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Multivalent dendrimeric and monomeric adenosine agonists attenuate cell death in HL-1 mouse cardiomyocytes expressing the A_3 receptor

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Abstract

Multivalent dendrimeric conjugates of GPCR ligands may have increased potency or selectivity in comparison to monomeric ligands, a phenomenon that was tested in a model of cytoprotection in mouse HL-1 cardiomyocytes. Quantitative RT-PCR indicated high expression levels of endogenous A_1 and A_{2A} adenosine receptors (ARs), but not of A_{2B} and A_3ARs. Activation of the heterologously expressed human A_3AR in HL-1 cells by AR agonists significantly attenuated cell damage following 4 h exposure to H_2O_2 (750 μM) but not in untransfected cells. The A_3 agonist IB-MECA (EC_{50} 3.8 μM) and the non-selective agonist NECA (EC_{50} 3.9 μM) protected A_3 AR-transfected cells against H_2O_2 in a concentration-dependent manner, as determined by lactate dehydrogenase release. A generation 5.5 PAMAM (polyamidoamine) dendrimeric conjugate of a N^6-chain-functionalized adenosine agonist was synthesized and its mass indicated an average of 60 amide-linked nucleoside moieties out of 256 theoretical attachment sites. It nonselectively activated the A_3AR to inhibit forskolin-stimulated cAMP formation (IC_{50} 66 nM) and, similarly, protected A_3–transfected HL-1 cells from apoptosis-inducing H_2O_2 with greater potency (IC_{50} 35 nM) than monomeric nucleosides. Thus, a PAMAM conjugate retained AR binding affinity and displayed greatly enhanced cardioprotective potency.

Keywords

cardioprotection; nucleoside; G protein-coupled receptor; dendrimers; polymeric drugs; HL-1 cells

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Supporting information available: Synthetic procedures for compounds **4** and **6** and mass spectra of the dendrimer derivatives, concentration dependence of H_2O_2-induced cytotoxicity in HL-1 cells.

Introduction

Four subtypes of adenosine receptors (AR), which belong to the rhodopsin family of G protein-coupled receptors (GPCR), are activated by the endogenous ligand adenosine as well as by the non-selective agonist 5′-*N*-ethylcarboxamidoadenosine (NECA **1**) [1]. There is increasing interest in the therapeutic potential of selective adenosine agonists for treating a wide range of diseases. For instance, the A_3AR is known to be overexpressed in peripheral blood mononuclear cells and synoviocytes of rheumatoid arthritis patients [2], and receptor activation is known to reduce lung injury following reperfusion in cats [3]. Numerous AR ligands are already in or heading toward clinical trials as drug candidates. For example, selective agonists of the A_3AR, N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (IB-MECA **2**) and 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (Cl-IB-MECA **3**), are in trials for autoimmune inflammatory diseases [4,5] and hepatocarcinoma [6,7], respectively.

A_3AR agonists have also displayed protective effects in ischemic models of the brain [8], nervous system [9], and skeletal muscle [10]. Activation of this receptor preconditions cardiomyocytes in culture [11,12], isolated hearts [13], and rabbit hearts *in vivo* to protect against the damaging effects of ischemia/hypoxia [14]. However, there is still uncertainty over the role of the A_3AR in cardioprotection. While the expression of A_1 and A_{2A}ARs in human (h) adult cardiomyocytes is known, direct evidence proving that the A_{2B} and A_3ARs are also expressed in these cells is lacking. In addition, the A_1 and A_{2A}ARs are implicated in cardioprotection, although the signaling pathways have not yet been determined [15].

While activation of ARs has pronounced cardioprotective properties, more work is needed to further elucidate the effects of small molecular and multivalent agonists for the A_3AR in models of cardioprotection. An immortalized atrial cardiomyocyte murine cell line, HL-1, is known to express all four ARs, although the levels of expression have not been reported [16]. These cells are able to continuously divide while maintaining a differentiated cardiac phenotype characterized by spontaneous action potentials and contractions [17]. HL-1 cells have been used to study pathophysiological conditions such as hypoxia and hyperglycemia [18], and can be transiently transfected using both transfection agents [19] and viral vectors [20]. A_1 and A_3ARs are involved in preconditioning HL-1 cells against damage from hypoxia and ischemic reperfusion [21,22]. Although cardioprotection induced by A_3AR agonists has been well explored in a variety of systems and species, we adapted here the mouse HL-1 cell culture model for use as a model system in which the expression of AR subtypes could be measured and manipulated.

The structure activity relationship (SAR) of nucleoside derivatives as agonists at the ARs has been extensively studied [1]. Recently this analysis has been extended to multivalent nucleoside conjugates of polyamidoamine (PAMAM) dendrimers [23-27], which we are terming GPCR Ligand-Dendrimer (GLiDe) conjugates. PAMAM dendrimers are peptide-like in structure and as such generally biocompatible. Assuming proper functionalization of GPCR ligands for covalent conjugation [24], such multivalent dendrimeric conjugates of these ligands have displayed dramatically increased potency or selectivity in comparison to the monomeric, small molecular ligands. A multivalent agonist of the A_{2A}AR effectively inhibited ADP-induced platelet aggregation [25]. A PAMAM dendrimeric conjugate (generation 2.5) **5** of a non-subtype selective adenosine agonist **4** (Figure 1A), which was chain-functionalized at the N^6 position, displayed enhanced selectivity for the A_3AR in both binding and functional assays [26]. Multivalent conjugates of the $P2Y_{14}$ receptor agonist UDP-glucuronic acid activated that nucleotide receptor with up to 800-fold enhanced potency in comparison to the corresponding monomeric ligands [27].

The theoretical ability of such dendrimeric conjugates to bridge multiple protomers in a homodimeric AR structure was shown [28]. It is hoped that this design approach can be used to prepare pharmacological probes to act as selective agonists and antagonists at homomultimeric and heteromultimeric GPCRs.

The aim of our study was to investigate the cardioprotective effects of a novel, newly-synthesized high molecular weight (>88,000 D), multivalent AR agonist acting at the A_3AR [18]. This agonist is similar to the A_3AR-selective agonist **5** structurally and in the presence of terminal carboxylate groups (Figure 1A), but is derived from a higher generation (G5.5) PAMAM dendrimer and is more highly conjugated with a strategically functionalized adenosine derivative. In this study, cell death was induced using hydrogen peroxide (H_2O_2) in HL-1 cultured cardiomyocytes, in which the expression of the A_3AR was controlled heterologously. A distinct protective effect of A_3AR activation by either known monomeric A_3AR agonists or a multivalent AR agonist was observed.

2. Materials and Methods

2.1. Materials

HL-1 mouse cardiomyocytes were a kind gift of Professor W.C. Claycomb, LSU Health Sciences Center, New Orleans, LA, USA [22]. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Rockville, MD). Plastic cellware was purchased from Becton Dickinson (Bedford, MA). 2-Chloro-N^6-cyclopentyladenosine (CCPA), and 2-[*p*-(2-carboxyethyl)phenylethyl-amino]- 5′-*N*-ethylcarboxamidoadenosine (CGS21680), and 3-iodobenzyl-5'-*N*-methylcarboxamidoadenosine (IB-MECA) were obtained from Tocris (Ellisville, MO). ADAC (N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl] adenosine), PAMAM dendrimers (ethylenediamine core, generation 5.5 as 10 wt. % solution in methanol), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC), 2-(*N*-morpholino) ethanesulfonic acid (MES), magnesium chloride, methanol, triethylamine, methyl sulfoxide-d_6 (DMSO-d_6), *N*,*N*-dimethylformamide (DMF), Claycomb Media, fibronectin, ascorbic acid, norepinephrine, H_2O_2, Triton-X, rolipram, and gelatin were purchased from Sigma (St. Louis, MO). Bio-Beads® SX-1 beads were purchased from Bio-Rad (Hercules, CA). Alexa-Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester, 5-isomer (AF488-TFP) was purchased from Invitrogen (Carlsbad, CA). [^{125}I]AB-MECA ([^{125}I]4-amino-3-iodobenzyl-5'-*N*-methylcarboxamidoadenosine, 2200 Ci/mmol), [^{3}H]CCPA (42.6 Ci/mmol), and [^{3}H] CGS21680 (40.5 Ci/mmol) were purchased from PerkinElmer (Waltham, MA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, DMEM/F12 medium, and 1 M Tris-HCl (pH 7.5) were purchased from Mediatech, Inc. (Manassas, VA).

2.2. Chromatographic separation and spectroscopy

The column for size exclusion chromatography (SEC) was prepared by suspending 100 g of Bio-Beads® SX-1 beads in 1 L of DMF. After 24 h to allow for equilibration and expansion, the beads were added to the column as described previously [25]. High Performance Liquid Chromatography (HPLC) purification was performed using an 1100 Series HPLC (Agilent, Santa Clara, CA) equipped with a Luna 5μ C18(2) 100A analytical column (250 x 10 mm; Phenomenex, Torrance, CA). Peaks were detected by UV absorption using a diode array detector. Proton nuclear magnetic resonance spectra (NMR) were recorded on a Bruker DRX-600 spectrometer after being optimized for each sample using DMSO-d_6 as a solvent unless otherwise noted. Electrospray ionization mass spectra (ESI MS) were taken using a LCT Premier mass spectrometer (Waters Corp., Waltham, MA). Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDITOF) spectra were obtained with a Waters Micro mass

spectrometer using Waters MassPREP Direct Ionization on Silica Desorption/ionization (DIOS) target plates.

The ESI MS data for the dendrimer complexes were obtained using a Waters LCT Premier TOF mass spectrometer. The mass spectrometer was operated in negative ion W mode with a resolution of 10,000 measured at half peak height. The capillary voltage was 2500 volts, the cone voltage was 40 volts, and the desolvation gas was dried nitrogen at 250 °C and a flow of 300 l/h. The sample was dissolved in a 1:1 solution of water:acetonitrile containing 0.2% formic acid and injected directly into the eluting stream flowing at 200 μl/min and consisting of 20:80 water:acetonitrile and 0.2% formic acid. The relevant spectra were summed using the MassLynx software, and the summed spectra were deconvoluted with the MaxEntI program (Waters).

2.3. Synthesis of dendrimeric derivatives PAMAMG5.5-1(AF488-ED) (7) and PAMAMG5.5-1 (AF488-ED)-60(*N*-(2-aminoethyl)-ADAC) (8)

This procedure was adapted from a similar procedure to synthesize PAMAMG2.5-1AF488-ED [26]. 1.07 μmol of G5.5 PAMAM stock solution (0.93 mM in methanol, 56.9 mg) was added to a flask, and the methanol was evaporated. The remaining residue containing the polymer and the Alexa-Fluor 488-ethylenediamine (ED) derivative **6** (procedure in Supporting Information, 1.4 mg, 2.5 μmol) were dissolved in 1.0 ml of 0.1 M MES buffer, pH 5. EDC (18 mg, 94 μmol) dissolved in 1.0 ml of 0.1 M MES buffer, pH 5, was added, and the reaction was stirred for 60 h. After exhaustive dialysis with water, the mixture was lyophilized to give 33.1 mg of product (0.69 μmol, 65% yield) and redissolved in D_2O for NMR measurements and further biological assays. The NMR spectrum was consistent with the assigned structure, but the signals resulting from AF488-ED could not be properly integrated due to the large G5.5 PAMAM peaks. Therefore, based on the MS results, it was assumed that each dendrimer **7** contained on average one moiety of **6**. *m/z* (ESI^- MS) calc: 53,435; found: 53,971.

Synthesis of PAMAMG5.5-1AF-ED-60(N-(2-aminoethyl)-ADAC) **8** 30.9 mg of **7** (0.65 μmol) was dissolved in 2.0 ml of 0.1 M MES, pH 5, and placed under a nitrogen atmosphere. *N*-(2-Aminoethyl)-ADAC **4** (procedure in Supporting Information, 28.7 mg, 46.4 μmol) was dissolved in 3.0 ml of DMSO and was added to the solution of **7**. Finally, 89 mg of EDC (146 μmol) was dissolved in 1 ml of 0.1 M MES, pH 5 and added to the mixture. After approximately 48 h, small molecule impurities were removed by filtration and extensive dialysis in water. After lyophilization, 26.8 mg (1.14 μmol, 46% yield) of product remained. The product was analyzed by MS, which indicated an average of 60 *N*-(2-aminoethyl)-ADAC moieties per dendrimer (of a possible 256 moieties). The NMR spectrum was consistent with the assigned structure, but the signals resulting from **7** could not be properly integrated due to the large G5.5 PAMAM peaks. *m/z* (ESI^- MS) calc: 88,771; found: 88,595.

2.4. Cell culture and membrane preparation

HL-1 cells (murine cardiomyocytes) were grown in Claycomb Media supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μmol/ml glutamine, and 0.1 mM norepinephrine previously dissolved in 0.3 mM ascorbic acid [17,18]. All cell culture plates for the HL-1 cells were coated with a 25 μg/ml fibronectin solution prepared in a 0.02% gelatin. The murine cardiomyocyte cells were transiently transfected with the hA_3AR gene expressed in the pcDNA5/FRT plasmid (Invitrogen) using GenJet (SignaGen Laboratories, Ijamsville, MD) as the transfection agent.

Chinese hamster ovary (CHO) cells stably expressing the recombinant hARs (except A2AAR, which was expressed in HEK293 cells) were cultured in Dulbecco's modified Eagle medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/ml penicillin,

100 μg/ml streptomycin, and 2 μmol/ml glutamine [26,29]. After harvesting, cells were centrifuged at 500*g* for 10 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The suspension was homogenized and recentrifuged at 20,000*g* for 20 min at 4 °C. The resultant pellets were resuspended in Tris-HCl buffer and incubated with adenosine deaminase for 30 min at 37 °C. The suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the BCA Protein Assay Kit from Thermo Fisher Scientific Inc. (Waltham, MA).

2.5. Radioligand membrane binding studies

Radioligand binding assays at A_1, A_{2A}, and A_3ARs were performed according to the procedures described previously [29]. Each tube in the binding assay contained 100 μL of membrane suspension (20 μg of protein), 50 μL of agonist radioligand, and 50 μL of increasing concentrations of the test ligands in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$. The concentrations of the dendrimer-ligand complexes are measured by the concentration of the dendrimer, not the ligand. Therefore, all binding K_i values of dendrimers are expressed as $K_{i\ app}$ (apparent inhibition constant). Nonspecific binding was determined using a final concentration of 10 μM NECA, a non-specific agonist, diluted with the buffer. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under a reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 5 ml of 50 mM ice-cold Tris-HCl buffer (pH 7.5). The radioactive agonists [^{3}H]CCPA and [^{3}H]CGS21680 were used for the A_1 and A_{2A} assays, respectively, while [^{125}I]AB-MECA was used for the A_3 assays. All of the filters were washed 3 times with Tris-HCl, pH 7.5. Filters for A_1 and A_{2A}AR binding were placed in scintillation vials containing 5 ml of Hydrofluor scintillation buffer and counted using a PerkinElmer Tricarb 2810TR Liquid Scintillation Analyzer. Filters for A_3AR binding were counted using a PerkinElmer Cobra II γ-counter. The K_i values were determined using Prism software (version 4.0, GraphPAD, San Diego, CA) for all assays.

2.6. cAMP assays

CHO cells expressing the A_1, A_{2A}, or A_3AR were seeded in 24 well plates and incubated at 37 °C overnight. The following day the medium was removed and replaced with DMEM containing 50 mM HEPES, 10 μM rolipram, 3 U/ml adenosine deaminase and increasing concentrations of the test compound. After an incubation of 30 min at 37 °C, 10 μM of forskolin was added to stimulate cAMP levels in the A_1 or A_3 assays, and the cells were incubated at 37 °C for an additional 15 min. The A_{2A} assay plates remained in the incubator for 45 min. Next, the medium was removed, and the cells were lysed with 200 μl of 0.1 M HCl. 100 μl of the HCl solution was used in the Sigma Direct cAMP Enzyme Immunoassay following the instructions provided with the kit. The results were calculated using an ELx808 Ultra Microplate reader (BioTek, Winooski, VT) at 405 nm and analyzed using Prism software.

2.7. Transfection of the A_3AR in HL-1 cells

150 μl of GenJet and 25 μg of pcDNA5 plasmid encoding the cDNA of the hA_3AR were each mixed in 750 μl of HL-1 media without serum or antibiotics. The solutions were combined together and added to 90% confluent HL-1 cells in a 75 cm^2 flask. GenJet solution with no plasmid was added to a second flask of HL-1 cells as a control. After 5 h, the medium was removed and replaced with HL-1 media containing serum and antibiotics. After 24 h, the cells were trypsinized and split to 24 and 6 well plates for qRT-PCR or assays of lactate dehydrogenase (LDH) and apoptosis.

2.8. Quantitative RT-PCR of ARs in HL-1 cells

Non-transfected and hA_3AR transfected HL-1 cells were grown in 6 well plates coated with fibronectin overnight. RNA from the cells was purified following the protocol of the RNeasy Kit (Qiagen Inc., Valencia, CA) with DNase I (Qiagen). Reverse transcription was completed using Superscript III First Strand Synthesis Supermix kit (Invitrogen). cDNA from ARs was quantified on a 7900HT Fast Real-Time PCR Machine (Applied Biosystems, Foster City, CA) instrument using SybrGreen PCR MasterMix (Sigma), 150 nM primers, and 50 ng/μl DNA (total volume is 20 μl). The following AR and β-actin primers were used: mA1-F: 5'-TGT GCC CGG AAA TGT ACT GG-3', mA1-R: 5'-TCT GTG GCC CAA TGT TGA TAA-3'; mA2A-F: 5'-TGC CTC TTC TTC GCC TGC TTT-3', mA2A-R: 5'-AAT CGC AAT GAT GCC CTT GCG C-3'; mA2B-F: 5'-GCG AGA GGG ATC ATT GCT GCT-3', mA2B-R: 5'-CCC CCA GTT CTG TGC AGT TG-3'; mA3-F: 5'-CAC CCA TGC TTC CAT CAT GTC-3', mA3-R: 5'-AGC CCC ACC AGA AAG GAA AC-3'; hA3-F: 5'-GGC CAA TGT TAC CTA CAT CAC C-3', hA3-R: 5'-CCA GGG CTA GAG AGA CAA TGA A-3'. Differential expression of the nontransfected and transiently transfected cell lines was compared using the ΔΔCt method [30].

2.9. LDH assay for quantification of cytotoxicity

Transiently transfected or non-transfected HL-1 cells were added to 24 well plates coated with fibronectin and left at 37 °C overnight. Increasing concentrations of each agonist, diluted with PBS with calcium and magnesium, were incubated with the cells at 37 °C for 1 h. If an antagonist was used, it was incubated with the cells 1 h prior to the addition of the agonist. Without removing the agonist, H_2O_2 in PBS was added to the cells at a final concentration of 750 μM. The cells were then kept at 37 °C for 4 h. In all cases, cells that did not receive H_2O_2 served as a negative control for 0% cytotoxicity, and cells that received 0.02% Triton X served as a positive control for 100% cytotoxicity. 100 μl of each cell supernatant was added to a 96 well plate in triplicate, and the LDH assay (Roche Applied Sciences, Indianapolis, IN) was run following the manufacturer's instructions. The results were determined using an ELx808 Ultra Microplate reader at 490 nm and 650 nm and analyzed using Prism software.

2.10. Luminescent caspase assay for quantification of apoptosis

A_3AR-transfected HL-1 cells were seeded in a 96-well opaque white bottom plate (30,000 cells/well) and incubated overnight at 37 °C. At the end of the incubation period, the media was replaced with DMEM with calcium and magnesium, which was used for the remainder of the experiment. The dendrimer compounds (10 μM) were added to the cells one hour prior to the addition of H_2O_2 and were left in the cell medium until the end of the experiment. Apoptosis was induced in the cells by the addition of 400 μM of H_2O_2, and cells were incubated for 3 h at 37 °C. The apoptosis induced by H_2O_2 was determined with the Caspase – Glo 3/7 assay kit (Promega Corporation, Madison, WI) which is based on the cleavage of the luminogenic caspase substrate. Equal volume of Caspase-Glo 3/7 reagent was added to the apoptosis induced cells, and the cells were incubated at room temperature for 90 min. After 90 min, caspase activity was quantitated using a 1420 Luminescence counter (PerkinElmer).

2.11. Statistical analysis of *in vitro* data

Pharmacological parameters were analyzed with Prism software. Data were expressed as mean ± standard error (n = 3). Statistical significance was calculated using the Student's t-test. There were 3 degrees of freedom (df) for the LDH assay, and 4 degrees of freedom for the caspase assay. *P* values less than 0.05 ($P<0.05$) were considered to be statistically significant. For the caspase assay, statistical significance between the results was analyzed by ANOVA followed by the Tukey-Kramer multiple comparison test.

3. Results

3.1. Synthesis of a G5.5 PAMAM dendrimer-nucleoside conjugate for AR activation

In order to introduce a fluorescent moiety on the parent dendrimer, we used compound **6**, an amine-functionalized derivative of Alexa Fluor 488 (AF488) that was synthesized in our previous study and which contained a terminal primary amine located on an ED moeity [26]. Alexa Fluor dyes have previously been shown to have a more stable fluorescent signal than fluorescein [31]. Thus, G5.5-PAMAM-AF488 (**7**) was synthesized by water-soluble carbodiimide coupling (EDC, in 0.1 M MES, pH 5) to attach compound **6** to the carboxylic-functionalized G5.5 dendrimer, as shown in Figure 1B.The unreacted EDC and urea byproduct were removed by dialysis. Next, the terminal amino group of AR agonist **4** was amide conjugated to the fluorescent-labeled G5.5 dendrimer **7** also using a carbodiimide coupling.

The dendrimer conjugates were purified using SEC and characterized using NMR and electrospray ionization (ESI) mass spectrometry (MS). The parent G5.5 dendrimer had a measured molecular weight of 53,813, which was ~1 % higher than the theoretical mass of 52,900, as shown in Figure S1 (Supporting Information). The extra molecular weight and the significant fragmenting of the peaks were probably caused by the excess sodium ions in the sample. NOESY NMR showed no significant backfolding of arms attached to the G5.5 dendrimer. The fluorescent conjugate increased in molecular weight by 158 D for a total molecular weight of 53,917, close to the theoretical weight of 53,435, as shown in Figure S2. NMR of **7** showed that AF488-ED was attached, but the peaks were too small in comparison to the parent dendrimer to be properly integrated [32].

The nucleoside conjugate **8** was also analyzed by NMR and ESI MS. After removal of the monomers by dialysis, MS showed that, on average, approximately 60 moieties of **4** were attached per dendrimer, as shown in Figure S3. The NMR analysis was noisy due to the small sample size, so the peaks could not be properly integrated. However, peaks corresponding to **4** were seen in the NMR spectrum.

3.2. Pharmacological characterization of a G5.5 PAMAM dendrimer-nucleoside conjugate in AR binding and cAMP assays

Standard radioligand binding assays were used to measure the affinity of the dendrimer conjugate at three of the subtypes of ARs [29]. The affinity of similar derivatives at the A_{2B}AR is very low [33]; thus, this subtype was not included in the assay. In radiologand saturation studies (data not shown), CHO cells stably transfected with the hA_1 or the hA_3AR had B_{max} values of 530 ± 210 fmol/mg protein or 253 ± 19 fmol/mg protein, respectively, showing that there is similar receptor expression in both stably transfected cell lines. HEK cells stably transfected with the hA_{2A}AR expressed 5000 ± 350 fmol/mg protein.

The hAR binding affinity of the functionalized congener **4** was prior to attachment to the dendrimers was previously reported [26]. Compound **4** displayed K_i values at the hA_1, A_{2A} and A_3ARs of 43 ± 5 nM, 300 ± 20 nM, and 9.5 ± 2.0 nM, respectively. In an assay measuring the accummulation of cAMP (Table 1), compound **4** was also a potent full agonist at the A_1 and A_3ARs (inhibition) and the A_{2A}AR (stimulation).

Although compound **5** displayed >100-fold selectivity for the A_3AR in comparison to the A_1 and A_{2A}ARs in both binding and functional cAMP assays [26], compound **8** was only slightly selective for the A_3AR in binding and nonselective in an assay of adenylate cylase inhibition (Table 1). The binding $K_{i\ app}$ values of compound **8** at the A_1, A_{2A} and A_3 ARs were 140, 80, and 15 nM, respectively. The control dendrimer **7** at a 10 μM concentration was inactive in an assay of A_3AR-mediated cAMP inhibtion and only weakly displaced radioligand at each of the three AR subtypes.

3.3. Quantification by qRT-PCR of the AR subtype gene expression in HL-1 cardiomyocytes

The levels of gene expression of all four mouse ARs in control HL-1 cells were measured using the ΔΔCT method of qRT-PCR using β-actin as an internal control. The endogenous level of the A_3AR gene expression was the lowest among the four ARs. qRT-PCR indicated that A_1 and A_{2A}ARs were expressed at 43 ± 18-fold and 56 ± 10-fold higher levels of expression than A_3AR, respectively, but the A_{2B}AR was only expressed at a 5-fold higher level than the A_3AR (Figure 2).

3.4. Cytoprotection by AR agonists in an *in vitro* model of mouse cardiomyocte cell death

We used the HL-1 mouse cardiomyocyte model, in which cell damage was induced using H_2O_2, to test the cytoprotective ability of AR agonists. The degree of death in nontransfected HL-1 cells was shown using an LDH assay to be dependent on the concentration of H_2O_2 (Figure S4). The half-maximal increase in cell death occurred at ~1 mM, and it reached a plateau thereafter. 750 μM H_2O_2 produced between 35 – 45% cell death following a 4 h incubation, and this concentration was selected for further protection experiments.

In order to test the effect of greatly increasing the level of expression of the A_3AR in the HL-1 cells on AR agonist-induced protection, we transfected the cells with cDNA coding for the receptor in the pcDNA5 plasmid. We measured the expression levels of the A_3AR in the HL-1 cells following transfection using qRT-PCR (data not shown). Although the transfection level significantly varied between multiple transfections, in each case there was at least a 500-fold increase in the expression level after transfection compared to the endogenous level of A_3AR. Therefore, all other ARs were expressed at a minimum of a 10-fold lower expression level than the A_3AR.

H_2O_2 has previously been shown to induce death in a primary neuronal cell culture after an incubation of 3 h [34]. In the non-transfected HL-1 cells, only the nonselective agonist NECA and the A_{2A} agonist CGS21680 protected against the H_2O_2–induced (750 μM) cytotoxicity as measured in an LDH assay, which reflects loss of cell membrane integrity. Interestingly, the nucleoside dendrimer **8** that bound to and activated the A_{2A}AR, although less potently than A_1 and A_3ARs, did not show cytoprotection in nontransfected cells. In various models, A_1, A_{2A}, and A_3ARs have all been shown to have cardioprotective properties.

A comparison of NECA, IB-MECA, or **8** in A_3AR-transfected HL-1 cells (Figure 3A) indicated that the dendrimeric derivative **8** was highly potent in cell protection. The results are tabulated as IC_{50} values in Table 2. The IC_{50} value of **8** was 100- to 200-fold less than those of the monomeric A_3AR agonists IB-MECA and Cl-IB-MECA. The IC_{50} value for NECA did not significantly change between transfected and non-transfected cells, while neither IB-MECA nor Cl-IB-MECA gave protection in non-transfected cells (Figure 3B).

In order to determine if IB-MECA was acting at the A_3AR, a selective A_3 antagonist, 1,4-dihydropyridine derivative MRS1191 (10 μM) [35], was added prior to treatment with IB-MECA (30 μM). The antagonist prevented the protection afforded by IB-MECA from the H_2O_2-induced cell death in the transfected HL-1 cells (Figure 4). The difference between the cytotoxicity induced by H_2O_2 alone and the cytotoxicity induced with coadminstration of MRS1191 and IB-MECA was not statistically different using a student t-test ($P = 0.05$, df = 3).

Finally, in order to determine if the cell death induced by the H_2O_2 was the result of apoptosis, an apoptosis-specific caspase 3/7 luminescent assay was used. There was an increase in the apoptosis signal in the control HL-1 cells when H_2O_2 was added (Figure 5). For the apoptosis assay, a lower concentration of H_2O_2 (400 μM) than in the LDH assay was sufficient. Apoptosis significantly decreased when **8**, the dendrimer-A_3 agonist conjugate was added prior to the

H_2O_2, but did not significantly decrease when the cells were was incubated with **7**, the control dendrimer ($P = 0.05$, df = 3).

4. Discussion

This study investigated the ability of A_3AR agonists, both multivalent GLiDe conjuates and monomers, to protect HL-1 cells against H_2O_2-induced cytotoxicity in HL-1 cells. While it was previously known that all ARs are expressed by HL-1 cells, the levels of expression were not known. Interestingly, the A_{2B} and A_3ARs are expressed at an almost 50-fold lower level than the A_1 and A_{2A}ARs. A_{2B} and A_3ARs have not been definitively found in adult cardiac myocytes [15]. However, the HL-1 cells are believed to be a hybrid of adult and embryonic cardiomyocytes [17]. Although the A_3AR may or may not be expressed in adult cardiac myocytes, protective effects in myocardial ischemia have been attributed to activation of the A_3AR [15].

Interestingly, the selective A_1AR agonist CCPA did not protect against H_2O_2-induced cell death in control HL-1 cells, while the A_{2A}AR agonist CGS21680 weakly protected with an IC_{50} value of 7.1 μM. Although this concentration is high with respect to the K_i value of CGS21680 in activation of cAMP accumulation through the mouse A_{2A}AR (7 nM) [36], the inactivity of A_1 and A_3AR agonists and the activity of NECA allow the conclusion that the A_{2A}AR is likely responsible for the protection in control HL-1 cells. The observed protection by A_{2A} but not A_1AR activation is in contrast to some previous reports. For instance, Germack et al. found that CGS21680 does not protect neonatal rat cardiomyocytes against ischemia/reperfusion, while CPA, an A_1AR agonist, does have protective effects [37]. However, H_2O_2-induced cell death may involve different signaling pathways than the damage resulting from ischemia/reperfusion. In fact, other studies have found that both the A_1 and A_{2A}ARs are believed to be involved in cardioprotection. There is also evidence of cross-talk between ARs and that multiple ARs may be activated by endogenous adenosine in order to achieve cardioprotection [15].

In order to determine if the A_3AR plays a role in cardioprotection in this cell culture model, we transfected the HL-1 cells with a plasmid coding for the hA_3AR to greatly increase the level of A_3AR expression. Although the transfection procedures remained constant, the levels of A_3AR expression measured by qRT-PCR varied greatly between transfections. However, the RNA level always increased at least 500-fold over the endogenous level of A_3AR RNA.There was not a significant difference in IC_{50} values of the A_3AR agonists in the LDH assay based on the expression level of the A_3AR.There are several explanations for why the IC_{50} values were not affected by the transfection level. For instance, the qRT-PCR measured the level of hA_3 mRNA, not the level of receptor expression, which may have been much lower in all of the experiments. Attempts to measure the protein expression level using an A_3-specific antibody were not successful due to non-specific binding by the available antibodies.

In the untransfected control cells, only NECA and CGS21680 were protective, likely through activation of the A_{2A}AR. Increasing the expression level of the A_3AR significantly revealed protection afforded by the A_3 agonists IB-MECA and Cl-IBMECA, although the protective effects of the nonselective agonist NECA did not change. In the transfected cells, NECA may be protective through activation of the A_{2A}AR, A_3AR, or some combination of ARs, while protection against H_2O_2-induced apoptosis by the A_3AR-selective agonists IB-MECA and Cl-IB-MECA is dependent on the expression of that subtype. This conclusion was further supported in the case of protection from cell death by the dendrimeric conjugate **8**, which was blocked by an A_3AR selective antagonist. Figure 6 shows a schematic representation of the basis for protection afforded to the HL-1 cells by compound **8**. After mouse HL-1 cells were transfected with the hA_3AR, the receptor protected the cells against H_2O_2-induced apoptosis

when the protein was activated by **8**. The A_3AR has significant and unique cardioprotective properties [11,12]. While most previous work used whole animals or cultured cardiomyocytes from various species [11,13], the HL-1 cell line can now be used to study the signaling pathways involved in cardioprotection caused by AR activation.

Dendrimeric conjugation of GPCR ligands is a means of modulating their pharmacokinetic and pharmacodynamic characteristics [25,26], assuming that the linking chemistry is done in a way that preserves or enhances the pharmacological properties of the ligand [24]. In general, the chemical and biological properties of multivalent drugs bound to nanocarriers may differ greatly from those of the corresponding monomeric agents [38]. This approach provides an opportunity to tune the pharmacokinetics and pharmacodynamics in an otherwise unattainable manner and to introduce reporter or targeting moieties. It is also conceivable that the nucleoside bound to a polymeric carrier would have a reduced rate of metabolism *in vivo* [39], while preserving or enhancing the potency and selectivity.

In the present study, we synthesized a G5.5 PAMAM dendrimeric conjugate **8** containing an N^6-chain-functionalized adenosine agonist amide-linked at approximately one quarter of the available carboxylic acid sites. The remaining terminal carboxylate groups afford it a negative charge. It was of much higher molecular weight than a previously synthesized G2.5 PAMAM conjugate of similar linkage chemistry, which was less densely substituted with ligand (~10% of the available sites) and selective in binding to and activating the A_3AR [18]. The larger conjugate was designed because of the expected longer half-life of higher molecular weight PAMAM dendrimers *in vivo* [40]. Conjugate **8** activated ARs nonselectively and, similarly to the monomeric agonists of the A_3AR, protected A_3 transfected HL-1 cells using both an LDH assay and an apoptosis assay. However, the dendrimer conjugate had significantly greater potency (IC_{50} 35 nM) than the corresponding monomeric nucleosides, which protected in the μmolar range. Thus, a multivalent conjugate retained binding affinity at the ARs and displayed greatly enhanced functional potency in an *in vitro* model of cardioprotection. GPCRs frequently exist as oligomeric complexes rather than single receptors [41], and the dendrimeric conjugate **8** could theoretically activate multiple receptor molecules in a dimer or higher order oligomer. While more research is necessary to determine the precise mechanism of the A_3AR–induced protection and if simultaneous binding of multiple receptors is the basis for the increased potency, this research provides the groundwork for the use of multivalent drugs in treating cardiac diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ADAC	N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl]adenosine
AF488	Alexa-Fluor® 488
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CHO	Chinese hamster ovary
Cl-IB-MECA	2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine

DMEM	Dulbecco's Modified Eagle Medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
ED	ethylenediamine
EDC	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
[^{3}H]CGS21680	2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
[^{125}I]AB-MECA	[^{125}I]-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine
IB-MECA	N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	mass spectrometry
NMR	nuclear magnetic resonance
PAMAM	polyamidoamine
qRT-PCR	quantitative real-time polymerase chain reaction

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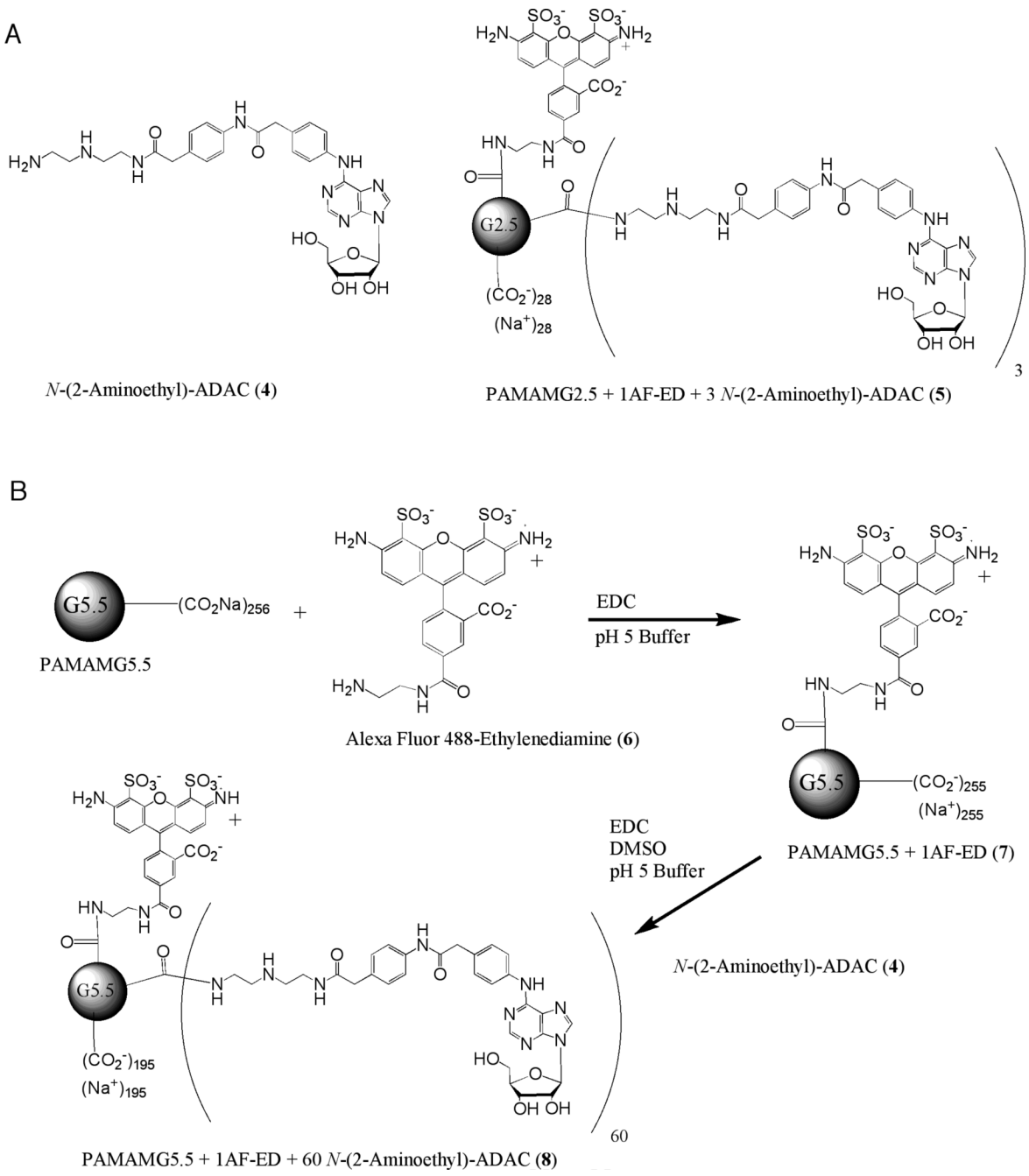

Figure 1.

(A) Structures of a non-selective amine-functionalized AR agonist (**4**) and an A_3 selective dendrimeric conjugate (**5**) as reported [26]. (B) Synthesis of **8**, a G5.5 PAMAM dendrimer with 1 AF488-ED and 60 N-(2-aminoethyl)-ADAC moieties. AF488-ED and *N*-(2-aminoethyl)-ADAC were conjugated to G5.5 PAMAM dendrimers using carbodiimide coupling.

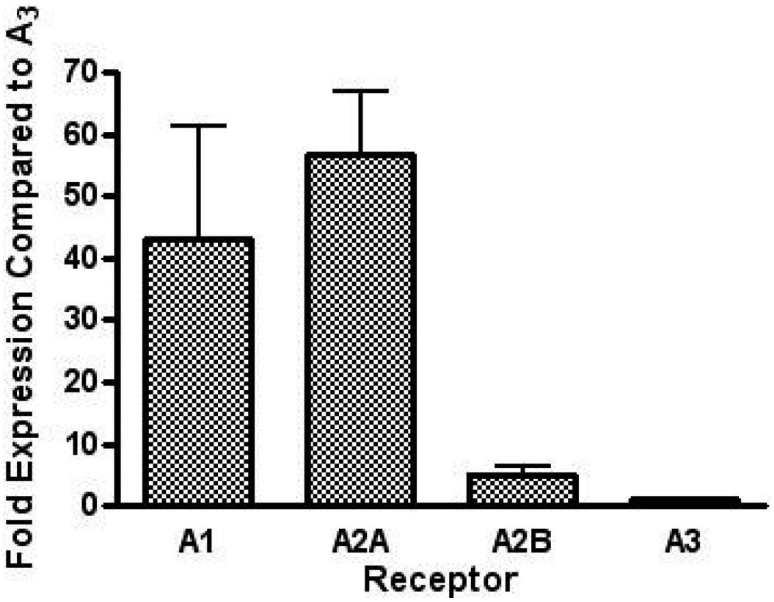

Figure 2.

Gene expression levels of the A_1, A_{2A}, and A_{2B} in HL-1 cells compared to A_3 ARs measured using qRT-PCR. In three separate experiments, the fold expression of each AR is measured and normalized to the A_3AR expression level, which is set to 1, using the ΔΔCT method.

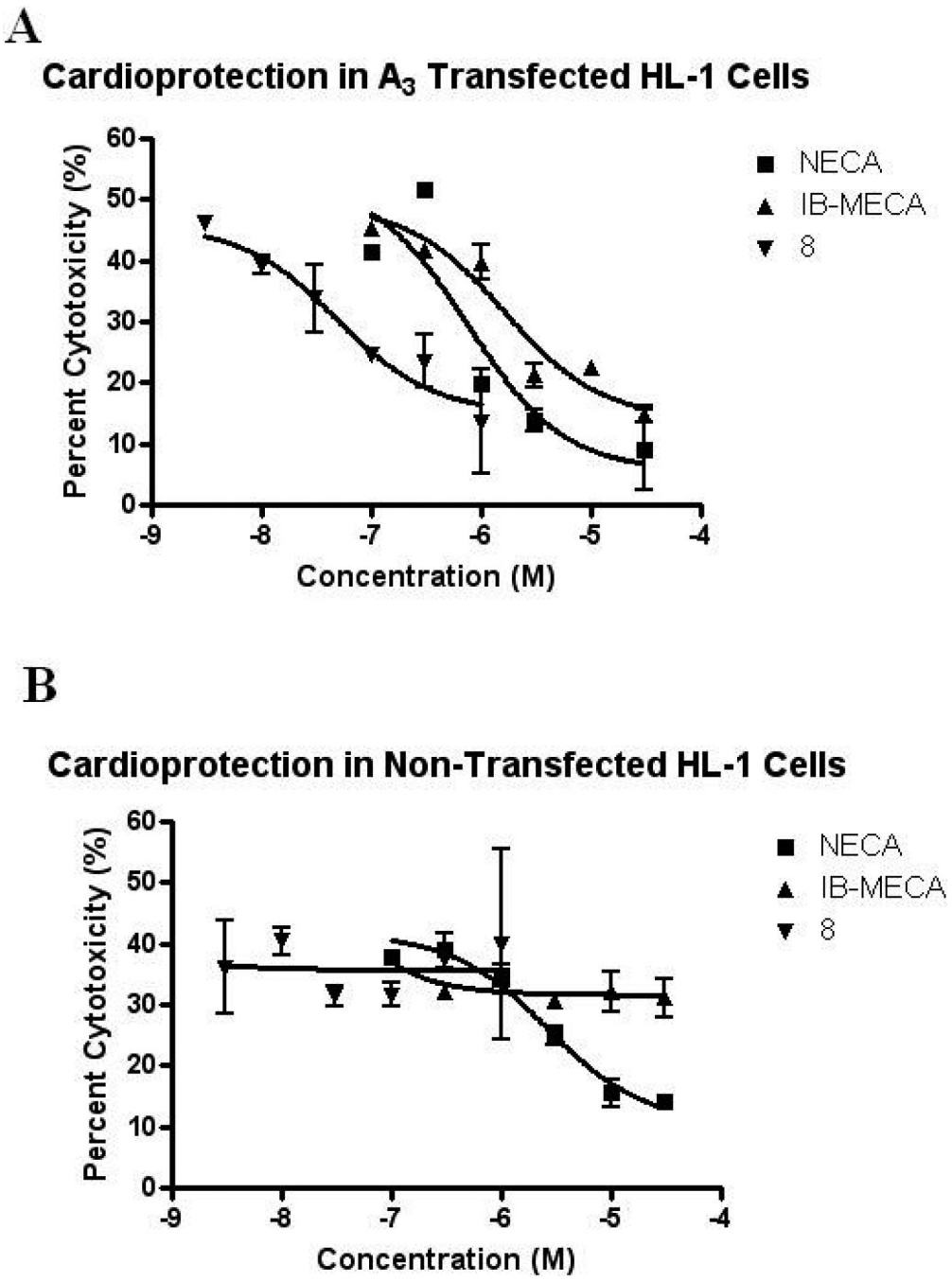

Figure 3.

Concentration-dependent protection against H_2O_2-induced cytotoxicity by A_3 selective and non-selective AR agonists (3 nM – 30 μM) in A_3AR-transfected (A) and non-transfected (B) HL-1 cells. HL-1 cells were pretreated with NECA (100 nM - 30 μM), IB-MECA (100 nM - 30 μM), or **8** (3 nM - 1 μM) for 1 h prior to the addition of H_2O_2 (final conc. 750 μM). After 4 h, 100 μl of media was added to 100 μl of the LDH measuring solution provided with the kit and incubated for 10 min. The results were analyzed with a microplate reader. Data shown are mean ± SD from three independent experiments in triplicate.

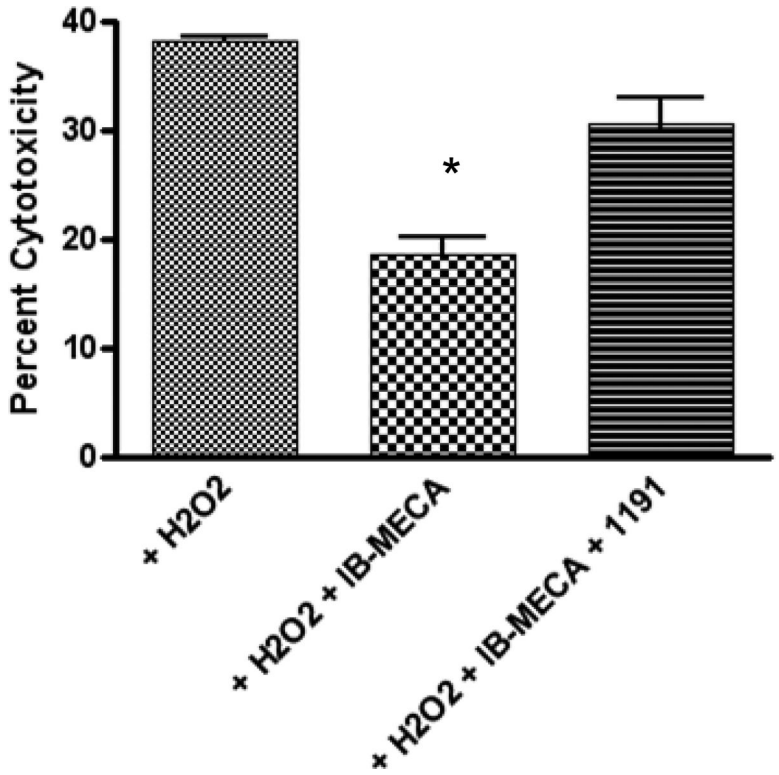

Figure 4.

Effect of the A_3 receptor antagonist MRS1191 on the protection by IB-MECA against H_2O_2-induced cell damage. HL-1 cells were pretreated with MRS1191 (10 μM), an antagonist of the A_3 receptor, 1 h before treatment with IB-MECA (30 μM). One h after the addition of the agonist, H_2O_2 (750 μM) was incubated with the cells for 4 h. 100 μl of media was then added to 100 μl of the LDH measuring solution provided with the kit and incubated for 10 min. The results were analyzed with a microplate reader. Data shown are mean ± SD from three independent experiments in triplicate. Groups labeled ***** are significantly different from H_2O_2 control ($P < 0.05$).

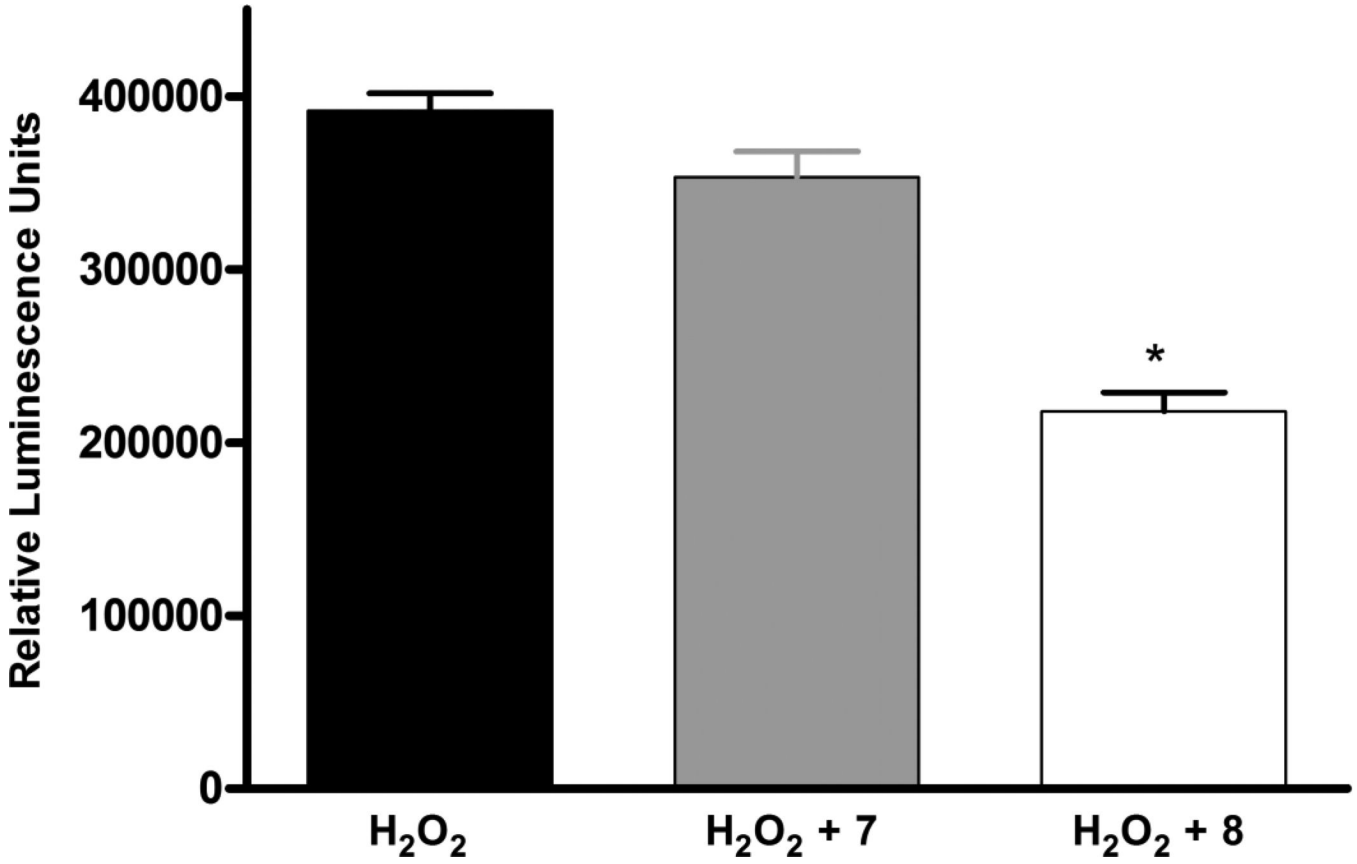

Figure 5.

Protection afforded HL-1 cells by **8**, a dendrimer-nucleoside conjugate, but not by **7**, a control dendrimer, against H_2O_2-induced apoptosis. Dendrimer compounds (10 μM) were added 1 h prior to the addition of H_2O_2 (400 μM). The luminescent caspase 3/7 reagent was added 3 h after the addition of H_2O_2. After 1.5 h, apoptosis was quantified using a luminometer. Data shown are mean ± SD from three independent experiments in triplicate. Groups labeled ***** are significantly different from control treated with H_2O_2 alone ($P < 0.05$). Control cells in the absence of H_2O_2 gave 182,000 ± 20,000 relative luminescence units.

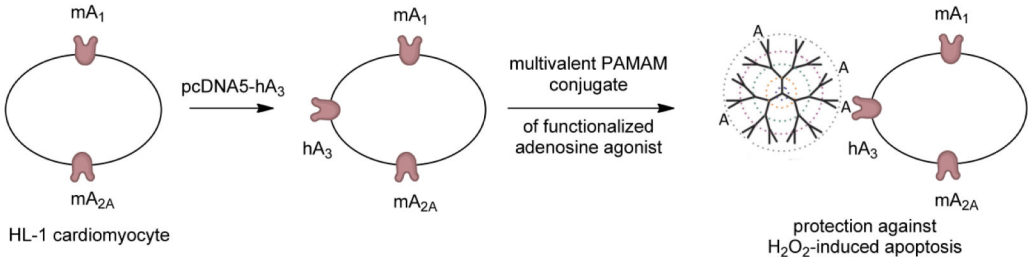

Figure 6.

Schematic description of the mechanism of protection afforded HL-1 cells by multivalent dendrimeric AR agonist **8**. The PAMAM dendrimer derivative is shown as a tree-like polymer with covalently attached nucleoside moieties "A". Mouse HL-1 cells, which express a high basal level of mA_1 and mA_{2A}ARs, were transfected with hA_3AR mRNA in order to increase the expression levels of this subtype. Following transfection, activation of the hA_3AR by conjugate **8** afforded significant protection to the HL-1 cells against H_2O_2-induced apoptosis.

Table 1

K_i or K_i apparent values for binding of nucleoside monomers and dendrimer conjugates and functional effects on cAMP at hA_1, A_{2A}, and A_3ARs.[a]

	Binding, $K_{i\ app}$ (nM) or % inhibition at 10 μM			**Effects on cAMP, EC_{50} (nM)**		
Compound	**A_1**	**A_2**	**A_3**	**A_1**	**A_2**	**A_3**
N-(2-Aminoethyl)-ADAC (**4**)	43 ± 5	300 ± 20	9.5 ± 2.0	89 ± 17	36 ± 13	35 ± 12
G5.5	25 ± 10%	23 ± 2%	26 ± 9%	NA[b]	NA[b]	NA[b]
G5.5 - 1 AF-ED (**7**)	24 ± 1%	37 ± 10%	55 ± 10%	NA[b]	NA[b]	NA[b]
G5.5 - 1 AF-ED – 60 *N*-(2-Aminoethyl)-ADAC (**8**)[c]	140 ± 65	80 ± 17	15 ± 4	100 ± 40	270 ± 90	66 ± 25

[a] Binding experiments were completed in stably transfected CHO cells (A_1, A_3) or HEK cells (A_{2A}). Cyclase experiments were completed in stably transfected CHO cells for all receptor types. Binding assays and functional assays using a cAMP kit were carried out as described in methods. Binding affinities are expressed as apparent inhibition constants ($K_{i\ app}$) and functional potencies as EC_{50} values (mean ± SEM, n = 3). As in previous studies, the results for the dendrimer derivatives are reported in dendrimer concentrations, rather than tethered monomer concentrations [18-20].

[b] NA, not active – 10 μM of compound gives less than 20% activation at receptor compared to NECA.

[c] MRS5212.

Table 2

Protection from cell death induced by H_2O_2 in HL-1 cells using an LDH quantification assay.[a]

Compound	Untransfected cells, IC_{50} (nM)[b]	Transfected cells, IC_{50} (nM)[b]
NECA (**1**)	3700 ± 1000	3900 ± 1800
CCPA	No protection	ND
CGS21680	7100 ± 2400	ND
IB-MECA (**2**)	No protection	3800 ± 1400
Cl-IB-MECA (**3**)	No protection	7900 ± 2300
G5.5 – 1 AF-ED (**7**)	No protection	No protection
G5.5 – 1 AF-ED – 60(*N*-(2-Aminoethyl)-ADAC) (**8**)	No protection	35 ± 8

ND not determined.

[a] Nucleoside derivative was administered 1 h prior to exposure to H_2O_2 and remained in the medium during the entire 4 h incubation in the presence of H_2O_2.

[b] Either control cells (not transfected to express the A_3AR) or cells transfected with cDNA for the hA_3AR. No protection indicates lack of significant inhibition of cell death by the compound at a conc. up to 30 μM, except for 7 which was tested up to 1 μM. As in previous studies, the results for the dendrimer derivatives are reported in dendrimer concentrations, rather than tethered monomer concentrations [18-20].