), indicating that like in mouse neutrophils, TRPM2 is activated by ADPR during chemotaxis and contributes to the Ca²⁺ signal by mediating Ca²⁺ entry downstream of chemoattractant receptors.

To analyze the effect of 8-phenyl-ADPR (S) on chemotaxis, we analyzed the migratory pattern of freshly isolated neutrophils from healthy donors. In the absence of a chemotactic gradient, the cells showed spontaneous migration that was largely nonvectorial as the center of gravity of all the cells were analyzed by ratiometric Ca²⁺ imaging. Cells were stimulated by fMLP or A5 peptide (A, gray line) or 1 μM fMLP and represent mean values of Fura-2 ratio (primary y-axis). Corresponding [Ca²⁺]i in nM are shown on secondary y-axis. The number of cells for each condition (n) is indicated in the inset legend.

Figure 11. Partial antagonism of Ca²⁺ signalling in human neutrophils upon preincubation with 8-Ph-ADPR (S). Primary human PMN (polymorphonuclear neutrophils) were loaded with Fura-2/AM and analyzed by ratiometric Ca²⁺ imaging. Cells were stimulated by addition of 10 μM A5 peptide (A, gray line) or 1 μM fMLP (B, gray line). To determine the effect of 5 on fMLP or A5 peptide induced Ca²⁺ signalling, cells were preincubated with 100 μM of S for 15 min at rt (A,B black line). Data of single cells were synchronized to the first maximum after addition of A5 peptide or fMLP and represent mean values of Fura-2 ratio (primary y-axis). Corresponding [Ca²⁺]i in nM are shown on secondary y-axis. The number of cells for each condition (n) is indicated in the inset legend.

that fMLP induced chemotaxis partially depends on a high extracellular Ca²⁺ concentration. Importantly, when the neutrophils were preincubated with 100 μM of S and then placed within the fMLP gradient, the decrease in directed migration was similar to migration in the experiment with EGTA (compare parts E to D of Figure 12). Taken together, these data demonstrate the importance of ADPR mediated TRPM2 activation in directing the chemotactic response of neutrophils.

8-Phenyl-ADPR (S) partially reduced Ca²⁺ signalling by the FPR1 ligand fMLP and the FPR2 specific agonist A5 peptide in human neutrophils and also partially inhibited the directional migration in a gradient of fMLP. These results demonstrate that the novel ADPR analogue S not only inhibits activation of TRPM2 directly on target in the “cell free” whole-cell patch clamp system but also affects TRPM2-dependent processes in a cellular environment, making it a useful tool for the investigation of ADPR/TRPM2 dependent processes in other cell systems and physiological processes.

To date, to the best of our knowledge, 8-Br-ADPR (4) has been the only specific antagonist available for activation of TRPM2 by ADPR. Therefore, for the first time, our systematic study of ADPR antagonism allows us to build up an early SAR profile for the inhibition of TRPM2 by ADPR analogues (Figure 13 and Table1) and provides more potent agents for the study of TRPM2.

**CONCLUSION**

In this study, we systematically derivatized the new second messenger ADPR and monitored the antagonist activity of the derivatives at TRPM2 during activation by ADPR. The replacement of bromine by the bulky, flat, and torsionally mobile, hydropic phenyl substituent at C8 of adenine together with a 2'-deoxy motif at the adenosine ribose converted the poor, but specific, antagonist 8-Br-ADPR (4) into the highly active specific NUDT9H-TRPM2 antagonist 8-Ph-2'-deoxy-ADPR (S6). We have established that the amino/imino groups at C6 and the N7 nitrogen within the adenosine base are important for antagonist activity. Likewise, the charges of the pyrophosphate bridge are critical for retaining antagonist activity because the bridge cannot be replaced by another noncharged linker with similar dimensions. However, substitution of the pyrophosphate by a sulfamate in Sal-AMS did lead to some antagonist activity and this compound is a promising lead for further development of neutral and more drug-like antagonists. Finally, we have also established that the terminal ribose is important but can be partially replaced by a cyclopentyl moiety. This SAR provides a key starting point for further development of potent modulators of TRPM2 and development of chemical biology tools to further probe this new signalling pathway.

**EXPERIMENTAL SECTION**

**Chemistry.** The purity of new tested compounds was determined to be ≥95% by analytical HPLC. HPLC analyses were carried out on a Waters 2695 Alliance module equipped with a Waters 2996 photodiode array detector (210–350 nm). The chromatographic system consisted of a Hichrom guard column for HPLC and a Phenomenex Synergi 4μ MAX-RP 80A column (150 mm × 4.60 mm), eluted at 1 mL/min with the following ion-pair buffer: 0.17% (m/v) cetrimide and 45% (v/v) phosphate buffer (pH 6.4) in MeOH.

**General Procedure A: NADase Hydrolysis of NAD⁺ Analogues.** NADase (from Neurospora crassa; Sigma; 0.9 U) in Tris-HCl buffer (2 mL, 0.1 M, pH 7.2–7.4) was added to a solution of the NAD⁺
analogue (30 μmol) in Tris-HCl buffer (1 mL, 0.1 M, pH 7.2–7.4). The reaction mixture was stirred at 37 °C until complete, followed by purification of the product by ion-exchange (Q-sepharose) chromatography eluting with a gradient (0–50%) of TEAB (1.0 M) in Milli-Q water. The appropriate fractions were collected and evaporated under reduced pressure. The residue was coevaporated several times with

Figure 12. Partial antagonism of chemotaxis in human neutrophils upon preincubation with 8-Ph-ADPR (S). Migration of freshly isolated primary human PMN was analyzed as detailed in the Experimental Section. (A) buffer, (B) buffer and 1.5 mM EGTA, (C) 50 nM fMLP gradient (indicated in green), (D) 50 nM fMLP gradient and 1.5 mM EGTA, (E) 50 nM fMLP gradient and S. All cells were standardized to the center of the coordinate system as a start point. Black lines indicate trajectories of single cells during measurement. Blue dots represent the position of each cell at the end of measurement. The center of mass as mean of the position at the end of the measurement of all cells is marked by a red cross with parallel to showing mean movement in the y direction along fMLP gradient and perpendicular to showing mean movement in the x-direction. Cells shifted in presence of fMLP (C) 49.4 μm, fMLP/EGTA (D) 32.6 μm, and fMLP/S (E) 34.4 μm in the parallel direction. In the absence of fMLP, cells showed less migration in the parallel direction (buffer control (A), 9.8 μm, buffer/EGTA; (B), 5.6 μm).
MeOH to remove excess TEAB to yield the desired ADPR analogue as a glassy solid in its triethylammonium (TEA) form.

General Procedure B: Suzuki Coupling. Cesium carbonate (0.24 mmol, 2.9 equiv) was added in one portion to a stirred solution of the corresponding boronic acid (0.103 mmol, 1.2 equiv), palladium acetate (0.004 mmol, 0.05 equiv), TPPTS (0.02 mmol, 0.24 equiv), and 8-Br-ADPR (TEA salt, 0.0823 mmol) in degassed MeCN–H₂O (1:2 v/v; 2.4 mL) under an argon atmosphere. The reaction mixture was heated at 125 °C for 5 min; the reaction mixture turned black and HPLC analysis confirmed the reaction was complete. The reaction mixture was cooled to room temperature, QuadraPure TU (~100 mg) added, and the mixture stirred for 16 h. The mixture was filtered and evaporated under reduced pressure to leave a crude product that was purified by ion-exchange (Q-Sepharose) chromatography eluted with a gradient (0–40%) of TEAB (1.0 M) in Milli-Q water followed by reverse phase (RP-18) column chromatography, eluted with 0–20% MeCN in TEAB (0.05 M) to isolate the desired 8-substituted ADPR product.

General Procedure C: Saturate Chemistry. To a solution of amine (0.443 mmol) and DIPEA (42 μL, 0.239 mmol) in EtOH (5 mL) was added diethylsilylane (72 μL, 0.487 mmol). The reaction was stirred at rt until TLC indicated completion of the reaction (ca. 1 h). The solvent was removed under reduced pressure, and the residue was purified on an Isco chromatographic system (DCM–acetone, 8:2 v/v) to yield the desired product.

General Procedure D: Isopropylidene Deprotection. The protected compound (0.1 mmol) was stirred in a 75%aq TFA solution (5 mL) at rt for 1 h. The solvents were evaporated under reduced pressure, and the residue was co-evaporated with MeOH to remove any residual TFA. The remaining residue was purified on an Isco purification system (DCM–MeOH, 8:2 v/v) to yield the desired compound as a white solid.

Synthesis of 8-Modified ADPR Analouges. 8-Phenyl Adenosine Diphosphoribosyl (8-Ph-ADPR, 5). Phenylboronic acid (0.103 mmol, 21 mg) and 8-Br-ADPR (4 TEA salt, 0.0823 mmol) were reacted under the general protocol B, yielding 8-Ph-ADPR (TEA salt, 6.0 by 1H NMR (18 mg, 14.3 mmol, 19%) as a colorless solid. H (400 MHz, D₂O) δ 8.14 (br s, 1H, H-2), 7.56–7.48 (br m, 5H, Ph), 5.78 (d, 1H, J = 5.9, H-1′), 5.16 (br, 0.4H, H-1′), 5.08 (br, 1H, H-2′), 5.05 (br, 0.6H, H-1′), 4.30 (br, 1H, H-2′), 3.82–4.18 (m, 8H, H-3′, H-4′, 2 × H₅, H-3′, H-4′ and 2 × H₅).

Figure 13. Early structure—activity relationship for ADPR antagonism.
Table 1. Summary of Analogues and Antagonist Activity at TRPM2

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Modification</th>
<th>Antagonism at TRPM2 (IC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8-Br-ADPR</td>
<td>8-H → 8-Br</td>
<td>300 μM</td>
</tr>
<tr>
<td>5</td>
<td>8-Ph-ADPR</td>
<td>8-H → 8-Ph</td>
<td>11 μM</td>
</tr>
<tr>
<td>6</td>
<td>8-thiophenyl-ADPR</td>
<td>8-H → 8-thiophenyl</td>
<td>51 μM</td>
</tr>
<tr>
<td>7</td>
<td>8-(3-Ac-Ph)-ADPR</td>
<td>8-H → 8-(3-Ac-Ph)</td>
<td>49 μM</td>
</tr>
<tr>
<td>12</td>
<td>8-NH&lt;sub&gt;2&lt;/sub&gt;-ADPR</td>
<td>8-H → 8-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>n.s.</td>
</tr>
<tr>
<td>13</td>
<td>IDPR</td>
<td>6-NH&lt;sub&gt;2&lt;/sub&gt; → 6-O</td>
<td>n.s.</td>
</tr>
<tr>
<td>14</td>
<td>7-deaza-IDPR, 6-O</td>
<td>7-NH&lt;sub&gt;2&lt;/sub&gt; → 7-CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>n.s.</td>
</tr>
<tr>
<td>15</td>
<td>7-deaza-8-Br-ADPR</td>
<td>7-NH&lt;sub&gt;2&lt;/sub&gt; → 7-CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>n.s.</td>
</tr>
<tr>
<td>16</td>
<td>2-F-ADPR</td>
<td>2-H → 2-F</td>
<td>n.s.</td>
</tr>
<tr>
<td>24</td>
<td>6-O-Me-ADPR</td>
<td>6-NH&lt;sub&gt;2&lt;/sub&gt; → 6-O-Me</td>
<td>n.s.</td>
</tr>
<tr>
<td>30</td>
<td>Acyclic-ADPR</td>
<td>8-R → 8-butyl</td>
<td>100 - 900 μM</td>
</tr>
<tr>
<td>33</td>
<td>2′-deoxy-ADPR</td>
<td>2′-OH → 2′-H</td>
<td>n.d.</td>
</tr>
<tr>
<td>43</td>
<td>3′-deoxy-ADPR</td>
<td>3′-OH → 3′-H</td>
<td>n.d.</td>
</tr>
<tr>
<td>47</td>
<td>ATPR</td>
<td>PP → PPP</td>
<td>n.s.</td>
</tr>
<tr>
<td>64</td>
<td>ASqR</td>
<td>PP → Sq</td>
<td>n.s.</td>
</tr>
<tr>
<td>65</td>
<td>Cyclopentyl-ASq</td>
<td>PP → Sq</td>
<td>n.s.</td>
</tr>
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<td>66</td>
<td>Butyl-ASq</td>
<td>PP → Sq</td>
<td>n.s.</td>
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<td>Hexyl-ASq</td>
<td>PP → Sq</td>
<td>n.s.</td>
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<td>73</td>
<td>8-Ph-methyl-ATrR</td>
<td>PP → Tr</td>
<td>n.s.</td>
</tr>
<tr>
<td>76</td>
<td>Sal-AMS</td>
<td>PP → SO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>n.s.</td>
</tr>
<tr>
<td>31</td>
<td>2′-deoxy-AMP</td>
<td>P → 8-R → deleted</td>
<td>100 - 900 μM</td>
</tr>
<tr>
<td>80</td>
<td>Cyclicpentyl-ADP</td>
<td>P → 8-R → deleted</td>
<td>&lt;900 μM</td>
</tr>
<tr>
<td>87</td>
<td>8-Cl-AMP</td>
<td>P → 8-R → deleted</td>
<td>n.s.</td>
</tr>
<tr>
<td>84</td>
<td>Cyclopentyl-8-Ph-ADPR</td>
<td>P → 8-R → deleted</td>
<td>100 - 900 μM</td>
</tr>
<tr>
<td>86</td>
<td>8-Ph-2′-deoxy-ADPR</td>
<td>2′-OH → 2′-H</td>
<td>15 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-H → 8-Ph</td>
<td>3 μM</td>
</tr>
</tbody>
</table>

4.35 (dd, 1H, J = 6.2, 5.1, H-3′), 3.88-4.09 (m, 4H, H-4′, H-5′ and NH₂CH₂), 3.26 (dd, 1H, J = 14.9, 5.3, NH₂CH<sub>2</sub>) and 3.08 (1H, obscured by Et₃N salt peak, NH₂CH<sub>2</sub>). 13C (100 MHz, D₂O) δ<sup>192.2</sup> (C=O), 155.1, 152.8, 152.6 (C-2), 150.1, 138.6, 129.9, 129.8, 126.9, 118.4, 88.6 (C-1′), 83.6 (C-4′), 70.0 (C-2′), 69.5 (C-3′), 63.4 (C-5′), 55.8 (NH₂CH₂) and 36.5 (ArCH₂). 31P (162 MHz, D₂O) δ<sup>-5.6</sup> (s).

HRMS (ES<sup>−</sup>) calcd for C₁₉H₂₂N₆O<sub>9</sub>P, 509.1191 M<sup>−</sup>; found, 509.1174; and R<sub>T</sub> = 6.96 min.

8-Amino-nicotinamide Adenine-5′-dinucleotide (8-NH₂-NAD<sup>+</sup>, 11). Triphenylphosphine (130 mg, 0.5 mmol), morpholine (92 mL, 1.06 mmol), and 2,2′-dipyridyldisulfide (110 mg, 0.5 mmol) were added to a solution of 8-NH₂-AMP<sub>10</sub> (55 mg, 0.15 mmol) in dry DMSO (600 μL). The mixture was stirred at rt for 4 h, and then the solution of sodium iodide in acetone (0.1 M) was added dropwise. The precipitate that formed was collected, washed with acetone, and redisolved in water and lyophilized to leave the crude morpholide intermediate (39 mg) as a pale-yellow solid. The morpholide was dissolved in a solution of MnCl₂ in formamide (1 mL, 0.2 M), MgSO₄ (48 mg, 0.64 mmol) and β-NMN<sup>+</sup> (67 mmol, 0.2 mmol) were added, and the mixture was stirred for 2 days. The crude product was precipitated from the reaction mixture by the dropwise addition of MeCN, and the precipitate was collected, washed with MeCN, and dried. The crude product was purified by reverse phase column chromatography, eluting with 0-20% MeCN in TEAB (0.05 M). The sample was then treated with Chelex 100 (sodium form) to remove any paramagnetic material and lyophilized to yield the 8-amino-NAD<sup>+</sup> (13 mg, 0.02 mmol, 13%) as a colorless solid.

HRMS (ES<sup>+</sup>) calcd for C<sub>21</sub>H<sub>29</sub>N<sub>8</sub>O<sub>14</sub>P<sub>2</sub>, 679.1273 M<sup>+</sup>; found, 679.1252; and R<sub>T</sub> = 3.03 min.

8-Aminoadenosine Diphosphoribose (8-NH₂-ADPR, 12). NADase (from Neurospora crassa; Sigma; 0.52 U) in Tris-HCl buffer (1 mL, 0.1 M, pH 7.2-7.4) was added to a solution of 8-NH₂-NAD<sup>+</sup><sup>+</sup> 11 (13 mg) in Tris-HCl buffer (4 mL, 0.1 M, pH 7.2-7.4). The reaction mixture was incubated at 37 °C for 20 min.

See ref 21. No significant inhibition at 100 μM; compounds were not evaluated further. Antagonistic effect observed but not statistically significant; see relevant figure. Could only be tested at 100 μM due to solubility difficulties. Partial inhibition was observed at 900 μM; not evaluated further. n.d. = not determined. n.s. = no significant antagonist effect observed.
was stirred at 35 °C and was monitored by HPLC. After 4 h, all of the starting material had been consumed, the reaction mixture was diluted with water until the conductivity <200 μS/cm, and the product purified by ion-exchange (Q-Sepharose) chromatography eluting with a gradient (0–50%) of TEAB (1.0 M) in Milli-Q. Subsequent purification by reverse phase column chromatography, eluting with 0–30% MeCN in TEAB (0.05 M), left the desired 8-NH2-ADPR product (4.5 mg, 7.65 μmol, 40%) as a colorless solid. 1H (400 MHz, D2O) δ 7.98 (s, 1H, H-2), 5.99 (d, 1H, J = 6.7, H-1'), 5.24–5.31 (br, 0.4H, H-1'), 5.11–5.17 (br, 0.6H, H-1'), 4.68–4.64 (br, 1H, H-2'), 4.38–4.44 (br, 1H, H-2'), 3.91–4.31 (m, 8H, H-3', 2×H-5', H-3', H-4', and 2×H-5'). HRMS (ES+) calc for C11H15N4O5P3, 573.0573 M+; found, 573.0775; and Rf = 12.2 min.

Synthesis of Purine Modified ADPR Analogues. Inosine-5'-diphosphoribose (IDPR, 13). NHD7 (30 μmol) and NADase were reacted under the general protocol A to afford IDPR as a glassy solid (40% yield). 1H (400 MHz, D2O) δ 8.44 (s, 1H, H-2), 8.19 (s, 1H, H-8), 6.61 (d, 1H, J1/2 = 6.1, H-1'), 5.31 (d, 1H, J1/2 = 4.1, H-1'). 1H (270 MHz, D2O) δ 8.03 (s, 1H, H-2), 6.66 (s, 1H, H-7), 6.17 (d, 1H, J1/2 = 6.1, H-1'), 5.21–5.17 (s, 2H, H-2' and H-4'), 5.40–4.50 (m, 1H, H-3, H-3') and 4.19–3.94 (m, 8H, H-5'). 13C (decoupled, 162 MHz, D2O) δ 102.0 (d, AB system, J = 18.8°), 10.7 (d, AB system, J = 18.8). HRMS (ES+) calc for C11H15N4O5P3, 559.0484 (M − H); found, 559.0480. UV (H2O, pH 7.2) λmax 248 nm (ε 14500).

7-Deaza-8-bromoadenosine Diphosphoribose (7-Deaza-8-BADPR, 15). Nicotinamide-7-deaza-8-bromo-adenosine-5′-dicinucleotide (7-deaza-8-bromo-NAD+) 15 was treated with NADase under the general procedure A to afford 7-deaza-8-Br-ADPR as a glassy solid (12.7 μmol, 85%). 1H (270 MHz, D2O) δ 8.03 (s, 1H, H-2), 6.66 (s, 1H, H-7), 6.17 (d, 1H, J1/2 = 6.1, H-1'), 5.21–5.17 (s, 2H, H-2' and H-4'), 5.40–4.50 (m, 1H, H-3, H-3') and 4.19–3.94 (m, 8H, H-5'). 13C (decoupled, 162 MHz, D2O) δ 105.0 (m and -10.7 (m). HRMS (ES+) calc for C11H15N4O5P3Br, 634.9797 (M − Br); found 634.9787. HRMS (ES+) calc for C11H15N4O5P3Br, 636.9776 (M + H−) δ 636.9776. UV (H2O, pH 7.1) λmax 277 nm (ε 13250).

6-O-Methylinosine Diphosphoribose (6-O-Me-IDPR, 24). 2′,3′,5′-Tri-O-acetyl-6-chloro adenosine 19. To a solution of 1,2,3,5-O-tetraacetate ribofuranoside 18 (4.7 g, 14.7 mmol), 6-chloropurine 1 (2.1 g, 100%), and MgSO4 (54 mg, 0.464 mmol) were dissolved in dry DMSO (2 mL) and coevaporated with dry DMF (5 × 3 mL). The residue was dissolved in DMSO (1 mL) to which was added morpholine (150 μL, 1.72 mmol), dipyriddilidilide (182 mg, 0.827 mmol), and triphenylphosphine (217 mg, 0.827 mmol), at which point the solution became bright yellow. It was stirred for 1 h at rt, after which HPLC analysis showed formation of a new peak. Precipitation of the product occurred by dropwise addition of solution of NaCl in acetone (0.1 M). The resulting precipitate was filtered and washed with acetone to yield the desired product as a pale-yellow solid, which was used in the next step without further purification. 6-O-Me-IMP morpholinate (0.14 mg, 0.232 mmol), β-NMN-2H2O (85 mg, 0.253 mmol), and MgSO4 (54 mg, 0.464 mmol) were dissolved in a 0.2 M solution of MeCN in formamide (1.7 mL) and stirred at rt for 16 h, after which HPLC analysis showed completion of the reaction (Rt (6-O-Me-NHDI) = 3.8 min). MeCN was added to precipitate the product, which was filtered, dissolved in Milli-Q, and applied to a reverse phase gradiclar column eluted with a gradient of 5–65% MeCN in 0.05 M TEAB. Further treatment with Chelex 100 to remove any paramagnetic particles afforded the desired product as the sodium salt (18 mg, 8%). 1H (400 MHz, D2O) δ 9.21 (s, 1H, H2), 9.07 (d, 1H, J1/2 = 6.3, H6), 8.67 (d, 1H, J1/2 = 8.2, H4), 8.39 (s, 1H, H8), 8.27 (s, 1H, H2), 8.09–8.06 (m, 1H, H5s), 5.96 (d, 1H, J1/2 = 5.9, H1'), 5.94 (d, 1H, J1/2 = 5.5, H1'), 4.62 (app t, 1H, J1/2 = J2/3 = 5.5, H2') and 4.38–4.40 (m, 9H, H3a). 13C (100 MHz D2O) δ 165.1 (C=O), 160.7 (C-6), 151.1 (C-8), 151.0 (C-4), 145.7 (C-5), 142.4 (C-6), 141.5 (C-2), 139.8 (C-2a), 133.6 (C-5), 128.6 (C-3), 120.4 (C-5), 79.9 (C-1'), 870.1 (C-4'), 86.8 (C-4', J = 9.2), 83.7 (C-4', J = 9.2), 77.4 (C-2'), 74.0 (C-2a), 70.4 (C-3'), 70.2 (C-3'), 64.3, 63.2 (2×CH) and 54.9 (CH3). 13C (100 MHz D2O) δ 11.4 (J = 20.7) and −11.7 (J = 20.7). MS (ES) m/z 678.2 [M + H]+, 100%. HRMS (ES+) calc for C11H15N4O5P2, 678.1093 ([M + H]+) found, 678.1088. UV (H2O, λmax 251 nm (ε 19200).

6-O-Methylinosine-5′-diphosphoribose (6-O-Me-IDPR, 24). 6-O-Me-NHD7 sodium salt 23 (17.3 mg, 25.5 μmol) was incubated with Apysia cyclus (40 μL) in a 25 mM HEPES buffer (35 mL, pH 7.4) at rt. After 4 h at rt, HPLC analysis showed completion of the reaction (Rt (6-O-Me-NHDI) = 1.7 min and Rt (product) = 15.9 min). The mixture was then applied to a Q-Sepharose ion exchange column eluted with 1 M TEAB buffer. The appropriate fractions were collected and evaporated under vacuum, and the residue was coevaporated with MeOH to afford the hydrolyzed product 6-O-Me-IDPR as a triethylammonium salt. 1H (270 MHz, D2O) δ 8.88 (s, 1H, H-2), 8.45 (s, 1H, H-8), 6.15
The residue was applied to a semi-preparative C18 column developed to a reverse phase column and the product eluted with a gradient of H2O, O-CH2-CH2). 31P (109 MHz, D2O) 1.4 (s).

MgSO4 (53 mg, 0.454 mmol) in a 0.2 M solution of MnCl2 in formamide (1.5 mL) at rt overnight, after which HPLC analysis showed no remaining starting material. The volatiles were evaporated under reduced pressure, and the residue was applied to a semipreparative C18 column developed with a linear gradient of 0.1 M TEAB against MeCN. The appropriate fractions were evaporated and the crude TEA salt was removed by coevaporation with MeOH to leave the desired ADPR analogue (2.6 μmol, 20%) as a glassy solid TEA salt. 1H (400 MHz, D2O) δ 8.37 (s, 1H, H-8), 5.16 (s, 1H, H-2), 5.03 (d, 1H, J3,2 = 5.0, H-1′), 5.20 (d, 1H, J1,4 = 4.1, H-α′, 5.10 (d, 1H, J1,2 = 2.2, H-1′), 4.71–4.63 (m, 2H, H-sugar), 4.23–3.85 (m, 7H, H-sugar), 2.35–2.69 (dd, 1H, J1,α = 10.0 and Jα+α = 5.8, H-α′) and 2.17–2.12 (m, 1H, H-3′). 13C (decoupled, 162 MHz, D2O) δ −11.1 (br m). HRMS (ES) calcd for C15H23N6O7P2, 542.0390 (M+H)−; found, 542.0389. UV (H2O, pH 7.3) λmax 259 nm (ε 15400).

**Synthesis of Adenylate Monosulfonamide (SALS).** Synthesis was carried out without protection of the 6-amino group to generate SALS. For details, see Supporting Information.

**Synthesis of Pyrophosphate Modified ADPR Analogues. Adenine Triphosphate (ATP).** A solution of cATPR 46 (5 μmol) in Tris HCl (100 mM, pH 7) was heated to 100 °C for 1 h, after which HPLC analysis showed conversion to a new product. The solution was applied to a reverse phase column eluted with a 5–65% gradient of MeCN in 0.05 M TEAB. The appropriate fractions were collected and evaporated to afford the desired triphosphate as its sodium salt (2.7 μmol, 54%). 1H (270 MHz, D2O) δ 8.54 (s, 1H, H-2), 8.26 (s, 1H, H-8), 6.11 (d, 1H, J2,3 = 5.8, H-1′), 5.31 (d, 0.4H, J1,2 = 4.1, H-1′), 5.15 (d, 0.6H, J3,2 = 2.3, H-1′), 4.55–4.52 (m, 1H, H-2′) and 4.37–3.96 (m, 9H, H-3′, H-4′, H-5′, H-2′, H-3′, H-4′ and H-5′). 13C (decoupled, 109 MHz, D2O) δ −11.6 (br s), −23.4 (br s, O-P-O). HRMS (ES) calcd for C16H24N6O7P2, 638.0307 (M+H)−; found, 638.0331. UV (H2O, pH 7.2) λmax 259 nm (ε 17180).

**Synthesis of Sulfonamide Analogues: Salicylic Adenosine Monosulfonamide (SALS).** Synthesis was carried out without protection of the 6-amino group to generate SALS. For details, see Supporting Information.

**Synthesis of Adenosine Squalar (Asq).** 2′,3′-O-isopropylidene-5′-amino-5′-deoxyadenosine 59. 10% Pd/C (110 mg) was added to a solution of 2′,3′-O-isopropylidene-5′-azido-5′-deoxyadenosine (1.0 g, 3.01 mmol) in EtOH. The mixture was stirred for 16 h under a hydrogen atmosphere, after which the palladium was filtered and the solvent was removed under vacuum, yielding the desired compound as a white solid (0.9 g, 95%). 1H (400 MHz, DMSO-d6) δ 8.34 (s, 1H, H-8), 8.14 (s, 1H, H-2), 7.29 (br s, 2H, NH2), 6.11 (d, 1H, J2,3 = 3.0, H-1′), 5.42 (dd, 1H, J1,2 = 6.2 and J1,3 = 3.0, H-2′), 5.01 (dd, 1H, J2,3 = 6.2 and J2,4 = 2.9, H-3′), 4.20–4.16 (m, 1H, H-4′), 2.91–2.81 (m, 2H, 2 × H-5′), 1.52 (s, 3H, CH3), and 1.30 (3H, CH3). 13C (100 MHz, DMSO-d6) δ 156.1 (C-6), 152.7 (C-8), 148.8 (C-4), 140.0 (C-2), 119.2 (C-5), 113.3 (C-9), 89.2 (C-1′), 87.80 (C-8′), 82.7 (C-2′), 81.6 (C-3′), 43.7 (C-5′), 27.0 and 25.2 (2 × CH3). HRMS (ES) calcd for C15H19N2O7S, 307.1513 (M+H)+; found, 307.1511.

**1-O-Methyl-2,3′-O-isopropylidene-5′-O-p-toluene sulfonamido-beta-D-ribofuranose 50.** To a solution of 1-O-methyl-2,3′-O-isopropylidene-beta-D-ribofuranose 49 (0.61 g, 2.989 mmol) in dry pyridine (1 mL), externally cooled with ice, was added p-tolumensulfonyl chloride (0.7 g, 3.668 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred at rt under nitrogen for 5 h. Water (0.3 mL) was added and stirring continued for 30 min. This mixture was extracted with chloroform (3 × 10 mL) and the combined organic phases washed sequentially with CuSO4 (10% w/v,aq satd), NaHCO3 (aq satd) and water and then dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was purified on an Isco chromatographic system (petrol–EtOAc, 7:3 v/v) to yield the desired compound as a white solid (0.92 g, 81%). 1H (400 MHz, CDCl3) δ 7.71 (d, 2H, J = 8.7 × Ar-H), 7.26 (d, 2H, J = 8.6, Ar-H), 4.83 (s, 1H, H-1′), 4.51 (dd, 1H, J1,2 = 6.0 and J1,3 = 3.44 (d, 1H, J1,2 = 6.0, H-1′), 4.21 (dt, 1H, J1,ε = 7.1 and Jε,γ = 0.6, H-4′), 3.93–3.91 (m, 2H, H-2′, H-3′), 3.14 (3H, 3H, OMe), 2.36 (3H, 3H, CH3), 1.35 (3H, 3H, CH3) and 1.19 (3H, 3H, CH3). 13C (100 MHz, CDCl3) δ 144.9 (C-SO2), 132.8 (C-Me), 129.8 (2C), 127.9 (2C) (all CH), 112.6 (C), 109.4 (C-1′), 84.8 (C-2′), 83.5 (C-2′), 81.3 (C-3′), 69.1 (C-5′), 54.9 (OMe), 26.2, 24.8.
(2 × CH₂) and 21.5 (CH₂-Ph). HRMS (ES') calc'd for C₉₁H₇₆NaO₁₈S, 381.0978 (MH') ; found, 381.0969.

1-O-Methyl-2,3-O-isopropylidene-5-azido-5-deoxy-β-D-ribofuranose 51. To a solution of 1-O-methyl-2,3-O-isopropylidene-5-O-toluenesulfonyl-β-D-ribofuranose 50 (2.4 g, 6.7 mmol) in DMF (20 mL) was added NaN₃ (5.2 g, 80.4 mmol), and the reaction mixture was stirred at 120 °C for 16 h. After cooling to rt, acetone (20 mL) was added and the solid was removed by filtration. The solvents were evaporated under reduced pressure, and the residue was dissolved in DCM (50 mL) and washed successively with water (50 mL), satd aq NaHCO₃ (50 mL), and water (50 mL). The organic layer was dried (MgSO₄), filtered, and concentrated under reduced pressure to yield a thick white solid.

1-β-Nosine-4-(cyclopentylamino)-cyclobut-3-ene-1,2-dione 60 (40 mg, 85%) was recrystallized from MeOH, 8:2 (v/v) to yield a white solid (30 mg, 60%).

1-β-Cardosino-4-(cyclopentylamino)-cyclobut-3-ene-1,2-dione 61. To a solution of 1-β-Cardosino-4-cyclopentylamino-cyclobut-3-ene-1,2-dione 55 (110 mg, 0.526 mmol) and DIPEA (50 μL, 0.284 mmol) in EtOH (3 mL) was added 2',3',5'-O-Isopropylidene-β-D-xylofuranosyl (4-cyclopentylamino)cyclobut-3-ene-1,2-dine 59 (177 mg, 0.578 mmol). The reaction mixture was stirred at rt for 18 h. The solvent was removed under reduced pressure, and the residue was purified on an Isgo chromatographic system (DCM–MeOH, 8:2 v/v) to yield the desired product as a white solid (60 mg, 43%).

1-O-Methyl-2,3- O-isopropylidene-5-amino-5-deoxy-β-D-ribofuranose 52 (PPh₃, 1.95 g, 7.45 mmol) was added to a solution of 1-O-methyl-2,3-O-isopropylidene-5-amino-5-deoxy-β-D-ribofuranose 51 (1.4 g, 6.11 mmol) in THF (7 mL). The reaction mixture was stirred at rt for 16 h. After cooling to 0 °C, the solvent was removed under reduced pressure, and the residue was stirred for another 7 h. Evaporation of the solvents followed by purification on an Isco chromatographic system (petrol–EtOAc, 1:1 v/v) yielded a thick white solid.

1-β-Cardosino-4-(cyclopentylamino)-cyclobut-3-ene-1,2-dione 61 (40 mg, 85%).

1-β-Cardosino-4-(cyclopentylamino)-cyclobut-3-ene-1,2-dione 61. To a solution of 1-β-Cardosino-4-cyclopentylamino-cyclobut-3-ene-1,2-dione 55 (110 mg, 0.526 mmol) and DIPEA (50 μL, 0.284 mmol) in EtOH (3 mL) was added 2',3',5'-O-Isopropylidene-β-D-xylofuranosyl (4-cyclopentylamino)cyclobut-3-ene-1,2-dine 59 (177 mg, 0.578 mmol). The reaction mixture was stirred at rt for 18 h. The solvent was removed under reduced pressure, and the residue was purified on an Isco chromatographic system (DCM–MeOH, 8:2 v/v) to yield the desired product as a white solid (60 mg, 43%).
3-Butylamino-4-ethoxy-3-ene-1,2-dione 56. Butylamine (135 μL, 1.367 mmol), DIPEA (141 μL, 0.811 mmol), and diethyl carbonate (322 μL, 1.503 mmol) were reacted under general protocol C to lead the desired product as a white foam (230 mg, 91%). 1H (400 MHz, CDCl3) δ 4.77 (q, 2H, J = 7.2, OCH2-CH2), 3.66 (t, 1H, CH2), 3.48 (t, 1H, J = 7.0, CH2), 1.69 – 1.62 (m, 2H, CH2), 1.53 – 1.00 (m, 5H, CH2), 1.37 – 1.01 (m, 5H, CH2), 1.26 (s, 6H, CH3). 13C (100 MHz, CDCl3) δ 189.9 (C-2), 184.5 (C-1), 177.6 (C-3), 174.7 (C-4), 70.7, 45.3, 33.7, 20.6 (4 × CH2), 16.2 (EtCH3) and 14.0 (BuCH3). HRMS (ES+) calc for C21H28N7O5, 481.2142 (MH)+; found, 484.2147.

Synthesis of Click Analogue: 8-Phenyladenosine-1,4-Triazole Ribose (8-PhTrAR). 1-O-Methyl-2,3-diisopropylamino-5-O-propargyl-β-D-ribofuranose 70. A solution of 1-O-methyl-2,3-diisopropylamino-5-O-propargyl-β-D-ribofuranose 49 (600 mg, 2.94 mmol) in DFM (40 mL) was cooled to 0 °C, and NaH (156 mg, 3.91 mmol, 60% in mineral oil) was added. The mixture was stirred at 0 °C for 10 minutes, after which TBAI (65 mg, 0.176 mmol) and propargyl chloride (0.25 mL, 3.528 mmol) were added. The reaction mixture was stirred at 25 °C for 16 h, the solvent was reduced under reduced pressure, and the residue was purified by column chromatography using an Isco chromatographic column (petrol–EtOAc, 1:0 → 0:1 v/v). The product was obtained as a colorless liquid (512 mg, 72%). 1H (400 MHz, CDCl3) δ 4.95 (s, 1H, H-1′), 4.65 (d, 1H, JX = 6.0, H-2′), 4.55 (d, 1H, JX = 6.0, H-3′), 4.33 – 4.30 (m, 1H, H-4′), 4.17 (d, 2H, J = 2.4, CH2), 3.85 (dd, 1H, J = 9.5 and J = 6.5, H-5′), 3.52 – 3.47 (m, 3H, H-6′, H-7′, and H-8′), 3.32 (3H, CH3, OMe), 2.42 (t, 1H, J = 6.3 Hz, CH), 1.46 (s, 3H, CH3) and 1.30 (3H, CH3). 13C (100 MHz, CDCl3) δ 112.3 (C-10′), 110.9 (C-8′), 84.8 (C-4′), 81.9 (C-3′), 79.3 (C-5′), 74.6 (HCC=O), 70.5 (C-7′), 58.3 (CH3), 54.8 (OCH3), 26.4 and 24.9 (2 × CH3). HRMS (ES+) calc for C29H32N10O11, 546.2146 (MH)+; found, 544.2157.

2′-O-Isopropylisopropylidene-5′-azido-8′-bromoadenosine 69. 2′,3′-O-Isopropylidene-5′-azido-8′-bromoadenosine 68 (100 mg, 0.30 mmol) was taken up in NaOAc buffer (pH 4, 1 M, 15 mL) and Br2 (12 μL, 0.45 mmol) added. The resulting solution was stirred in the dark for 24 h and then a solution of NaHSO3 (4 M,aq) added until the solution was colorless. All solvents were evaporated and the residue purified by column chromatography using an Isco chromatographic column (petrol–EtOAc, 2:1 v/v). The title compound was obtained as an off-white solid (123 mg, 99%). 1H (400 MHz, CDCl3) δ 8.27 (s, 1H, H-2′), 6.20 (d, 1H, JX = 1.8 Hz, H-1′), 5.99 (br s, 2H, NH), 5.68 (dd, 1H, JX = 6.3 and J = 1.8 Hz, H-2′), 5.15 (dd, 1H, JX = 6.3 and J = 3.6 Hz, H-3′), 4.36 – 4.31 (m, 1H, H-4′), 3.54 – 3.43 (m, 2H, 2 × H-5′), 1.61 (s, 3H, CH3) and 1.39 (s, 3H, CH3). 13C (100 MHz, CDCl3) δ 154.6 (C-6′), 152.8 (C-2′), 150.3 (C-4′), 127.5 (C-8′), 120.1 (C-5′), 114.5 (C-1′), 91.2 (C-1′), 86.4 (C-2′), 83.4 (C-3′), 82.4 (C-4′), 52.1 (C-5′), 27.0 and 25.3 (2 × CH3). HRMS (ES+) calc for C27H24N13O4Br, 511.0523 (MH)+; found, 511.0522; and calc for C27H24N13O4Br, 510.0503 (MH)+; found, 510.0522. Rf = 0.58 (DCM–acetone, 3:2 v/v).

1′-O-Isopropylidene-5′-deoxy-8′-bromoadenosine 71. To a solution of 2′,3′-O-Isopropylidene-5′-deoxy-8′-bromoadenosine
Syntesis of Terminal Ribose Modification: Synthesis of Cyclopentyl-ADP. Cyclopentyl-dibenzyolphosphoramide 78. To a solution of tetrazole (81 mg, 1.16 mmol) and disopropyl-dibenzyolphosphoramide (300 mg, 0.871 mmol) in DCM (10 mL) was added cyclopentanol 77 (50 mg, 0.58 mmol). The reaction mixture was stirred at rt for 20 min, after which TLC analysis showed total conversion of starting material to a single phosphate (petrol–EtOAc, 6:4 v/v, Rf = 0.32). The solution was cooled to 0 °C, and mCPBA (200 mg, 1.16 mmol) was added in one portion. The mixture was warmed up to rt, diluted with EtOAc (20 mL), and washed with 10% Na2SO4 (20 mL), satd aq NaHCO3 (20 mL), and brine (20 mL). The organic phase was collected, dried (Na2SO4), filtered, and evaporated to dryness. The residue was purified with an Isco chromatography system (petrol–EtOAc, 7:3 v/v) to yield the title compound as a colorless oil (173 mg, 86%). \( \text{H} \) (400 MHz, CDCl3) δ 7.36–7.20 (m, 10H, H-benzyl), 5.06–4.97 (m, 4H, 2 × CH2), 4.90–4.85 (m, 1H, CH–O) and 1.86–1.50 (m, 8H, 4 × CH). \( ^{31} \text{P} \) (161 MHz, CDCl3, decoupled) δ −1.6 (s).

Cyclopentyl-monophosphate 79. The above material (78, 173 mg, 0.5 mmol) was dissolved in a mixture of MeOH–H2O–cyclohexane (10:1:5 v/v/v, 16 mL) to which was added Pd(OH)2/C (20%). The solution was heated to 80 °C for 2 h, after which the palladium was removed by filtration through Celite and the filtrate was evaporated under reduced pressure, leaving a residue which was used directly in the next step.

Cyclopentyl-ADP 80. AMP-Na+ salt (190 mg, 0.547 mmol) was passed through a small Dowex column (TEA form) and eluted with Milli-Q water. The solvent was evaporated to leave a residue, which was dissolved in DMSO and coevaporated with DMF (3 × 3 ml). The residue obtained was dissolved in DMSO (3 mL) and morpholine (0.25 mL, 2.845 mmol), dipiperyldisulfide (301 mg, 1.367 mmol), and triphenylphosphine (358 mg, 1.367 mmol) were added in this order. The resulting yellow solution was stirred for 90 min, after which a 0.1 M solution of NaCl in acetone was added. The precipitate obtained was collected by filtration and used directly in the next step. To a solution of AMP-morpholidente (154 mg, 0.350 mmol) and cyclopentane monophosphate 79 (64 mg, 0.380 mmol) in 0.2 N NaN3 in formamide (2 mL) was added MgSO4 (82 mg, 0.70 mmol), and it was stirred for 16 h at which time HPLC analysis showed product formation. Precipitation of the product occurred on addition of MeCN and was purified on RP-18 afforded (after treatment with Chelex 100) the desired dinucleotide as a glassy solid (55 mg, 14% over 2 steps). \( \text{H} \) (400 MHz, D2O) δ 8.44 (s, 7H, H-2), 8.19 (s, 1H, H-8), 8.07 (s, 1H, H-1′), 4.73–4.71 (1H, CH–O), 4.66 (br s, 1H, H-2′), 4.48 (br s, 1H, H-3′), 4.32 (br s, 1H, H-4′), 4.15 (br s, 2H, 2 × H-5′), 1.62 (d, 6H, 2 × H-6′), 1.32–1.50 (2H, 2 × CH2), and 1.36–1.34 (2H, 2 × CH2).

Cyclopentyl-phosphate 81. A flask containing 8-bromo-2′,3′-isopropylidene-adenosine 82 (200 mg, 0.519 mmol), NaCl/Pd (5 mol %), TPPTS (25 mol %), Pb(OH)2 (190 mg, 1.562 mmol), and NaN3 (165 mg, 1.557 mmol) was purged with argon, and a degassed mixture of MeCN–H2O (1:1 v/v, 6 mL) was added. The resulting mixture was refluxed for 1 h, then water (6 mL) was added and the solution neutralized with 1 M HCl. The white precipitate obtained was collected by filtration and dried under vacuum (161 mg, 81%). \( \text{H} \) (400 MHz, DMSO-d6) δ 8.16 (s, 1H, H-2′), 7.73–7.71 (m, 2H, Ar–H), 7.60–7.58 (m, 3H, Ar–H), 7.44 (s, 2H, NH2), 5.84 (d, 1H, J2′,2 = 3.4, H-3′), 5.56 (dd, 1H, J2′,2 = 6.1 and J2′,3 = 3.4, H-2′), 5.36–5.36 (m, 1H, H-5′,OH), 5.03 (dd, 1H, J2′,2 = 6.1 and J2′,3 = 2.5, H-3′), 4.17 (dd, 1H, J2′,3 = 5.1 and J2′,4′ = 2.5, H-4′), 3.63 (dd, 1H, J2′,4′ = 11.5 and J2′,5′ = 5.1, H-5′) and 3.56–3.51 (m, 2H, 2 × CH2).

Cyclopentyl-ADP 82. 8-Phenyl-2′,3′-isopropylidene-adenosine. A mixture of 154 mg, 0.350 mmol) and cyclopentane monophosphate 79 (64 mg, 0.380 mmol) in 0.2 N NaCl in formamide (2 mL) was added MgSO4 (82 mg, 0.70 mmol), and it was stirred for 16 h at which time HPLC analysis showed product formation. Precipitation of the product occurred on addition of MeCN and was purified on RP-18 afforded (after treatment with Chelex 100) the desired dinucleotide as a glassy solid (55 mg, 14% over 2 steps).
CH3). HRMS (ES+) calc'd for C16H17N5O7, 422.0871 (M+); found, 422.0868.

8-Phenyladenosine-monophosphate 83. 8-Phenyl-2′,3′-O-isopropylidene-adenosine (82, 150 mg, 0.39 mmol) was deprotected under general protocol D to yield the desired compound as a white solid which was used directly in the next step. 8-Phenyl-adenosine (0.39 mmol) was dissolved in triethylphosphate (1 mL) by heating with a heat gun. The resulting colorless solution was cooled to 0 °C, and water (2 μL) was added followed by POCl3 (0.15 mL, 1.56 mmol), then stirred at 0 °C until disappearance of the starting material and formation of a single peak was observed as shown by HPLC. After 1 h, the reaction was quenched by addition of ice-water (15 mL) and the mixture was stirred for 15 min at 0 °C, after which it was warmed to rt. Triethylphosphate was extracted with EtOAc (6 × 6 mL) and the aqueous phase was neutralized with 2 N NaOH. The resulting yellow solution was stirred for 90 min, after which a 0.179 of 8-Ph-AMP-morpholidate (31 mg, 0.060 mmol) and cyclopentane (0.478 mmol) in 0.2 N MnCl2 in H2O (5 mL) was added followed by triethylphosphate (1 mL) and the residue obtained was dissolved in DMSO (90 μL) and the residue was applied to a C18 semipreparative column eluted 65% gradient of MeCN (100 mM, pH 7.4). Precipitation of the product occurred on addition of MeCN (10 mL) and the residue was collected by filtration and used directly in the next step. To a solution of 8-Ph-AMP-morpholidate (82, 31 mg, 0.066 mmol) in 0.2 N MnCl2 in H2O (5 mL), 1H (500 MHz, DMSO-d6) δ 8.21 (s, 1H, H-2), 7.67 (d, 1H, J = 6.2), 7.60–7.56 (m, 2H), 7.28 (d, 2H, J = 8.1) (5 × ArH), 5.82 (d, 1H, J = 6.4, H-1′), 5.15 (app t, 1H, H-2′), 4.31 (dd, 1H, J = 6.4, 4.5, H-3′), 4.08–3.98 (m, 4H, H-4′, 2 × H-5′, CH—O), 1.62–1.60 (m, 4H), 1.52–1.50 (m, 2H) and 1.36–1.34 (m, 2H) (4 × CH3). 31P (161 MHz, D2O) δ 115.1 (m). 13C (100 MHz, DMSO-d6) δ 158.3 (C-6′), 152.1 (C-8′), 149.3 (C-4′), 140.1 (C-2′), 132.4 (2C), 129.7 (2C), 128.6 (5 × ArCH), 113.2 (C-5′), 87.2 (C-1′), 83.8 (C-4′), 79.7 (CH3), 73.9 (C-2′), 70.4 (C-3′), 65.3 (C-5′), 33.6 and 22.9 (2C). HRMS (ES+) calc'd for C21H26N5O13P2, 618.1008 (M+ – H); found, 618.1013. UV (H2O, pH 7.4) λmax 276 nm (ε 16700).

Pharmacology. Human neutrophils. BSA (bovine serum albumin), Fura-2/AM, NaCl, EGTA, NMDG, Tris base, and Histopaque-1119 were purchased from Sigma Aldrich (München, Germany). KCl, MgSO4, MgCl2, CaCl2, NaH2PO4, d-glucose, i-ascorbic acid, Tween, ionomycin, and EDTA were obtained from Merck (Darmstadt, Germany). HEPES was procured from Biomol (Hamburg, Germany). Fibronecctin, DMEM, and penicillin/streptomycin were supplied by Invitrogen (Darmstadt, Germany). FBS (fetal bovine serum) and G418 sulfate were purchased from Biochrom (Berlin, Germany). The anti-TRPM2 antibody was procured from Novus Biologicals (Littleton, USA). The goat antirabbit antiserum was purchased from Dianova (Hamburg, Germany). PVDF membrane was acquired from Millipore (Darmstadt, Germany). Percoll and ECL western blotting detection reagents were supplied by GE Healthcare (Uppsala, Sweden).

Cell Culture. HEK293 cells were maintained in DMEM medium containing Glutamax 1% (100 mM, pH 7.4) complemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in the presence of 5% CO2. HEK293 clones expressing TRPM2/EGFP (or EGFP for control) were cultured under the same conditions, while the medium was supplemented with 400 μg/mL G418 sulfate.

Transfection of HEK293 Cells and Generation of Cell Lines. HEK293 wild-type cells were transfected with two different expression vectors coding either for human TRPM2 and EGFP (pIRE2-EGFP-TRPM2) or for EGFP alone (pIRE2-EGFP) as described previously.58,59 Cells carrying the expression constructs were selected by addition of 400 μg/mL G418 sulfate (BIOchrom) to the culture medium. Clonal cell lines with stable expression were generated using the limiting dilution technique.

Patch Clamp Measurements. Cells were seeded at low density the day before use. During the experiments, cells were kept at room temperature in bath solution (1 mM CaCl2, 140 mM NMDG, 5 mM KCl, 3.3 mM MgCl2, 1 mM CaCl2, 5 mM d-glucose, 10 mM HEPES, pH 7.4). Patch pipets with resistances of 1.7–3.5 MΩ were pulled from 1.5-mm diameter borosilicate glass capillaries and filled with pipet solution (120 mM KCl, 8 mM NaCl, 1 mM MgCl2, 10 mM HEPES, 10 mM EGTA, 5.6 mM CaCl2), resulting in 200 mM free [Ca2+] as calculated by CaBuf software (G. Droogmans, formerly available from ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip). Patch clamp experiments were carried out in the whole-cell configuration.60 Data were acquired with an EPC10 amplifier and PatchMaster software (HEKA Elektronik, Germany) and were compensated for fast and slow capacity transients. The cells were held at −50 mV and current was measured during 140 ms voltage ramps from −85 to −20 mV every 5 s over a period of 450 s. Series resistance was compensated by 70%. For activation of TRPM2, ADPR was added to the pipet solution at a concentration of 100 μM. Antagonist activity of the ADPR analogues was tested by adding them at different concentrations to a pipet solution with 100 μM ADPR. During some experiments, the pipet solution contained 0.1% DSMO because stock solutions of the more lipophilic ADPR analogues were prepared in DMSO.

Purification of Human Neutrophils. Fresh blood with EDTA as anticoagulant was obtained from indiscriminately selected volunteers. Neutrophils were isolated as described elsewhere.61 In brief, the blood was fractionated with a Histopaque-1119 density gradient and subsequently neutrophils were further purified by the use of Percoll layers ranging from 65 to 85% in density. After a final washing step, the cells were resuspended in Ca2+ measurement buffer (140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM NaH2PO4, 4 mM glucose, 20 mM HEPES, pH 7.4) and kept on ice until use. To avoid premature activation of the neutrophils, all buffers used during the isolation were supplemented with 2 mM EDTA, cell concentrations exceeding 5 × 106 cells/mL were avoided, and only endothoxin free

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materials and solutions were used. All experiments were performed within 6 h of blood donation.

**Ratiometric Ca^{2+} Imaging of Human Neutrophils.** Neutrophils were incubated with 4 μM Fura2/AM for 30 min at 37 °C in the dark, washed twice, and resuspended in Ca^{2+} measurement buffer (see above) at a concentration of 1 × 10^6 cells/mL. For each measurement, 5 × 10^6 cells were transferred to a small chamber consisting of a rubber O-ring fixed with silicone grease on a glass coverslip coated subsequently with 25 ng of BSA and 250 ng of fibronectin. The cells were incubated for 15 min at ambient temperature in the presence of 10 mM L-ascorbic acid (pH 7.4) and, if applicable, varying concentrations of 8-phenyl-ADP-R. The loaded coverslip was mounted on the stage of a PerkinElmer/Imrovision imaging system built around a Leica DM IRE 2 fluorescence microscope. Approximately 70 s after the beginning of the measurement, the cells were stimulated by addition of fMLP (final concentration 1 μM) or AS peptide (final concentration 10 μM). Ratiometric Ca^{2+} imaging was performed as described previously.52

**Chemotaxis Measurement of Human Neutrophils.** The migration of neutrophils was observed microscopically in microfluidic devices (μ-Slide Chemotaxis, Ibidi, Martinsried, Germany). First the μ-Slides were coated with 50 μg/mL fibronectin for 30 min at ambient temperature before washing three times and drying. Isolated neutrophils were resuspended to a concentration of 3 × 10^6 cells/mL in Ca^{2+} measurement buffer supplemented with 10% (v/v) plasma obtained from the same donor. If applicable, 8-phenyl-ADP-R or EGTA was added and the whole slide was loaded according to the manufacturer’s instructions and incubated at room temperature for 15 min. Adding 18 μL of fMLP (125 nM) to the upper reservoir resulted in a chemotactical gradient from 0 to 50 nM fMLP across the observation chamber. Plasma supplemented buffer without chemotactic agent was used in control experiments. The slide was mounted on the stage of the imaging system, and the main chamber observed at 10 times magnification in bright-field mode. After a 5 min resting period, greyscale images with a resolution of 672 × 510 pixels were recorded every 30 s for 1 h using Openlab Software 4.0.4. Cell migration was tracked manually with a 5 × 5 pixel maximum intensity centering correction using the manual-tracking plugin for ImageJ (1.45e). Migrational parameters were calculated from the movement paths using the Chemotaxis and Migration Tool software (v2.0, Ibidi GmbH).

## ASSOCIATED CONTENT

### Supporting Information

General experimental information, experimental procedures for known compounds, and ^1^H NMR spectra for compounds 5, 6, 7, 84, and 86. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written by contributions from all authors and all authors have approved the final version. C.M and T.K. contributed equally.

### Notes

The authors declare no competing financial interest.

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## REFERENCES


## ABBREVIATIONS USED

ACA, N-(p-aminocinnamoyl)anthranilic acid; ADP, adenosine 5′-diphosphate; ADPR, adenosine 5′-diphosphoribose; ADPRC, ADPR cyclase; AIBN, azobisobutyronitrile; AMP, adenosine 5′-monophosphate; ASQ, adenosine squaryl ribose; ATTR, adenosine triazole ribose; [Ca^{2+}]+, free cytosolic Ca^{2+} concentration; cADPR, cyclic adenosine 5′-diphosphoribose; cATPR, cyclic adenosine 5′-triphosphate ribose; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, N,N′-dicyclohexylcarbodiimide; DIPEA, N,N-diisopropylethylamine; FFA, fufenic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; IDPR, inosine 5′-diphosphoribose; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD°, nicotinamide adenine 5′-dinucleotide; NHD°, nicotinamide hypoxanthine 5′-dinucleotide; NMDG, N-methyl-D-glucamine; β-NMN°, β-nicotinamide 5′-mononucleotide; NUDT9H, NudT9-homology domain; PARC, poly-ADP-ribose-glycodylhydrolase; PARP, poly-ADP-ribose-polymerase; 4-Ph-ala, 4-phenylalanine; SAR, structure–activity relationship; Sal-AMS, salicyl-adenosine monosulfamate; TEAB, triethylammonium bicarbonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TEP, triethylphosphate; TPPTS, trisodium tris(m-sulfonatophenyl)phosphine; TRP, transient receptor potential channels; TRPM2, transient receptor potential channel, subfamily melastatin, member 2.


