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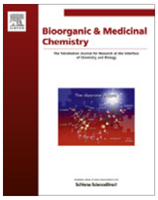

Effect of adenosine modified with a boron cluster pharmacophore on reactive oxygen species production by human neutrophils

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ABSTRACT

Methods for the synthesis of adenosine/boron cluster conjugates are proposed and the potential of the obtained derivatives to modulate neutrophil activity, especially reactive oxygen species (ROS) production in vitro, is described. An efficient inhibition of ROS production in activated neutrophils by adenosine modified at the 2′-C and 6-N positions with a *para*-carborane cluster ($C_2B_{10}H_{11}$) was discovered. The high affinity of the selected compounds for adenosine receptor A_{2A} was established. These results are in agreement with the possible involvement of receptor A_{2A} in the biological activities of adenosine/boron cluster conjugates. This study extends the range of innovative molecules available for testing as agents affecting inflammatory processes.

1. Introduction

The role of polymorphonuclear leukocytes (PMNs, neutrophils) in the innate immune response to invading pathogens and in inflammation is well established.[1–3] These cells protect the host from invading microorganisms through the ability to adhere, phagocytose and destroy engulfed pathogens. Neutrophil-derived reactive oxygen species (ROS, oxygen burst) and granule proteins not only kill and eliminate microbes and activate immune cells such as dendritic cells and lymphocytes but also damage surrounding tissue at sites of inflammation.[4] The inhibition of the harmful side effects of neutrophil activity in acute inflammatory processes may therefore prevent tissue injury and the spread of inflammation.

Adenosine is constitutively present in the extracellular space at a low concentration, but its extra- and intra-cellular levels significantly increase under unfavourable conditions including hypoxia, ischemia and inflammation. It was also shown that neutrophils themselves produce adenosine during activation at sites of inflammation and infection.[5–7] Adenosine and adenosine derivatives play an important role in the regulation of normal immune processes and also act as endogenous anti-aggregatory and anti-inflammatory agents. Adenosine is considered to be one of the most important agents that exerts both pro- and anti-inflammatory effects on neutrophils through, among others, inhibition of ROS production by formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated neutrophils.[8–10] It exerts its biological functions through interactions with numerous surface receptor subtypes classified as A_1, A_{2A}, A_{2B} and A_3 that are present on cells involved in immune and inflammatory reactions.[8–10] The A_{2A} receptors are the primary adenosine receptors responsible for anti-inflammatory effects during inflammatory processes[11,12] and play an important role in mediating the inhibition of neutrophil ROS production and adhesion.[13] The A_1 receptors, when occupied, promote neutrophil chemotaxis,[14] phagocytosis,[15] and adhesion.[16] It was also shown that A_3 receptors, which are expressed on neutrophils, together with A_{2A} receptors are involved in the reduction of neutrophils superoxide anion production.[17,18]

The therapeutic importance of exogenous adenosine is limited due its short half-life in circulation and its rapid uptake into adjacent cells.[19] Alterations in the structure of synthetic adenosine derivatives have been shown to modulate receptor-binding potency, selectivity and biological activity and to contribute to the improved therapeutic potential of these compounds.[20–23]

Carboranes are caged boron compounds that have been widely investigated in medicinal chemistry.[24] Carboranes, such as icosahedral dicarba-closo-dodecaboranes ($C_2B_{10}H_{12}$), are characterised by rigid geometry, high chemical and biological stability and an exceptional hydrophobic character. These characteristics support the use of these compounds in designed molecules based on hydrophobic target/ligand interactions.

The recent appeal of boron clusters to the pharmaceutical industry lies in that these clusters are abiotic and therefore chemically and biologically orthogonal to native cellular components (i.e., they are stable and inert in biological environments) and resistant to catabolism, which is a desirable property for biological

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applications. In addition, boron clusters may be used to target biological receptors that appear to be unaffected by nonboron-containing organic molecules by interacting with these receptors through diverse mechanisms.[25,26]

In a previous paper, we described the synthesis of new adenosine derivatives modified with boron clusters (Fig. 1, compounds **10** and **11**) and their effects on human blood platelet activity.[27] The modification of adenosine at the 2′-C position with a 1,12-dicarba-*closo*-dodecaboran-1-yl-(*para*-carboranyl) group results in the efficient inhibition of platelet function, including aggregation, protein secretion and P-selectin expression induced by thrombin or adenosine diphosphate (ADP).[27] In the present work we focus on modulating the effect of this new type of adenosine derivative on immune cells, such as neutrophils. New adenosine conjugates containing boron clusters at the 2′-C, 6-N and 2-C positions were synthesised and, together with compounds obtained previously (Fig. 1), were examined as possible modulators of human neutrophil activity in vitro. These compounds were applied exogenously to human neutrophils at different concentrations, and the respiratory burst (release of ROS) upon stimulation by fMLP was investigated.

2. Results and discussion

2.1. Chemistry

Medicinal chemistry is still dominated by organic chemistry, and most marketed drugs are purely organic molecules. The original stimulus for the development of the bioorganic and medicinal chemistry of boron clusters came from their high boron content and catabolic stability, both of which are necessary for boron neutron capture therapy (BNCT) for the treatment of cancer.[26,28] More recently, the less explored advantages of boron clusters and their use as pharmacophores or lipophilic components in design of bioactive molecules has attracted growing attention.[24–26,29–31] To expand the range of boron cluster-nucleoside conjugates available for biological screening, we developed several methods for the synthesis of this type of bioorganic-inorganic conjugates. These new methods can be used to produce pyrimidine as well as purine nucleoside derivatives, which were not accessible earlier.

The newly synthesised adenosine derivatives containing boron clusters (**4**, **7** and **9**, Fig. 1) and previously described compounds (**10** and **11**, Fig. 1), containing boron clusters such as 1,12-dicarba-*closo*-dodecaborane and 7,8-dicarba-*nido*-undecacarborane, were tested to determine their effects on neutrophil function. These compounds were prepared according to the following methods: 1) attachment of the carborane moiety at the 2′ position of the nucleoside via a formacetal linkage (compound **10**),[32,33] 2) tethering of the boron cluster to the nucleobase moiety of the nucleoside via dioxane ring opening of the oxonium derivative of the boron cluster (compound **11**)[27] and 3) a 'click chemistry' approach based on a modified Huisgen 1,3-dipolar cycloaddition (compound **4**).[34–36] Two new approaches useful in the preparation of adenosine-boron cluster conjugates modified at the nucleobase have also been proposed: (1) Sonogashira-type cross coupling between 2-iodoadenosine and a boron cluster donor containing a triple bond (compound **7**) and (2) substitution of chlorine at carbon 6 of

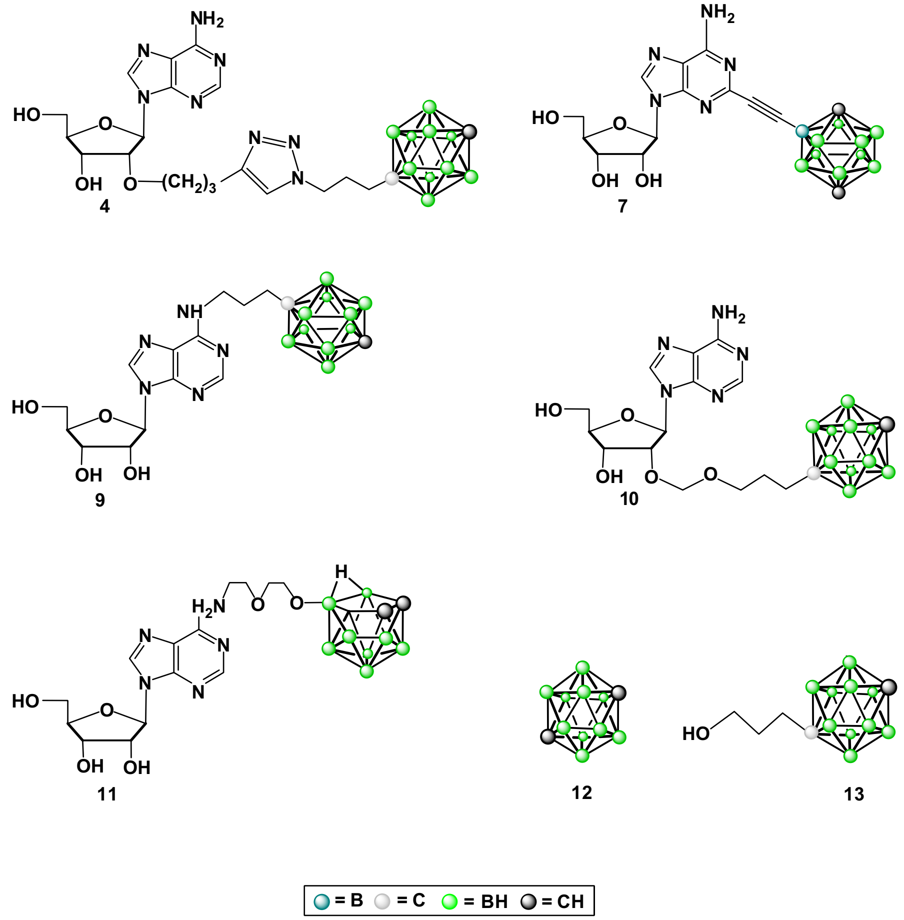


Figure 1. Structures of 2′-*O*-{[3-propyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)]-1-*N*-1,2,3-triazol-4-yl}propyladenosine (**4**), 2-ethynyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)adenosine (**7**), 6-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]adenosine (**9**), 2′-*O*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propyleneoxymethyl]adenosine (**10**), 6-*N*-[(3-oxapentoxy)-7,8-dicarba-*nido*-undecaboran-10-yl]adenosine (**11**), 1,12-dicarba-*closo*-dodecaborane (**12**) and 1-(3-hydroxypropanyl)-1,12-dicarba-*closo*-dodecaborane (**13**).

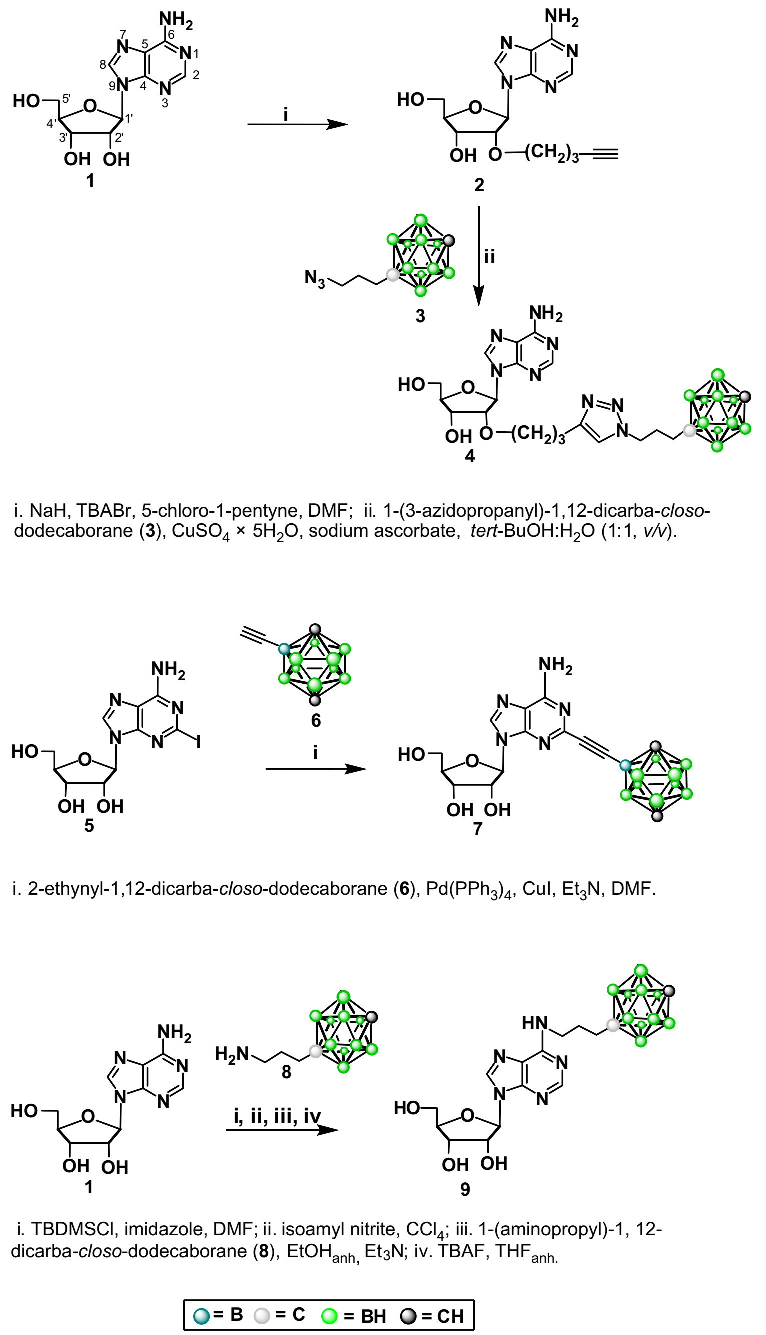
adenosine with a boron cluster containing an alkylamine (compound **9**).

The diversity and flexibility of the proposed methodologies make possible the further expansion of the present library of adenosine boron cluster conjugates for biological screening and hit optimisation.

A previously described 'click chemistry' approach was successfully applied to the synthesis of new adenosine derivatives modified at the 2′ position of the sugar residue (compound **4**, Scheme 1). The boron cluster acceptor, 2′-*O*-(1-pentyn-5-yl)adenosine (**2**), bearing a terminal triple bond, was obtained according to the literature method, whereas the boron cluster donor 1-(3-azidopropanyl)-1,12-dicarba-*closo*-dodecaborane (**3**) was synthesised via the reaction of 1-(3-hydroxypropanyl)-1,12-dicarba-*closo*-dodecaborane (**13**) and sodium azide in the presence of triphenylphosphine according to the general procedure for the synthesis of azides from alcohols.[34,36–40]

To prepare 2-ethynyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)-adenosine (**7**), an adenosine modified at carbon 2, a Sonogashira-type reaction between a boron cluster acceptor, 2-iodoadenosine (**5**) and a triple bond-containing boron cluster donor (compound **6**) was developed (Scheme 1). Compound **5** was prepared according to the literature procedures.[41–43] 2-Ethynyl-1,12-dicarba-*closo*-dodecaboran (**6**) was prepared as described.[44] The Sonogashira coupling was performed under the same conditions as used for the synthesis of 8-ethynyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)-2′-deoxyadenosine.[27]

The modification of adenosine at position 6 was achieved via the substitution of the chlorine in 6 chlorinated nucleosides with a boron cluster bearing an alkylamine (compound **9**). In this



i. NaH, TBABr, 5-chloro-1-pentyne, DMF; ii. 1-(3-azidopropanyl)-1,12-dicarba-*closo*-dodecaborane (**3**), $CuSO_4 \times 5H_2O$, sodium ascorbate, *tert*-BuOH:H_2O (1:1, *v/v*).

i. 2-ethynyl-1,12-dicarba-*closo*-dodecaborane (**6**), $Pd(PPh_3)_4$, CuI, Et_3N, DMF.

i. TBDMSCl, imidazole, DMF; ii. isoamyl nitrite, CCl_4; iii. 1-(aminopropyl)-1, 12-dicarba-*closo*-dodecaborane (**8**), $EtOH_{anh}$, Et_3N; iv. TBAF, $THF_{anh.}$

Scheme 1. Synthesis of adenosine modified with boron cluster at carbon 2′ (compound **4**), carbon 2 (compound **7**) and nitrogen 6 (compound **9**).

approach, 2′,3′,5′-tri-*O*-(*tert*-butyldimethylsilyl)-6-chloroadenosine was used as the boron cluster acceptor and 1-(3-aminopropyl)-1,12-dicarba-*closo*-dodecaborane (**8**) as the boron cluster donor. Amine **8** was obtained from azide **3** according to the published procedure with the exception that only methanol was used as a solvent. The substitution of chlorine at position 6 with compound **8** was performed by analogy to the reaction of 6-chloroadenosine with other amines, providing conjugate **9**.[45–47] All described compounds were fully characterised by chromatographic and instrumental methods before biological evaluation.

2.2. Biological results and discussion

2.2.1. Effects on ROS production

The effect of the synthesised adenosine derivatives on the ROS production of neutrophils in vitro were examined and compared with the effect of unmodified adenosine. Prior to functional studies, the effect of the synthesised compounds on neutrophil viability was determined using an exclusion test with propidium iodide (PI).[48,49] PI is excluded by viable cells but can penetrate the cell membranes of dying or dead cells. The presence of more than 10% nonviable neutrophils in the sample was interpreted to be indicative of a toxic effect of the compound. Based on the above test no toxicity for compounds **4**, **7**, **9**, **10** and **11** was observed.

The functional activity of mature neutrophils can be affected and/or regulated in vivo and in vitro by numerous agents, such as pro-inflammatory and anti-inflammatory cytokines, factors and signals.[1,50] The excessive and uncontrolled function of neutrophils can propagate inflammation, and therefore, agents that limit the intensity and time-course of this process are in constant demand.

The effects of adenosine and modified adenosine derivatives on the ROS production of human neutrophils stimulated with fMLP (10 μM) were determined using a luminol-enhanced chemiluminescence (CL) method.[51] Measurement of CL allows the detection of intracellularly and extracellularly released nicotinamide adenine dinucleotide phosphate (NADPH)-derived ROS. At the concentrations used (0.001–20 μM), the tested compounds did not interfere with luminol and did not affect ROS production in non-stimulated neutrophils (data not shown). The ability of these compounds to inhibit ROS production in neutrophils was defined as percentage values of the total relative light intensity (RLU) of fMLP-stimulated neutrophil CL in the absence of compounds. Figure 2 shows the dose-response curves for compounds **4**, **7**, **9**, **10**, **11** and adenosine effects on ROS production.

As shown in Figure 2, adenosine and adenosine modified with 1,12-dicarba-*closo*-dodecaborane at the 2-C, 6-N and 2′-C positions (compounds **7**, **9** and **10**, respectively) efficiently inhibited ROS production by fMLP-stimulated neutrophils. It is noteworthy that the level of inhibition of ROS production in the presence of compound **9** (6-N modified adenosine) was significantly higher than that in the presence of adenosine (Fig. 2). The ROS production in the presence of compound **9** at the concentrations of 5 μM and 10 μM dropped to the 25 ± 4% and 22 ± 5% as compared to the value of neutrophils stimulated with fMLP only and it was twofold lower than in the presence of adenosine (Fig. 2). This compound even at the concentrations of 0.01 and 0.1 μM, significantly inhibited ROS production, whereas adenosine did not (Fig. 2, log scale of concentration). The half maximal inhibitory concentration (IC_{50}) value of compound **9** was significantly lower (0.72 ± 0.22) than that of adenosine (1.81 ± 0.31; $p < 0.01$).

The 6-N adenosine derivative modified with the charged 7,8-dicarba-*nido*-undecacarborane (compound **11**) had no effect on neutrophil function (Fig. 2). This result was the opposite of the result for the 6-N adenosine derivative modified with the neutral 1,12-dicarba-*closo*-dodecaborane (compound **9**). In contrast to adenosine derivative **9**, which bears a neutral *para*-carborane modification at 6-N, compound **11**, which bears a charged 7,8-dicarba-*nido*-undecacarborane cage, was ineffective as an ROS production inhibitor in activated neutrophils.

The presence of the hydrophobic boron cluster 1,12-dicarba-*closo*-dodecaborane (**12**) gave the modified nucleoside a highly hydrophobic character.[31] It has consequently been hypothesised that the hydrophobic carborane modification might intensify the interaction of the adenosine derivative with receptors through hydrophobic domains of the protein and therefore enhance receptor selectivity and modulate immune cell function.[52] We hypothesized that the presence of a negative charge, in addition to steric effects, may be the reason for the observed differences in activity between compounds **9** and **11**. The effect of boron clusters such as **12** and **13** alone (Fig. 1), on the neutrophil oxidative response was also examined (data not shown). No effect on ROS production was observed for either compound **12** or **13**. We concluded that boron clusters do not interact efficiently with neutrophils and that they can affect the neutrophil function only if delivered to the cells as part of an adenosine/boron cluster conjugate. The 2-C and 2′-C conjugates of adenosine with neutral 1,12-dicarba-*closo*-dodecaborane (compounds **7** and **10**) reduced ROS production in activated neutrophils similarly to adenosine (Fig. 2). The IC_{50} values of compounds **7**, **10** and adenosine were 2.28 ± 0.32, 1.90 ± 0.39 and 1.81 ± 0.31, respectively.

In our previous study[24] we observed that compound **10** significantly and strongly inhibited platelet function (thrombin- and ADP-stimulated aggregation, protein secretion and P-selectin expression). Herein, we have shown for the first time that adenosine modified with boron cluster at the 2′-position, such as in compound **10**, may also exert biological effects on neutrophils. Interestingly, compound **4**, another 2′-C adenosine derivative modified with 1,12-dicarba-*closo*-dodecaborane (containing a longer linker than compound **10**) did not affect ROS production in fMLP-stimulated neutrophils. We presumed that the length of the linker may play a role in the biological activity of 2′-C derivatives because compound **4**, with a longer linker than compound **10**, was not active against fMLP-stimulated neutrophil ROS production.

2.2.2. Contribution of A_{2A} receptor

The A_{2A} receptors are the primary adenosine receptors responsible for inhibition of fMLP-activated ROS production in neutrophils by adenosine. To determine whether the effect of compounds **9** and **10**, most effectively inhibiting ROS production, is mediated by the occupation of A_{2A} adenosine surface receptors, an assay using highly selective A_{2A} receptor antagonist 2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-e][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH 58261) was performed.[53]

As shown in Figure 3, the effects of leading compounds **9** and **10** on ROS production were abolished by selective A_{2A} receptor antagonist SCH 58261. The half maximal effective concentration (EC_{50}) values of SCH 58261 for compounds **9** and **10** were 0.17 μM and 0.02 μM, respectively. The above results suggest that the inhibitory effects of **9** and **10** are mediated via the A_{2A} receptor.

To confirm that compounds **9** and **10** indeed interact with the A_{2A} receptor and to determine compounds' affinities for this receptor, a radioligand binding assay was carried out with the specific A_{2A} receptor agonist [3H]–3-[4-[2-[[6-amino-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]-ethyl]phenyl]propanoic acid hydrochloride ([3H]CGS 21680).[54] The radioligand [3H]CGS 21680 bound to the neutrophil membrane with a dissociation constant (K_d) of 27.8 ± 4.8 nM (18.3–35.4 nM), matching that reported previously.[55] For both compounds **9** and **10**, high affinities for the A_{2A} receptor in the low nanomolar range were found. Compounds **9** and **10** displaced radioligand [3H]CGS 21680 with inhibitory constants (K_i) of 9.9 nM

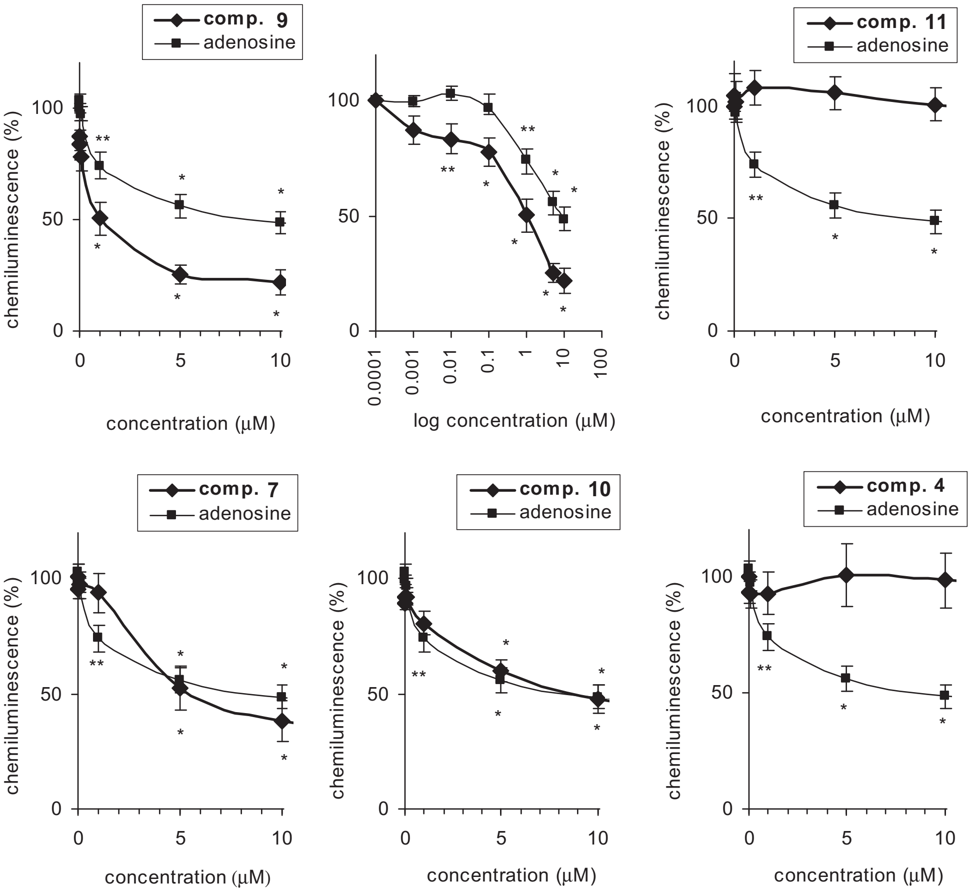


Figure 2. The effects of compounds **4**, **7**, **9**, **10**, **11** and adenosine, on reactive oxygen species (ROS) production by neutrophils stimulated with fMLP. Neutrophils (1.5 × 10^6/mL in HBSS) were pre-incubated with compounds for 10 min at 37 °C and then stimulated with fMLP (1 μM) in 96-well plates. The chemiluminescence (CL) reading was recorded for 30 min at 2 min intervals. The CL intensity was presented in relative light units (RLU) as described in the Methods section. The data are expressed as percentages of the CL intensities of the fMLP-stimulated neutrophils in the absence of compounds. The RLU value for the fMLP-stimulated neutrophils was 367 ± 37, and these values were set to 100%. The data represent the mean ± SEM for 8–10 healthy donors. $^{*}P < 0.01$, $^{**}p < 0.05$ (compound + fMLP vs fMLP only, Wilcoxon's signed rank test).

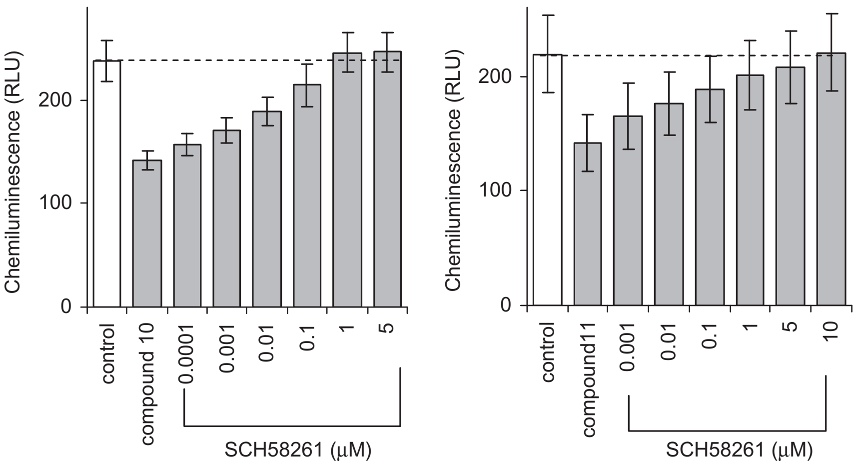


Figure 3. The effect of SCH 58261, A_{2A} receptor antagonist, on ROS inhibition by compounds **9** (left) and **10** (right). Neutrophils (1.5 × 10^6/mL in HBSS) were incubated with an SCH 58261 (0.0001–10 μM) for 5 min at 37 °C, and then compounds **9** or **10** were added (5 μM). The cells were then incubated for 10 min. The fMLP (1 μM) was added to stimulate a respiratory burst (ROS production) by the neutrophils. The CL reading was recorded for 30 min with 2 min intervals. The CL intensity is given in relative light units (RLU) as described in the Methods section. The data represent the mean ± SEM for eight to ten healthy donors. $^{*}P < 0.02$, $^{**}P < 0.05$ (compounds + fMLP vs fMLP, Wilcoxon's signed rank test). The half maximal effective concentrations (EC_{50}) of SCH 58261 for reducing the inhibitory effects of **9** and **10** were 0.02 and 0.17 μM, respectively.

(4.58–22.2 nM) and 1.04 nM (0.55–1.99 nM), respectively and displayed 3- and 26-fold and higher affinity for the A_{2A} receptor than CGS 21680 did.

As mentioned in the introduction, the involvement of the A_3 receptor subtype in the inhibition of ROS production should be also taken into consideration.[18,19] However, under experimental condition used we did not observe the involvement of A_3 receptors. The inhibition of ROS production by compounds **9** and **10** was not affected in the presence of highly selective antagonist for the human A_3 receptor, 8-ethyl-1,4,7,8-tetrahydro-4-methyl-2-(2,3,5-trichlorophenyl)-5*H*-imidazo[2,1-*i*]purin-5-one monohydrochloride (PSB 10 hydrochloride) at the concentrations of 0.001–10 μM (data not shown). Thus, we conclude that observed by us inhibition of ROS production by these compounds was mainly dependent on A_{2A} receptor activation.

3. Conclusions

In summary, new approaches to the synthesis of adenosine boron cluster conjugates have been proposed. We show that the adenosine derivatives containing boron clusters modulated neutrophil function in a structure-dependent manner. The inhibition of ROS production by 2′-C and 6-N adenosine derivatives in activated neutrophils may reduce the harmful effects of overactive neutrophils accompanying inflammatory processes. These compounds might therefore be considered as new type of agents useful in neutrophil-targeted research focused on anti-inflammatory activity.

4. Experimental section

4.1. Chemistry

4.1.1. Materials

Chemicals were obtained from Aldrich Chemical Company and were used without further purification unless otherwise stated. Adenosine was purchased from Pharma-Waldhof GmbH (Düsseldorf, Germany). *tert*-Butyldimethylsilyl chloride was from ABCR GmbH&Co.KG (Karlsruhe, Germany), and sodium hydride (60% suspension in mineral oil) was purchased from Lancaster (Morecambe, England). Flash chromatography was performed on silica gel 60 (230–400 mesh, Aldrich Chemical Company). R_f values refer to analytical TLC performed using pre-coated silica gel 60 F254 plates purchased from Sigma–Aldrich (Steinheim, Germany) and developed in the solvent system indicated. Compounds were visualised with UV light (254 nm) or a 0.5% acidic solution of $PdCl_2$ in HCl/methanol for boron-containing derivatives. The yields are not optimised.

4.1.2. General information

^1H NMR, ^{13}C NMR and ^{11}B NMR spectra were recorded on a Bruker Avance DPX 250 MHz spectrometer equipped with a BB inverse probe-head. The spectra for ^1H, ^{13}C, and ^{11}B nuclei were recorded at 250.13 MHz, 62.90 MHz, and 80.25 MHz, respectively. Tetramethylsilane was used as the standard for ^1H NMR and ^{13}C NMR; $BF_3/(C_2H_5)_2O$ was used as the standard for ^{11}B NMR. Alternatively, spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with a direct ATM probe. These spectra for ^1H, ^{13}C, and ^{11}B nuclei were recorded at 600.26 MHz, 150.94 MHz and 192.59 MHz, respectively. Deuterated solvents were used as standards for ^1H NMR and ^{13}C NMR; $BF_3/(C_2H_5)_2O$ was used as the standard for ^{11}B NMR. All chemical shifts are reported in ppm (δ) relative to external standards. The following abbreviations are used to denote the multiplicities: s = singlet, d = doublet, dd = double doublet, t = triplet, dt = double triplet, q = quartet, quin = quintet, bs = broad singlet, m = multiplet. Coupling constants are reported in hertz.

Fast atom bombardment (FAB, Gly) mass spectra were recorded on a Finnigan MAT (Bremen, Germany). The MS *m*/*z* values were measured in positive and negative modes. Calculation of the theoretical molecular mass was performed using the 'Show Analysis Window' option in ChemDraw 8.0. The calculated *m*/*z* corresponds to the average mass of the elements consisting of natural isotopes.

UV measurements were performed on a GBC Cintra10 UV–vis spectrometer (Dandenong, Australia). For UV experiments, samples were dissolved to an A_{260} of approximately 0.5 ODU in 96% C_2H_5OH. The measurements were performed at ambient temperature.

Infrared absorption spectra were recorded with a Nexus (Thermo-Nicolet) Fourier-transform, infrared spectrometer or an ATI Mattson Infinity series MI-60, equipped with a silicon carbide (SiC) air-cooled source for the IR range, a caesium iodide beam splitter, and deuterated triglycine sulphate (DTGS) detectors. Samples were prepared by dilution with potassium bromide (70–140 mg of KBr per sample) followed by pressing in a stainless steel die to form discs 0.8 cm in diameter. Alternatively, the spectra were taken in Nujol mulls.

HPLC analysis of compounds **7** and **11** was performed on a Hewlett–Packard 1050 system using an Econosil C18 (5 μm, 4.6 × 250 mm) column or on a Dionex Ultimate 3000 system using an Agilent Zorbax-SB-C18 (5 μm, 4.6 × 250 mm) column for compound **9**. All analyses were run at ambient temperature. HPLC conditions were as follows: 20 min from 0% D to 100% D, 5 min 100% D, and 5 min to 0% D. Buffer A contained 0.1 M TEAB (pH 7.0) in a mixture of acetonitrile and water (1:49, v/v), and buffer D contained 0.1 M TEAB (pH 7.0) in a mixture of acetonitrile and water (3:2, v/v). HPLC analysis of compounds **4** and **10** was performed on the Dionex Ultimate 3000 system using an Agilent Zorbax-SB-C18 column (5 μm, 4.6 × 250 mm); the following gradient conditions were used: 50 min from 0% D to 100% D, 5 min 100% D, and 5 min to 0% D. The composition of buffers A and D was as described above. UV detection was performed at λ = 268 nm.

4.1.3. Synthesis of 2′-*O*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propyleneoxy-methyl]adenosine (10) and 6-*N*-[(3-oxapentoxy)-7,8-dicarba-*nido*-undecacarboran-10-yl]adenosine (11)

These compounds were prepared according to previously described procedures.[27,32,33]

4.1.4. Synthesis of 2′-*O*-{[3-propyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)]-1-*N*-1,2,3-triazol-4-yl}propyladenosine (4)

2′-*O*-(1-Pentyn-5-yl)adenosine (**2**) (16.70 mg, 0.050 mmol) and the boron cluster donor bearing an azido-group, 1-(3-azidopropanyl)-1,12-dicarba-*closo*-dodecaborane (**3**, 12.61 mg, 0.055 mmol, 5% molar excess), were dissolved in a mixture of *tert*-butanol and water (1 mL, 1:1 v/v). Next, a freshly prepared solution of $CuSO_4 \times 5H_2O$ (0.0025 mmol, 5 mol %, 25 μL of a 100 mM solution) and potassium ascorbate (0.005 mmol, 10 mol %, 50 μL of a 100 mM solution) were added simultaneously. The reaction mixture was stirred at room temperature, and the progress of the reaction was monitored by TLC (CH_2Cl_2/MeOH, 8:2 v/v or CH_2Cl_2:MeOH, 9:1 v/v). After reaction completion (usually 24 h), the solvent was evaporated under vacuum. The crude product was purified by silica gel column chromatography using a gradient of methanol in methylene chloride as the eluent. Pure compound **4** was lyophilised from a mixture of *tert*-butanol and water (1:1, v/v) giving the title compound as a white solid. Yield: 17.00 mg (60%); TLC (CH_2Cl_2:MeOH, 9:1 v/v): R_f = 0.56; FT-IR (KBr, cm^{-1}): 3363, 3266, 3211 (OH), 2957, 2930, 2872 (CH_2), 2607 (BH); UV–vis (95% C_2H_5OH, nm): λ_{max} = 259.70; ^1H NMR (CH_3OD, ppm): δ = 8.34 (s, 1H, H-2), 8.17 (s, 1H, H-8), 7.49 (s, 1H, $H^{triazole}$), 6.06 (d, 1H, $^3J_{HH}$ = 5.75, H-1′), 4.52–4.44 (m, 2H, NCH_2), 4.22–4.13 (m, 4H, OCH_2, H-2′, H-3′), 3.92–3.72 (m, 2H, H-5′), 3.63–3.39 (m, 1H, H-4′), 3.12 (br s, 1H, $CH^{carborane}$), 3.0–0.5 (bm, 10H, BH), 2.78–2.57 (m, 2H, CH_2), 1.88–1.58 (m, 4H, 2 × CH_2), 1.0–0.85 (m, 2H, CH_2-*para*-carborane); ^{11}B {^1H BB} NMR (CH_3OD, ppm): δ = −11.20 (s, 5B), −13.46 (s, 5B), ^{11}B NMR (CH_3OD, ppm): δ = −11.27 (d, 5B,

J_{BH} = 172.46), −13.43 (d, 5B, J_{BH} = 174.38); MS (FAB): m/z = 562.40 $(M+2)^+$, $C_{20}H_{36}B_{10}N_8O_4$ [560.39].

4.1.5. Synthesis of 2-ethynyl-(1,12-dicarba-*closo*-dodecaboran-1-yl)adenosine (7)

This synthesis was performed under anhydrous conditions in an argon atmosphere. 2-Iodoadenosine (**6**, 50 mg, 0.13 mmol), 2-ethynyl-1,12-dicarba-*closo*-dodecaborane (**7**, 23.56 mg, 0.14 mmol), CuI (3.06 mg, 0.016 mmol) and $Pd(PPh_3)_4$ (6.36 mg, 0.0055 mmol) were placed in a flame-dried flask and dissolved in anhydrous DMF (0.9 mL) and Et_3N (0.3 mL). The resultant solution was stirred for 75 min at room temperature and then for 70 min at 80 °C. After the reaction was complete, the solvents were evaporated under reduced pressure, and the residue was dissolved in ethyl acetate (12 mL). The resultant solution was extracted with deionised water (2 × 6 mL). The organic fractions were collected, washed with 0.5% aqueous EDTA (1 × 2 mL), dried over anhydrous magnesium and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (2 g, 230–400 mesh) using a linear gradient of CH_3OH in CH_2Cl_2 (0–10%) as the eluent. Yield: 37.68 mg (68%). TLC (CH_2Cl_2:CH_3OH, 9:1 v/v): R_f = 0.11; UV–vis (95% C_2H_5OH, nm): λ_{min} = 280, 251, λ_{max} = 300, 270, 239; ^1H NMR (($(CD_3)_2CO$, ppm): δ = 8.31, (s, 1H, H-8), 6.81 (br s, 2H, NH_2), 5.97 (d, 1H, $^1J_{1',2'}$ = 7.50, H-1′), 5.10–5.05 (m, 1H-5′, 1H-OH), 4.82–4.73 (m, 2H, 1H-2′, 1H-OH), 4.39–4.37 (m, 2H, 1H-3′, 1H-OH), 4.16–4.14 (m, 1H, H-4′), 4.16–4.14 (m, 1H, H-4′), 3.84–3.71 (m, 3H, 2H-5′, 5″, C(1)$H^{carborane}$), 3.51 (br s, 1H, C(12)$H^{carborane}$), 3.50–1.00 (m, 9H, $BH^{carborane}$); ^{13}C NMR ($(CD_3)_2CO$, ppm): δ = 157.02 (C-6), 150.00 (C-2), 146.21 (C-4), 142.15 (C-8), 121.00 (C-5), 90.38 (C-1′), 87.81 (C-4′), 75.17 (C-2′), 72.38 (C-3′), 67.53 ($CH^{carborane}$), 64.90 ($CH^{carborane}$), 63.20 (C-5′); ^{11}B NMR ($(CD_3)_2CO$, ppm): coupled δ = −12.18 (s, 2B), −13.90 (s, 4B), −15.90 (s, 4B); MS (FAB): m/z = 435.40 $(M+2)^+$, 497.4 $(M+Na+K+2)^+$, $C_{14}H_{23}B_{10}N_5O_4$ [433.28].

4.1.6. Synthesis of 6-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]adenosine (9)

2′,3′,5′-tri-*O*-(*tert*-butyldimethylsilyl)-6-chloroadenosine (100 mg, 0.16 mmol) and 1-(3-aminopropyl)-1,12-dicarba-*closo*-dodecaborane (**8**, 42 mg, 0.16 mmol) were placed in a 10 mL round bottom flask equipped with a magnetic stirrer together with absolute ethanol (2 mL) and triethylamine (100 μL). The reaction mixture was stirred at room temperature for 24 h and then at 70 °C for 8 h. The reaction progress was monitored by TLC. After reaction was complete, the solvents were evaporated under reduced pressure, and the residue was dissolved in chloroform (3 mL). The solution was washed with H_2O (3 × 5 mL), and the organic phases were collected, dried over magnesium sulphate and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using CH_2Cl_2 as the eluting solvent to afford chromatographically pure 2′,3′,5′-tri-*O*-(*tert*-butyldimethylsilyl)-6-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]adenosine as pale yellow solid (72 mg, 57%).

To remove the TBDMS protecting groups, the 2′,3′,5′-*O*-protected **9** (70 mg, 0.08 mmol) was dissolved in tetrahydrofuran (1 mL) and treated with tetrabutylammonium fluoride (TBAF, 1 M solution in THF, 350 μL, 0.35 mmol). The reaction mixture was stirred at room temperature, and the progress of the reaction was monitored by TLC. After reaction was complete (usually 3 h), the solvent was evaporated under reduced pressure, the residue was dissolved in methylene chloride (4 mL), and the resultant solution was washed with water (3 × 2 mL). The organic phase was dried over anhydrous magnesium sulphate and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using a linear gradient of methanol in methylene chloride (0–5%) to give product **9** as white solid. Yield: 31 mg (78%). TLC (CH_2Cl_2:CH_3OH, 9:1 v/v): R_f = 0.63; FT-IR (KBr, cm^{-1}): 3421 (OH), 2927, 2866 (CH_2), 2607 (BH); UV–vis (95% C_2H_5OH, nm): λ_{max} = 267.49; ^1H NMR (CH_3OD, ppm): δ = 8.23 (s, 1H, H-2), 8.19 (s, 1H, H-8), 5.93 (d, 1H, $^3J_{HH}$ = 6.50, H-1′), 4.72 (2 ×d, 1H, $^3J_{HH}$ = 6.50, $^3J_{HH}$ = 6.48, H-2′), 4.30 (2 × d, 1H, $^3J_{HH}$ = 5.07, $^3J_{HH}$ = 5.04, H-3′), 4.16 (part X of ABX spin system, 1H, H-4′), 3.91–3.75 (part AB of ABX spin system, 2H, H-5′), 3.20 (br s, 1H, $CH^{carborane}$), 1.80–1.74 (m, 2H, CH_2NH), 1.57–1.52 (m, 2H, $CH_2CH_2CH_2$), 1.50–1.20 (m, 2H, CH_2-*para*-carborane), 3.0–0.5 (bm, 10H, BH); MS (FAB): m/z = 452.30 $(M+1)^+$, $C_{15}H_{29}B_{10}N_5O_4$ [451.32].

4.2. Biological assays

4.2.1. Materials

Hanks′ balanced salt solution (HBSS), bovine serum albumin (BSA), formyl-methionyl-leucyl-phenylalanine (fMLP), propidium iodide (PI), Tris, NaCl, $MgCl_{2,}$ Total Protein Kit Micro Lowry and Triton X-100 were purchased from Sigma–Aldrich (St Louis, MO, USA). The A_{2A} receptor agonist 3-[4-[2-[6-amino-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]-phenyl]propanoic acid hydrochloride (CGS 21680), the A_{2A} receptor antagonist 2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine) (SCH 58261), the antagonist for the human adenosine A_3 receptor 8-ethyl-1,4,7,8-tetrahydro-4-methyl-2-(2,3,5-trichlorophenyl)-5*H*-imidazo[2,1-*i*]-purin-5-one monohydrochloride (PSB 10 hydrochloride) and adenosine non-selective receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) were from Tocris Bioscience (Ellisville, MO, United States). Polymorphprep was purchased from AXIS-SHIELD PoC AS (Oslo, Norway). The A_2-selective agonist radioligand [3*H*]–3-[4-[2-[6-amino-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid hydrochloride ([3H]CGS 21680) was obtained from Perkin Elmer (USA).

The purities of compounds **4**, **7**, **9**, **10** and **11** were established by HPLC and exceeded 95%. **4**, t_R = 52.62 min; **7**, t_R = 21.28 min; **9**, t_R = 18.88 min; **10**, t_R = 38.35 min; **11**, t_R = 16.80 min (for HPLC conditions).

4.2.2. Preparation of stock solutions

Adenosine and **4**, **7**, **9**, **10** and **11** were dissolved in DMSO to 25 mM and then stored at −20 °C until use. The stock solution was diluted with PBS prior to use. The final DMSO concentration in the tested samples did not exceed 0.08%.

4.2.3. Isolation of blood neutrophils

Heparinised peripheral blood was obtained from healthy volunteers, and the neutrophils were isolated by centrifugation of blood samples on Polymorphprep (500 *g* for 30 min, room temperature) as described previously.[56] Briefly, the top band containing mononuclear cells was removed, and the lower band, containing polymorphonuclear cells, was collected. The neutrophils were washed twice with PBS. The cell viability (95%) and cell purity (95%) were assessed by trypan blue and May-Grünwald-Giemsa staining, respectively. The Regional Commission for Ethics in Research approved the research protocol. All donors gave informed consent.

4.2.4. Cell viability assay

To determine if adenosine and its synthesised derivatives had direct cytotoxic effects on neutrophils, cells were incubated with varying concentrations of adenosine, **4**, **7**, **9**, **10** and **11** compounds (1–40 μM, 10 min, 37 °C) or left untreated and then washed with PBS twice. Next, 10 μL of propidium iodide (PI) solution (20 μg mL^{-1}) was added to 190 μL of cells (1.5×10^6 cells mL^{-1}) following incubation in the dark at room temperature for 10 min. Then, cells were kept at 4 °C in the dark until analysis. PI is not excluded by necrotic cells and stains the nuclei of cells with ruptured membranes.[48,49] The red PI fluorescence of individual nuclei was measured using a Beckman Coulter (Brea, CA USA) flow cytometer equipped with a Cytomics FC 500 MPL System running MXP Software for cell acquisition and data analysis.

4.2.5. Production of reactive oxygen species (ROS) assay

The production of ROS by neutrophils was assessed with a luminol-enhanced chemiluminescence (CL) method.[51] Neutrophils (1.5×10^6 cells mL^{-1} in HBSS) were pre-treated for 10 min with compounds that were assessed as non-toxic: adenosine, compounds **4**, **7**, **9**, **10** and **11**. In some experiments neutrophils were also treated with the selective A_{2A} receptor antagonist SCH 58261 and the selective antagonist for the human adenosine A_3 receptor PSB 10 for 5 min at 37 °C or were left untreated before the addition of compounds. After that, cells were distributed into white 96-well plates (1×10^5 cells per well). Then, fMLP (1 μM) were added to the cells to initiate ROS production, and luminol (10 μM) was added to enhance the CL. The CL reading was recorded for 30 min at 2 min intervals on a Fluoroskan Ascent FL fluorometer (Labsystems, Finland). The CL intensity was given in relative light units (RLU). The RLU total values were calculated as the area under the curve of CL versus assay time. The data were expressed as percentage values of the RLU total values of stimulated neutrophil CL in the absence of compounds according to the formula:

$$\%CL = 100 \times (CL_{compound+stimuli} - CL_{withoutstimuli})/(CL_{stimuli} - CL_{withoutstimuli})$$

The IC_{50} values for the studied compounds were calculated and expressed as means ± SEM for 8–11 donors. The SEM values reflected variability of IC_{50} among donors. The p values were calculated for derivatives versus adenosine. Statistical significance was defined as $p < 0.05$. The compounds that did not affect ROS production in neutrophils at concentrations below 10 μM were regarded as 'not effective' (IC_{50} of compounds **4** and **11** were >100 μM).

4.2.6. Preparation of neutrophil membranes

Preparation of neutrophil membranes was performed according to the method of Varani et al.[57] Human neutrophils were resuspended in Tris–HCl buffer (50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$) and centrifuged at 11,000*g* for 15 min at 4 °C. The pellet was recentrifuged in Tris–HCl buffer (16,000*g* for 15 min at 4 °C). Finally, the pellet was resuspended in 50 mM Tris–HCl pH 7.4 at 10 mg protein mL^{-1}. The protein concentration in the homogenate was determined using Total Protein Kit Micro Lowry according to the manufacturer's protocol with bovine serum albumin as the standard.

4.2.7. [3H]-CGS 21680 binding assay

The A_{2A} receptor binding assay was performed according to the method published by Bazzichi et al.[55] In saturation studies, neutrophil membranes (0.2–0.3 mg protein) were incubated with 8 to 10 different concentrations of the radiolabelled A_{2A} receptor agonist [3H]CGS 21680 (0.5–100 nM) at a final volume of 0.35 mL of Tris buffer (50 mM Tris HCl, pH 7.4, containing 10 mM $MgCl_2$) for 60 min at 4 °C.[54] Nonspecific binding was determined in the presence of 100 μM NECA. The binding reaction was terminated by vacuum filtration using a 96-well microplate filtration system (Millipore MultiScreen MAFC). Radioactivity was counted with a MicroBeta Trilux instrument (Perkin Elmer, USA). The assay was performed in triplicate. Competition experiments were carried out with 15 nM [3H]CGS 21680 and 8–10 different concentrations of selected compounds (0.1 nM–100 μM). The dissociation constant (K_d), the maximum number of binding sites (B_{max}) and the inhibitor binding constant (K_i) were calculated by the non-linear fitting program GraphPad Prism 5.04. Non-specific binding accounted for approximately 48% of total binding. In the saturation analysis, the K_d of the radioligand [3H]CGS 21680 was 27.8 ± 4.8 nM (18.3–35.4 nM), and the B_{max} was 39.4 ± 5.3 fmol mg^{-1} protein (32.1–46.7 fmol mg^{-1} protein).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.039.

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