Pharmacologic characterization of novel adenosine A_{2A} receptor agonists in equine neutrophils

Wan-chun Sun, PhD; James N. Moore, DVM, PhD; David J. Hurley, PhD; Michel L. Vandenplas, PhD; Joel M. Linden, PhD; Thomas F. Murray, PhD

Objective—To evaluate anti-inflammatory effects of several novel adenosine receptor agonists and to determine their specificity for various adenosine receptor subtypes on neutrophils, cells heterologously expressing equine adenosine receptors, or equine brain membranes.

Sample Population—Neutrophils isolated from 8 healthy horses.

Procedures—Radioligand binding experiments were performed to compare binding affinities of adenosine receptor agonists to equine adenosine A_1, A_{2A}, and A_3 receptor subtypes. Effects of these agonists on endotoxin-induced production of reactive oxygen species (ROS) by equine neutrophils and roles of specific adenosine receptor subtypes and cAMP production in mediating these effects were determined.

Results—Radioligand binding experiments yielded a ranked order of affinity for the brain equine A_{2A} receptor on the basis of 50% inhibitory concentrations (IC_{50}) of the agonists as follows: ATL307 (IC_{50} = 1.9nM) and ATL313 > ATL309 and ATL310 > ATL202 > 2-([p-2-carboxyethyl] phenylethylamino)-5′-*N*-ethylcarboxyamidoadenosine > 5′-N-ethylcarboxamidoadenosine. Furthermore, ATL313 had approximately 100-fold greater selectivity for A_{2A} over A_1 and A_3 receptors. In functional assays with equine neutrophils, the compounds inhibited endotoxin-induced ROS production and stimulated production of cAMP with the same ranked order of potency. Results of experiments performed with selective adenosine receptor antagonists indicated that functional effects of ATL313 were via stimulation of A_{2A} receptors.

Conclusions and Clinical Relevance—Results indicated that activation of A_{2A} receptors exerted anti-inflammatory effects on equine neutrophils and that stable, highly selective adenosine A_{2A} receptor agonists may be developed for use in management of horses and other domestic animals with septic and nonseptic inflammatory diseases. (*Am J Vet Res* 2007;68:981–987)

Polymorphonuclear neutrophils are the most abundant circulating leukocytes in horses and are the first to migrate to sites of infection, where they participate in the initial phases of the inflammatory response. At those sites, they ingest invading microorganisms and cell debris, generate ROS, and release proteolytic enzymes.[1] Although these functions of neutrophils are important in limiting the spread of pathogens, these same inflammatory products also are capable of causing tissue damage at the site of infection and exacerbating inflammation in pathologic conditions such as endotoxemia, arthritis, and myocardial infarction.[2]

Adenosine, a ubiquitous endogenous purine nucleoside released during ATP metabolism, has anti-inflammatory effects and may be an important part of the natural dampening mechanism for the inflammatory response.[3,4] Cellular signaling by adenosine is through 4 subtypes of adenosine receptors (ie, A_1, A_{2A}, A_{2B}, and A_3), all of which are G-protein–coupled receptors.[5,6]

Abbreviations

ROS	Reactive oxygen species
LPS	Lipopolysaccharide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
NECA	5′-N-ethylcarboxamidoadenosine
CGS21680	2-([p-2-carboxyethyl]phenylethylamino)-5′-N-ethylcarboxyamidoadenosine
$[^{125}I]$AB-MECA	4-amino-3-$[^{125}I]$iodobenzyl-5′-N-methylcarbamoyladenosine
AFU	Arbitrary fluorescence unit
EC_{50}	Effective concentration that induces 50% of the maximum response
K_B	Dissociation constant
K_D	Equilibrium dissociation constant
IC_{50}	50% inhibitory concentration

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From the Departments of Large Animal Medicine (Sun, Moore, Hurley, Vandenplas), Physiology and Pharmacology (Moore), and Population Health (Hurley), College of Veterinary Medicine, University of Georgia, Athens, GA 30602; the Department of Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22908 (Linden); and the Department of Pharmacology, School of Medicine, Creighton University, Omaha, NE 68178 (Murray).
Supported by a grant from the USDA Small Business Innovation Research Program and the White Fox Farm Research Fund.
Address correspondence to Dr. Moore.

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Results of studies[2,7-9] indicate that activation of the adenosine A_{2A} receptor subtype exerts anti-inflammatory effects by several mechanisms, including suppressing the production of ROS by activated neutrophils. A common problem associated with the administration of adenosine receptor agonists is the lack of selectivity of compounds for the various adenosine receptor subtypes and the concomitant development of important adverse cardiovascular effects, such as heart block mediated by activation of the adenosine A_1 receptor.[6]

The goal of our laboratory group's research in this area is to identify highly selective and potent adenosine analogues for A_{2A} receptors that may be useful in the management of inflammation in horses. In the study reported here, a series of novel adenosine A_{2A} receptor agonists were investigated. We compared their selectivity for binding to equine receptor subtypes, ability to reduce LPS-induced production of ROS by equine neutrophils, and selectivity for exerting this effect via adenosine A_{2A} receptors. Results obtained in this study may serve as a foundation for the development of a new class of pharmaceutical agents that can be used to manage septic and nonseptic inflammatory responses in horses and other domestic animals.

Materials and Methods

Sample population—Blood samples for all experiments were obtained from 8 healthy adult horses. Brain tissue was obtained from a single cadaveric horse. Use of these horses was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Isolation of neutrophils—Blood samples were obtained from the jugular vein from each horse into syringes that contained EDTA as an anticoagulant, and RBCs were allowed to settle for approximately 20 minutes. Leukocyte-rich plasma was layered onto a solution of polysucrose and sodium diatrizoate[a] and centrifuged at 400 × *g* for 30 minutes at 20°C. The RBCs that contaminated the resulting pellet were lysed with distilled water, and tonicity was then restored by the addition of 2× PBS solution.

Granulocytes (> 95% neutrophils) were washed 3 times with PBS solution and then suspended at a final concentration of 3×10^7 cells/mL in RPMI 1640 medium containing 10% fetal bovine serum[b] (without phenol red), 2mM L-glutamine, 2mM sodium pyruvate, and gentamicin (50 µg/mL). Cells were then incubated in 5% carbon dioxide at 37°C for 90 minutes to reduce signal induced by exposure to the solution of polysucrose and sodium diatrizoate. After that incubation, it was determined by use of trypan blue dye exclusion that viability of the neutrophils was ≥ 98%. Neutrophils were diluted by the addition of the aforementioned medium to achieve a final concentration of 3×10^6 cells/mL.

Radioligand binding assays with equine A_1, A_{2A}, and A_3 receptors—Specific binding of [^{3}H]DPCPX,[c] an A_1-selective antagonist radioligand, to equine cerebellar membranes was determined by use of a rapid filtration assay. The cerebellum was removed from the calvarium of a euthanized horse and dissected into blocks. These blocks were then immediately frozen on dry ice and stored in sealed containers at –80°C until use (tissues were used within 1 month after collection). On the day of the assay, cerebellar tissue was thawed, weighed, and homogenized[d] in 100 volumes of ice-cold 50mM Tris-HCl buffer. The homogenate was centrifuged (48,000 × *g* for 10 minutes); pellets were then suspended in the same volume of ice-cold buffer and homogenized again. The homogenate was centrifuged, and the pellet was suspended in 100 volumes of 50mM Tris-HCl buffer containing 2 U of adenosine deaminase[e]/mL. After incubation at 22°C for 30 minutes, the homogenate was cooled on ice and centrifuged; the pellet was then suspended in 100 volumes of ice-cold 50mM Tris-HCl by use of the homogenizer.

Equilibrium binding reactions were performed in triplicate. In equilibrium competition binding assays, aliquots (0.175 mL) of the brain membrane suspension were incubated at 22°C for 90 minutes with 0.2nM [^{3}H]DPCPX and increasing concentrations of adenosine analogues (ATL202,[f] ATL307,[g] ATL309,[h] ATL310,[i] or ATL313[j]), NECA,[k] or CGS21680.[l] Aliquots of the membrane suspension were incubated with 25 µL of [^{3}H]DPCPX (120 Ci/mmol) and 25 µL of buffer or N^6-cyclopentyladenosine (final concentration, 10µM) to define nonspecific binding. Aliquots of the membrane suspension were dissolved in 0.5 N NaOH for protein concentration determination. Total binding minus nonspecific binding with N^6-cyclopentyladenosine[m] was designated as specific binding, which was approximately 95% of the total binding. Binding reactions for all 3 radioligands were terminated by adding 2 mL of ice-cold 50mM Tris-HCl buffer followed by solution filtration with filter strips[n] and in a 24-well cell harvester.[o] Filter strips were soaked in 0.5% polyethyleneamine before use to reduce nonspecific adsorption of ligand. The filter strips were rinsed 4 times with 4 mL of ice-cold 50mM Tris-HCl buffer. For [^{3}H]-labeled ligands, filter strips were placed in 7-mL vials that contained 3.5 mL of scintillation cocktail, which was followed by measurement of radioactivity in a scintillation counter.[p]

For determination of specific binding to A_{2A} receptors in equine brain tissues, striatal membranes were prepared by Dounce homogenization (20 strokes) of dissected equine striatum in ice-cold 50mM Tris-HCl buffer (pH, 7.7) and centrifuged at 40,000 × *g* for 20 minutes. Membrane pellets were then resuspended in buffer, centrifuged, and washed 2 additional times prior to performing the binding assays. For the equilibrium competition assays, 100 µg of equine striatal membranes were incubated with approximately 1.0nM of the selective A_{2A} receptor antagonist [^{3}H]ZM241385[q] and increasing concentrations of adenosine analogues (ATL202, ATL307, ATL309, ATL310, or ATL313), NECA, or CGS21680. Aliquots of membrane suspension were incubated with [^{3}H]ZM241385, with or without the addition of 10µM 6-amino-2-chloropurine riboside,[r] to define nonspecific binding. Binding reactions were allowed to proceed at 22°C for 45 minutes.

For determination of specific binding to equine adenosine A_3 receptors, HEK293 cells that heterologously but stably expressed these receptors were used. Cell membranes were harvested from equine A_3–expressing HEK293 cells, as described elsewhere.[10] Briefly, 25 µg of the transfected HEK293 cell membranes were incubated with 0.25nM [^{125}I]

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AB-MECA[s] and increasing concentrations of adenosine analogues (ATL202, ATL307, ATL309, ATL310, or ATL313), NECA, or CGS21680. The binding reaction was allowed to proceed at 22°C for 45 minutes. Equine A_3 receptor–expressing HEK cell membranes were incubated with [^{125}I] AB-MECA alone or with 10µM 4-(3-[cyclopentyloxy]-4-methoxyphenyl)-2-pyrrolidinone[t] to define nonspecific binding. The binding reaction was terminated via rapid filtration onto filter strips[n] by use of a 24-well cell harvester.[o] Radioactivity was then determined by use of an automatic gamma counter.[u]

Measurement of ROS production by isolated neutrophils—Production of ROS by the neutrophils (3×10^5 cells/well) was monitored in 96-well flat-bottom tissue culture plates. Selected wells contained specific adenosine A_{2A} receptor agonists with or without adenosine deaminase and with or without *Escherichia coli* O55:B5 LPS.[v] Phorbol myristate acetate,[w] a direct activator of protein kinase C, was used to determine the maximal amount of ROS production. To identify the involvement of specific adenosine receptor subtypes, selected wells also contained ZM241385,[x] MRS1706,[y] or MRS1220.[z] To detect ROS, 10 µL of nonfluorescent dihydrorhodamine dye[aa] (final concentration, 10µM) was added to each well. The dye oxidizes to green-fluorescent rhodamine in response to hydrogen peroxide produced by neutrophils. Plates were incubated in a humidified atmosphere of 5% carbon dioxide at 37°C for 2 hours. Fluorescence was measured by use of a fluorescent plate reader[bb] with a 485-nm excitation filter and 538-nm emission filter. Values were reported as the number of AFUs. All reagents were diluted with RPMI 1640 medium not containing phenol red, but that did contain 10% fetal bovine serum, 2mM L-glutamine, 2mM sodium pyruvate, and gentamicin (50 µg/mL).

To permit comparison of data among experiments, fluorescence in each group of wells was adjusted relative to that obtained for unstimulated cells and thus was standardized for endogenous production of ROS. The degree of inhibition of LPS-induced responses was evaluated by use of the following equation:

$$\text{Percentage inhibition} = ([\text{AFU LPS} - \text{AFU treatment}]/[\text{AFU LPS} - \text{AFU cells}]) \times 100$$

where AFU LPS is the value for cells incubated with LPS alone, AFU treatment is the value for cells incubated with the particular treatment being evaluated and with LPS, and AFU cells are the basal value for unstimulated cells.

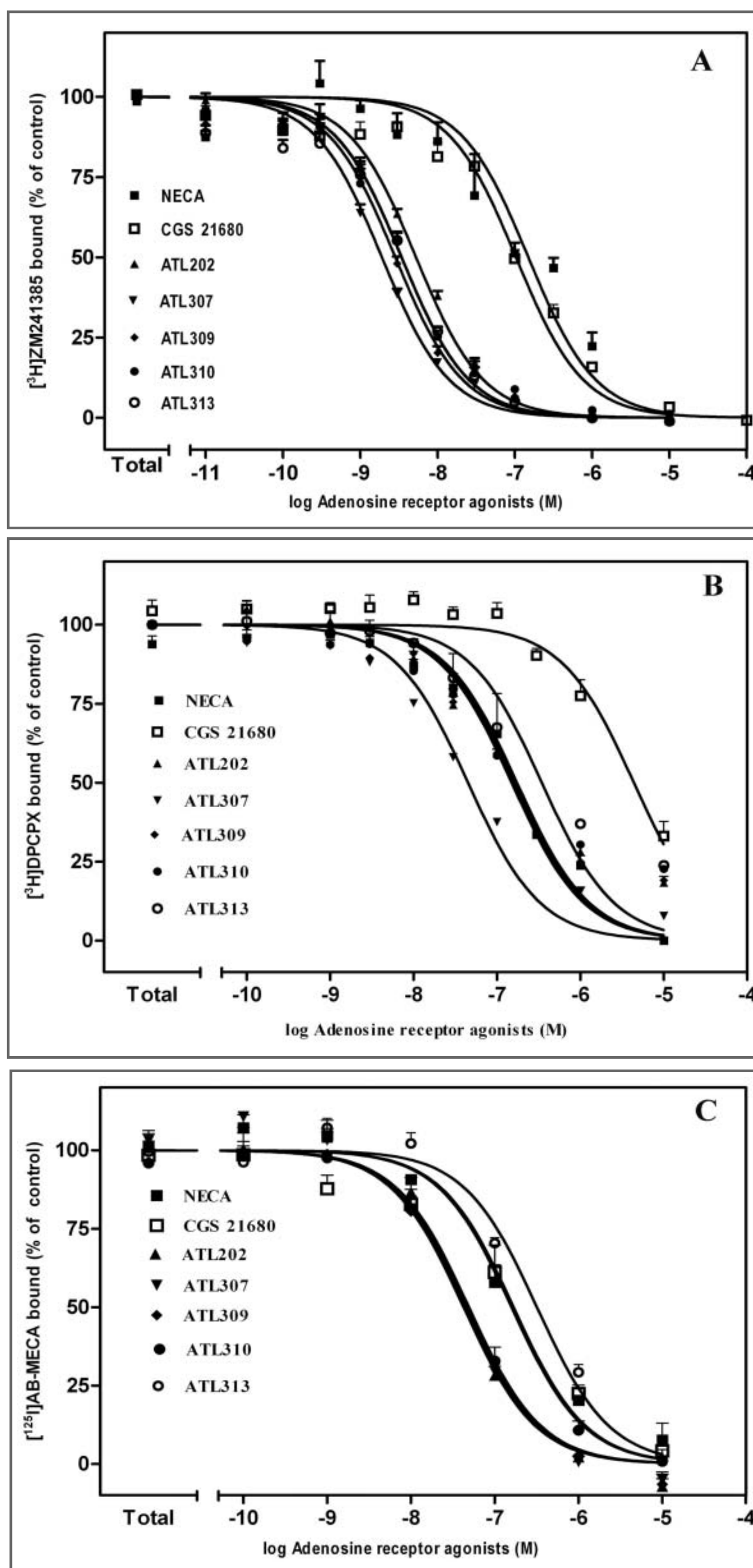

Figure 1—Competitive binding of adenosine receptor agonists incubated with [^{3}H]ZM241385 (A_{2A} receptors; A), [^{3}H]DPCPX (A_1 receptors; B), and [^{125}I]AB-MECA (A_3 receptors; C). Each point represents the mean ± SEM for 3 to 6 replicates. Results are reported as the percentage of radioligand binding without addition of an agonist. Membranes for binding of [^{3}H]DPCPX were prepared from equine cerebellum, membranes for binding of [^{3}H]ZM241385 were prepared from equine corpus striatum, and membranes for binding of [^{125}I]AB-MECA were prepared from HEK293 cells stably transfected with equine A_3 adenosine receptors. Similar results were obtained in 3 additional experiments.

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Table 1—Binding of various receptor agonists to adenosine receptors in equine brain membranes.

Receptor agonist	IC_{50} (nM)* A_1	A_{2A}	A_3	A_1/A_{2A}	A_3/A_{2A}
NECA	174.0 (123.0–246.0)	149.5 (106.0–211.0	158.7 (109.0–232.0)	1.2	1.1
CGS21680	4,393.0 (3,312.0–5,825.0)	103.3 (84.2–127.0)	167.5 (100.0–280.0)	42.5	1.6
ATL202	156.7 (99.5–246.0)	5.3 (4.7–5.9)	45.9 (31.6–66.6)	29.8	8.7
ATL307	45.1 (34.1–59.5)	1.9 (1.7–2.2)	42.5 (29.3–61.7)	23.6	22.3
ATL309	150.5 (97.7–232)	2.8 (2.5–3.2)	44.6 (34.5–57.6)	53.0	15.7
ATL310	164.3 (95.5–286.0)	3.4 (2.9–4.0)	49.0 (35.9–66.8)	48.2	14.4
ATL313	345.4 (214.0–559.0)	3.4 (2.8–4.2)	318.9 (230.1–442.0)	100.4	92.7

*Values are expressed as mean (95% confidence interval).

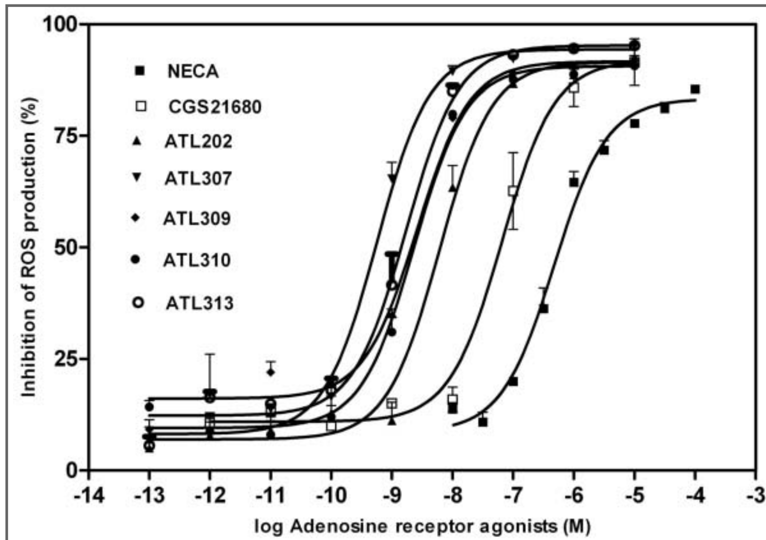

Figure 2—Inhibition of LPS-induced neutrophil ROS production by various adenosine receptor agonists. Each point represents the mean ± SEM for 6 replicates. Results are reported as the percentage of inhibition of ROS production induced by LPS. Similar results were obtained in 3 additional experiments.

Table 2—Mean (95% confidence interval) values of the LPS-induced ROS production for neutrophils obtained from 8 healthy horses after neutrophils were incubated with various analogues of A_{2A} receptor agonists with and without the A_{2A} receptor antagonist ZM241385.

Receptor agonist	IC_{50} (nM)* 0nM ZM241385	100nM ZM241385
NECA	111.0 (46.4–265.7)	4,885.0 (2,104.0–11,340.0)
CGS21680	65.7 (40.9–105.6)	4,222.0 (685.1–26,010.0)
ATL202	13.9 (6.8–28.4)	673.8 (343.5–1,322.0)
ATL307	2.5 (1.9–3.2))	214.5 (82.9–555.0)
ATL309	5.3 (4.1–6.8)	690.5 (575.7–828.3)
ATL310	5.4 (3.3–8.8)	609.6 (381.8–973.4)
ATL313	4.9 (3.6–6.7)	1,130.0 (733.4–1,740.0)

*Values are expressed as mean (95% confidence interval).

To provide a quality-control index, a parallel set of wells was included in each experiment in which ROS production was determined for unstimulated cells incubated with polymyxin B.[cc] If ROS production by those cells was < 90% of ROS production by cells incubated without polymyxin B, we concluded that there was LPS contamination at some point during the experiment and that experiment was excluded from the study.

In preliminary experiments, we did not observe a significant effect of the addition of adenosine deaminase on ROS production. However, adenosine deaminase (1 U/mL) was included in the experiments reported here to counter possible effects of endogenously produced adenosine and minimize variability.

Measurement of total cellular cAMP concentration—Neutrophils (2×10^6 cells/mL) were suspended in RPMI 1640 medium that contained adenosine deaminase (1 U/mL) and 50µM 4-(3-[cyclopentyloxy]-4-methoxyphenyl)-2-pyrrolidinone,[dd] a type IV–specific phosphodiesterase inhibitor. Aliquots (160 µL) of the cell suspensions were placed into duplicate wells of 96-well plates. Various concentrations of adenosine analogues (ATL202, ATL307, ATL309, ATL310, or ATL313) were added in 20 µL of RPMI 1640 medium with or without the A_{2A} receptor antagonist ZM241385 or the A_{2B} receptor antagonist MRS1706. After incubation in a humidified atmosphere of 5% carbon dioxide at 37°C for 20 minutes, reactions were terminated by the addition of 20 µL of stop solution to each well. Concentrations of cAMP were then determined by use of a commercially available EIA kit[ee] performed in accordance with the manufacturer's protocol.

Data analysis—All experiments were repeated 3 times, and the ROS production assay was performed in quadruplicate. Concentration-response data were analyzed by use of nonlinear regression analysis with commercially available software.[ff] The EC_{50} values were expressed as mean and 95% confidence intervals. Best-fit values for 3 variables (log EC_{50} and the ROS values at the top and bottom plateaus of the curves) were compared in a pairwise manner by use of the *F* test to identify significant differences between concentration-response curves generated after incubation with and without each of the receptor antagonists. Significance was defined at values of $P < 0.05$.

Results

Comparative binding affinities of adenosine A_{2A} receptor agonists—Equilibrium competition experiments performed with the selective adenosine A_{2A} receptor antagonist [^{3}H]ZM241385 yielded a ranked order of agonist affinities of ATL307 > ATL309 and ATL310 and ATL313 > ATL202 > CGS21680 > NECA (**Figure 1**; **Table 1**). To determine the selectivity of these compounds for equine adenosine A_1, A_{2A}, and A_3 recep-

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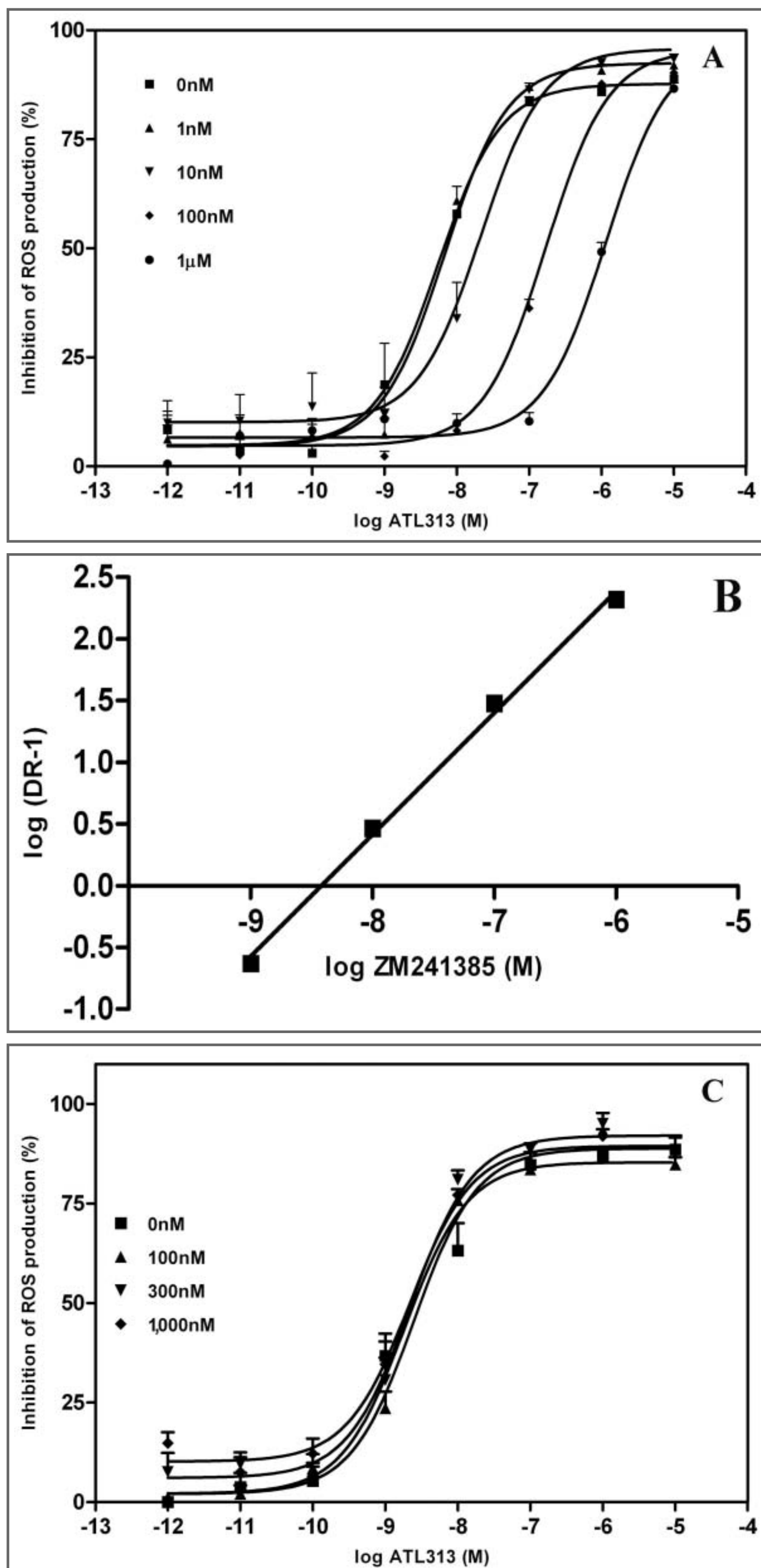

Figure 3—Results of LPS-induced neutrophil ROS production when incubated with ATL313 and various concentrations of ZM241385 (A), a Schild plot of those results (B), and effects of MRS1706 on LPS-induced ROS production (C). For the Schild plot, the negative logarithm of K_D (ie, PA_2) was 8.419. Results in panels A and C represent mean ± SEM of 6 replicates and are reported as the percentage of inhibition of LPS-induced ROS production. Similar results were obtained in 3 additional experiments. DR=EC_{50} with the addition of various concentrations of receptor antagonist)/EC_{50} without the addition of a receptor antagonist.

tors, equilibrium competition experiments were also performed with selective adenosine A_1 and A_3 receptor ligands, [^{3}H]DPCPX and [^{125}I]AB-MECA, respectively. All 5 adenosine analogues were highly selective for equine adenosine A_{2A} receptors, compared with selectivity for equine adenosine A_1 and A_3 receptors, with ATL313 having approximately 100- and 93-fold greater selectivity for adenosine A_{2A} receptors than for A_1 or A_3 receptors, respectively.

Functional effects of adenosine A_{2A} receptor agonists—Experiments evaluating the inhibitory effects of the adenosine A_{2A} receptor agonists on LPS-induced production of ROS by equine neutrophils yielded a ranked order of potency of ATL307 > ATL309 and ATL310 and ATL313 > ATL202 > CGS21680 > NECA (**Figure 2**; **Table 2**). This ranked order was the same as that obtained for the radioligand binding experiments. Inhibition of LPS-induced ROS production was reduced significantly by a selective adenosine A_{2A} receptor antagonist, ZM241385, which suggested that this effect of the adenosine analogues used in the study was predominantly via activation of adenosine A_{2A} receptors.

To more fully evaluate the involvement of various adenosine receptor subtypes in the inhibition of LPS-induced production of ROS, experiments were performed with different consentrations of ZM241385 as well as the selective adenosine A_{2B} receptor antagonist MRS1706. Inhibition of LPS-induced ROS production by ATL313 was reduced in a concentration-dependent manner by the A_{2A} antagonist ZM241385. A Schild plot of these data yielded a ZM241385 K_B value of 3.8nM, which is consistent with the K_D value reported elsewhere[11] for radioligand binding experiments performed with HEK293 cells expressing equine A_{2A} receptors (**Figure 3**). Furthermore, incubation of neutrophils with the adenosine A_{2B} receptor antagonist MRS1706 did not significantly alter the calculated IC_{50} value for inhibition of ROS production by ATL313. Collectively, these findings indicated that ATL313-mediated inhibition of LPS-induced ROS production was via activation of A_{2A} receptors.

cAMP production and activation of adenosine A_{2A} receptors—In other species, activation of adenosine A_{2A} receptors is coupled to G-protein–mediated responses that lead to the accumulation of cAMP. Results obtained from experiments in which equine neutrophils were incubated with the adenosine A_{2A} receptor agonists yielded a ranked potency order of ATL307 and ATL313 > ATL309 and ATL310 >

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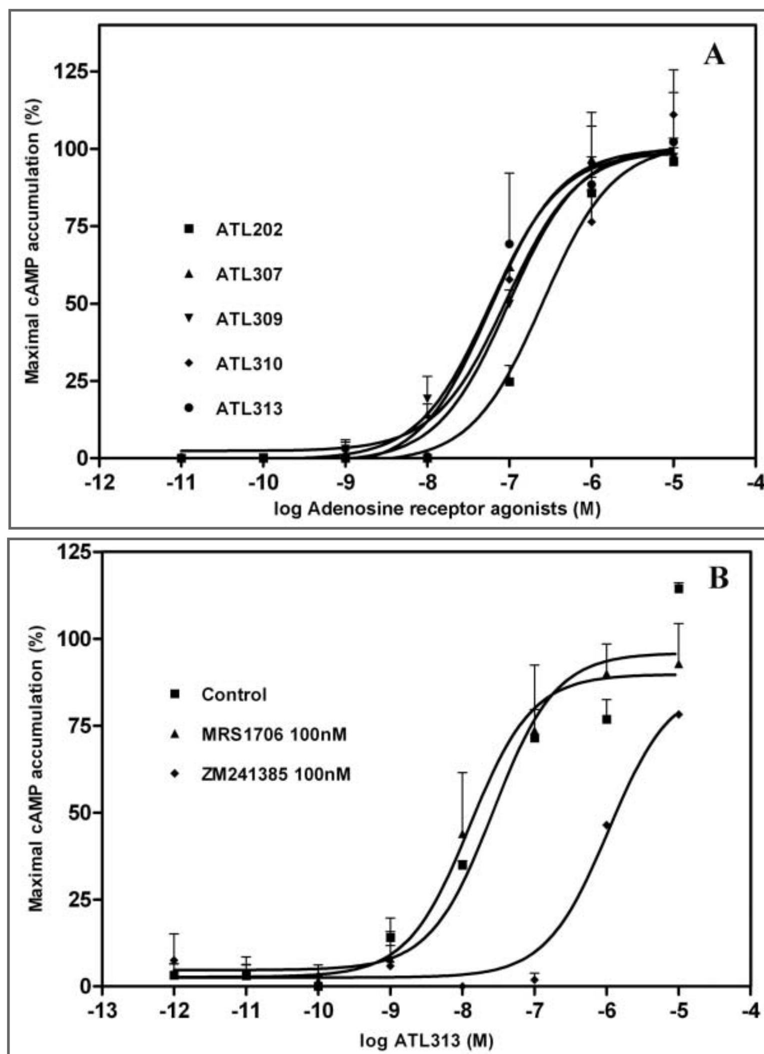

Figure 4—Concentration of cAMP in equine neutrophils induced by 5 adenosine A_{2A} receptor agonists (A) and effects of ZM241385 and MRS1706 on ATL313-induced accumulation of cAMP (B). Each point represents the mean ± SEM of 3 replicates. Values for the control samples were obtained without addition of receptor antagonists.

ATL202 (**Figure 4**). This order was nearly identical to that obtained for both A_{2A} receptor binding and inhibition of ROS production.

To determine the adenosine receptor subtypes responsible for the increase in cAMP production, experiments were performed in which equine neutrophils were incubated with the A_{2A} receptor antagonist ZM241385 or the A_{2B} receptor antagonist MRS1706. Incubation of neutrophils with ZM241385 (100nM) significantly increased the calculated EC_{50} value for ATL313-induced cAMP accumulation (Figure 4). In contrast, incubation of neutrophils with MRS1706 (100nM) did not affect the EC_{50} value for ATL313. These findings indicated that the ATL313-mediated increase in cAMP accumulation was via activation of A_{2A} receptors.

Discussion

Adenosine exerts its biological effects by interacting with the 4 known adenosine receptor subtypes. Results of most of the conducted studies[12-15] indicate that activation of A_{2A} receptors is responsible for the anti-inflammatory effects attributed to adenosine. In 1 study,[9] our laboratory group determined that NECA inhibits ROS production in LPS-stimulated equine neutrophils via activation of adenosine A_{2A} receptors.

In a previous study reported here, we expanded on the other studies conducted by our laboratory group by characterizing the adenosine receptor signature and functional effects of an array of novel synthetic adenosine analogues. Similar to information reported for other species, NECA had equivalent binding affinities for A_1, A_{2A}, and A_3 receptors, whereas CGS21680 had greater affinity for A_{2A} and A_3 receptors than for A_1 receptors. More importantly, however, we found that all 5 adenosine analogues tested in these experiments were highly selective for equine adenosine A_{2A} receptors, relative to their selectivity for equine A_1 and A_3 receptors. Of the adenosine analogues tested here, ATL313 had approximately 100-fold greater affinity for the equine adenosine A_{2A} receptor than for the A_1 or A_3 receptors. The other 4 adenosine analogues tested were less selective for A_{2A} receptors than for A_3 receptors. These analogues vary only in the constituent at the 2 position of adenine, which suggests that modification in this region influences potency for A_{2A} and A_3 receptors.

Although neutrophils express all 4 adenosine receptor subtypes,[16-18] analysis of results of the study reported here suggested that adenosine A_{2A} receptors were primarily responsible for the anti-inflammatory effects of ATL313. For example, the inhibitory effects of ATL313 on LPS-induced ROS production by equine neutrophils were prevented by coincubation of the cells with the adenosine A_{2A} receptor antagonist ZM241385. In addition, a Schild plot of the data yielded a K_B value of 3.8nM for ZM241385. This latter finding is consistent with the reported[11] affinity of ZM241385 for equine adenosine A_{2A} receptors. Furthermore, the effect of ATL313 on LPS-induced ROS production was not affected by coincubation with an antagonist for the adenosine A_{2B} receptor. These findings are consistent with the effects of stimulation of adenosine A_{2A} receptors on oxidative activity of human neutrophils.[8]

Considerable evidence exists[19] to support the concept that agents that increase intracellular concentrations of cAMP also modulate neutrophil function. Analysis of results of the study reported here revealed that incubation of equine neutrophils with adenosine receptor agonists increased cellular cAMP concentration. In addition, the ranked order of potency of these compounds for initiating this response was the same as the ranked order for both binding affinity to equine A_{2A} receptors and inhibition of LPS-induced ROS production. By use of ATL313 (ie, the compound with the highest binding selectivity

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for equine A_{2A} receptors), we found a concentration-dependent increase in cellular cAMP that was prevented by coincubation with ZM241385 but not by incubation with MRS1706. The lack of an effect of MRS1706 indicated that the increase in cellular cAMP concentration did not involve activation of the other adenosine receptor subtype that also activates adenylyl cyclase (ie, the adenosine A_{2B} receptor).[8]

Analysis of results of the study reported here indicated that stimulation of adenosine A_{2A} receptors on equine neutrophils inhibited LPS-induced ROS production by increasing intracellular concentrations of cAMP. These findings suggested that synthetic adenosine receptor agonists that have high selectivity for A_{2A} receptors may prove to be useful as anti-inflammatory agents in horses.

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a. Histopaque 1077, Sigma-Aldrich, St Louis, Mo.
b. Fetal bovine serum, Hyclone, Logan, Utah.
c. [^{3}H]DPCPX, PerkinElmer Life Sciences, Wellesley, Mass.
d. Polytron, Brinkmann Instruments Inc, Westbury, NY.
e. Adenosine deaminase, Roche Diagnostics Corp, Indianapolis, Ind.
f. ATL202, Adenosine Therapeutics LLC, Charlottesville, Va.
g. ATL307, Adenosine Therapeutics LLC, Charlottesville, Va.
h. ATL309, Adenosine Therapeutics LLC, Charlottesville, Va.
i. ATL310, Adenosine Therapeutics LLC, Charlottesville, Va.
j. ATL313, Adenosine Therapeutics LLC, Charlottesville, Va.
k. NECA, Tocris Bioscience, Ellisville, Mo.
l. CGS21680, Tocris Bioscience, Ellisville, Mo.
m. N^6-Cyclopentyladenosine, Sigma-Aldrich, St Louis, Mo.
n. Whatman GF/C filter strips, Whatman Inc, Sanford, Me.
o. Brandell cell harvester, Brandell, Gaithersburg, Md.
p. Beckman LS 6000 counter, Beckman Coulter Inc, Fullerton, Ca.
q. [^{3}H]ZM241385, Tocris Bioscience, Ellisville, Mo.
r. 2-CADO, Sigma-Aldrich, St Louis, Mo.
s. [^{125}I]AB-MECA, PerkinElmer Life Sciences, Wellesley, Mass.
t. IB-MECA, Sigma-Aldrich, St Louis, Mo.
u. PerkinElmer 1470 automatic gamma counter, PerkinElmer Life Sciences, Downers Grove, Ill.
v. *Escherichia coli* O55:B5 LPS, List Biologics, Campbell, Calif.
w. Phorbol myristste acetate, Sigma-Aldrich, St Louis, Mo.
x. ZM241385, Tocris Bioscience, Ellisville, Mo.
y. MRS1706, Tocris Bioscience, Ellisville, Mo.
z. MRS1220, Tocris Bioscience, Ellisville, Mo.
aa. DHR123, Invitrogen-Molecular Probes, Carlsbad, Calif.
bb. Fluoroskan Ascent FL, Thermo Labsystems, GMI Inc, Albertville, Minn.
cc. Polymyxin B, Bedford Laboratories, Bedford, Ohio.
dd. Rolipram, Sigma-Aldrich, St Louis, Mo.
ee. cAMP Biotrak EIA kit, Amersham Biosciences, Piscataway, NJ.
ff. Prism software, version 4.0, GraphPad Software Inc, San Diego, Calif.

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