Research article

Suppression of inflammation by low-dose methotrexate is mediated by adenosine A2A receptor but not A3 receptor activation in thioglycollate-induced peritonitis

M Carmen Montesinos1,2, Avani Desai2 and Bruce N Cronstein2

1Department of Pharmacology, Universidad de Valencia, Burjassot, Valencia, Spain
2Department of Medicine, New York University School of Medicine, New York, USA

Corresponding author: M Carmen Montesinos, m.carmen.montesinos@uv.es

Received: 13 Sep 2005  Revisions requested: 26 Oct 2005  Revisions received: 7 Feb 2006  Accepted: 8 Feb 2006  Published: 6 Mar 2006

Arthritis Research & Therapy 2006, 8:R53 (doi:10.1186/ar1914)

This article is online at: http://arthritis-research.com/content/8/2/R53

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Abstract

Prior studies demonstrate that adenosine, acting at one or more of its receptors, mediates the anti-inflammatory effects of methotrexate in animal models of both acute and chronic inflammation. Both adenosine A2A and A3 receptors contribute to the anti-inflammatory effects of methotrexate treatment in the air pouch model of inflammation, and the regulation of inflammation by these two receptors differs at the cellular level. Because different factors may regulate inflammation at different sites we examined the effect of low-dose weekly methotrexate treatment (0.75 mg/kg/week) in a model of acute peritoneal inflammation in adenosine A2A receptor knockout mice and A3 receptor knockout mice and their wild-type littermates. Following intraperitoneal injection of thioglycollate there was no significant difference in the number or type of leukocytes, tumor necrosis factor alpha (TNF-α) and IL-10 levels that accumulated in the thioglycollate-induced peritoneal exudates in adenosine A2A knockout mice and A3 receptor knockout mice and their wild-type littermates. Following intraperitoneal injection of thioglycollate there was no significant difference in the number or type of leukocytes, tumor necrosis factor alpha (TNF-α) and IL-10 levels that accumulated in the thioglycollate-induced peritoneal exudates in adenosine A2A knockout mice or wild-type control mice. In contrast, there were more leukocytes, TNF-α and IL-10 in the exudates of the adenosine A3 receptor-deficient mice. Low-dose, weekly methotrexate treatment increased the adenosine concentration in the peritoneal exudates of all mice studied, and reduced the leukocyte accumulation in the wild-type mice and A3 receptor knockout mice but not in the A2A receptor knockout mice. Methotrexate reduced exudate levels of TNF-α in the wild-type mice and A3 receptor knockout mice but not the A2A receptor knockout mice. More strikingly, IL-10, a critical regulator of peritoneal inflammation, was increased in the methotrexate-treated wild-type mice and A3 knockout mice but decreased in the A2A knockout mice. Dexamethasone, an agent that suppresses inflammation by a different mechanism, was similarly effective in wild-type mice, A2A mice and A3 knockout mice. These findings provide further evidence that adenosine is a potent regulator of inflammation that mediates the anti-inflammatory effects of methotrexate. Moreover, these data provide strong evidence that the anti-inflammatory effects of methotrexate and adenosine are mediated by different receptors in different inflammatory loci, an observation that may explain why inflammatory diseases of some organs but not of other organs respond to methotrexate therapy.

Introduction

Low-dose weekly methotrexate has become the mainstay treatment of rheumatoid arthritis and psoriasis, and it is the gold standard by which other systemic medications are measured in both disorders [1,2]. Methotrexate has been used to treat other inflammatory diseases including ankylosing spondylitis, multiple sclerosis and inflammatory bowel disease, but its efficacy in the therapy of these conditions is far less impressive [3-7].

An increasing body of evidence indicates that adenosine mediates, at least in part, the anti-inflammatory effects of methotrexate [8-13]. All known adenosine cell surface receptors (A1, A2A, A2B and A3) contribute to the modulation of inflammation, as demonstrated by many in vitro and in vivo pharmacologic studies (reviewed in [14,15]). We have previously demonstrated pharmacologically, using nonselective antagonists, that the anti-inflammatory effect of methotrexate is mediated by more than one subtype of adenosine receptor in the adjuvant arthritis model in the rat [16], and, using mice ren-
dered deficient in A2A or A3 adenosine receptors, we found
that both receptor subtypes are critical for the anti-inflam-
matory effects of methotrexate in the murine air pouch model
of inflammation [17]. Since inflammation at different loci
can be regulated by different cellular mechanisms, we
determined whether the A2A and A3 receptors played similar roles in regu-
lat ing inflammation in the peritoneum.

We examined the pharmacologic mechanism by which meth-
otrexate diminishes inflammation in the thioglycollate-induced
peritoneal inflammation model of acute inflammation in the
mouse. We report here that, similar to the air pouch, meth-
otrexate treatment increases peritoneal exudate adenosine
concentrations in wild-type mice, A2A receptor knockout mice
and A3 receptor knockout mice but, in contrast to the air pouch
model, diminishes leukocyte accumulation only in the perito-
neal exudates of A2A receptor knockout and wild-type mice, not
of A2A knockout mice. Similarly, methotrexate decreased exu-
date tumor necrosis factor alpha (TNF-α) levels and increased
IL-10 levels in wild-type mice and A3 knockout mice, but only
marginally decreased TNF-α levels and significantly decreased IL-10 levels in A2A knockout mice.

Materials and methods
Materials
Thioglycollate medium (FTG) was obtained from Sigma Chem-
ical Co. (St Louis, MO, USA). Methotrexate was purchased
from Immunex (San Juan, PR, USA). All other materials were
the highest quality that could be obtained.

Animals
Mice with a targeted disruption of the gene for the adenosine
A2A and A3 receptor have been described in detail elsewhere
[18,19]. The mice used in these experiments were derived
from four original heterozygous breeding pairs for each mouse
strain. Mice described as wild type were specific for the
related receptor knockout mice, since their background was
different. Confirmation of mouse genotype was performed by
PCR as previously described [17]. Mice were housed in the
New York University animal facility, fed regular mouse chow
and given access to drinking water ad libitum. All procedures
described in the following were reviewed and approved by the
Institutional Animal Care and Use Committee of New York Uni-
versity Medical Center and were carried out under the super-
vision of the facility veterinary staff.

Peritoneal inflammation
Animals were given weekly intraperitoneal injections of either
methotrexate (0.75 mg/kg, freshly reconstituted lyophilized
powder) or vehicle (0.9% saline) for 4 weeks and the experi-
ments were carried out within 3 days of the final dose of meth-
otrexate. Dexamethasone (1.5 mg/kg) was administered by
intraperitoneal injection 1 hour prior to induction of inflamma-
tion in the peritoneum. Thioglycollate peritonitis was induced
by intraperitoneal injection of 0.5 ml sterile solution of thiogy-
collate medium (10% w/v in PBS) [20]. After 4 hours the ani-
mals were sacrificed by CO2 narcosis and their peritoneal
cavities were lavaged with 3 ml cold PBS. The peritoneal area
was massaged before withdrawing the lavage fluid. Exudates
were maintained at 4°C until aliquots were diluted 1:1 with
methylene blue (0.01% w/v in PBS) and cells were counted in
a standard hemocytometer chamber. The concentration of
adenosine and TNF-α in inflammatory exudates was quantified
by HPLC and ELISA, respectively [17]. The IL-10 concentra-
tion in cell-free inflammatory exudates was quantified by ELISA
(R&D Systems, Minneapolis, MN, USA) following the manufac-
turer’s instructions.

Statistical analysis
All statistical analyses were performed by SigmasStat software
(SPSS, Inc., Chicago, IL, USA). Differences between groups
were analyzed by one-way analysis of variance.

Results
Since previous studies carried out in our laboratory showed
that adenosine receptors play a pivotal role in the formation of
the granulation tissue lining the air pouch [21], in a manner
that might alter the inflammatory response, we sought to fur-
ther evaluate the role of adenosine receptors in methotrexate-
mediated suppression of inflammation in tissue that had not
previously undergone injury or disruption. We therefore deter-
mined whether methotrexate inhibits acute leukocyte accumu-
lation in thioglycollate-induced peritoneal inflammation in wild-
type mice, adenosine A2A receptor knockout mice and adeno-
sine A3 receptor knockout mice. Similar numbers of leukocytes
accumulated in peritoneal inflammatory exudates of A2A knock-
out mice and their corresponding wild-type controls (Table 1).
In contrast, there was a significant increase (20%) in the
number of leukocytes that accumulated in peritoneal exudates
of A3 knockout mice as compared with the wild-type controls
(Table 1).

Treatment with methotrexate increased the exudate adenosine
concentration in wild-type mice, A2A knockout mice and A3

Table 1
Leukocyte accumulation in inflammatory exudates

| Mouse group            | Peritoneal exudate (× 10⁶ cells ± SEM) |
|------------------------|---------------------------------------|
| A2A wild type          | 9.3 ± 0.6 (n = 14)                     |
| A2A knockout           | 9.2 ± 0.8 (n = 14)                     |
| A3 wild type           | 10.6 ± 0.5 (n = 19)                    |
| A3 knockout            | 12.5 ± 0.4* (n = 23)                   |

Inflammatory exudates were induced in the peritoneum of knockout
and wild-type mice, as described. After 4 hours the exudates were
collected and the leukocytes quantitated. The wild-type control mice
were derived from the same heterozygous breeding pairs and were
matched for age and sex. There was no difference in the number of
leukocytes accumulating in the exudates of male vs female mice in
either the knockout mice or wild-type mice. *P < 0.005 vs A3 wild-
type mice, Student’s t test.
knockout mice (Table 2) and reduced the leukocyte accumulation in A2A wild-type mice by 30 ± 5% (P < 0.01 vs control, n = 7; Figure 1a), but reduced the leukocyte accumulation in the A2A knockout mice by only 7 ± 5% (P = not significant vs wild-type control, n = 6; Figure 1a). In contrast to the A2A knockout mice, methotrexate was not less effective as an anti-inflammatory agent in A3 receptor knockout mice (23 ± 5% inhibition, P < 0.001 vs A3 knockout control, n = 12; Figure 1b) than in A3 wild-type mice (22 ± 5% inhibition, P < 0.001 vs A3 wild-type control, n = 10; Figure 1b).

To determine whether the diminished anti-inflammatory effect of methotrexate in the A2A knockout mice was specific, we tested the effect of the potent steroidal anti-inflammatory agent dexamethasone in this model. Dexamethasone diminished leukocyte accumulation similarly in A2A wild-type mice, A2A knockout mice, A3 wild-type mice and A3 knockout mice (39 ± 9%, 38 ± 13%, 35 ± 4% and 36 ± 4% inhibition, P < 0.005, P < 0.05, P < 0.001 and P < 0.001 vs control, n = 4, n = 3, n = 9 and n = 9, respectively; Figure 1). Under the conditions studied there was no difference in the type of white cells that accumulated in the peritoneal cavities of either treated or untreated wild-type mice or knockout mice (>90% polymorphonuclear leukocytes).

In general, TNF-α accumulation in peritoneal exudates was much lower than previously reported in other models of inflammation, including carrageenan-induced inflammation in the air pouch and zymosan-induced peritoneal inflammation [17,22]. Similar to leukocyte accumulation, we found comparable levels of the proinflammatory cytokine TNF-α in peritoneal exudates of wild-type mice and A2A knockout mice, but significantly increased accumulation of TNF-α in peritoneal exudates of A3 knockout mice (Table 3). Methotrexate nevertheless inhibited TNF-α accumulation in peritoneal exudates of wild-type mice and A3 knockout mice more markedly than leukocyte accumulation (by 67% and 59%, respectively), and had a modest effect on TNF-α accumulation in peritoneal exudates of A2A knockout mice (Table 3). These findings are consistent with the prior observation that both A2A and A3 receptors modulate TNF-α production [23].

The cytokine IL-10, released by resident peritoneal macrophages, plays a regulatory anti-inflammatory role in the recruitment of leukocytes in murine models of peritoneal inflammation [22,24]. Since adenosine receptor activation modulates the release of IL-10 by different inflammatory cells [25-27] and methotrexate-treated rheumatoid arthritis patients have shown increased serum levels of this cytokine [28,29], we determined whether constitutively or methotrexate-modified IL-10 accumulation in the inflammatory exudate was altered in adenosine receptor-deficient mice. We found that, similar to the leukocyte infiltration and the TNF-α concentration, A3 knockout mice had significantly higher IL-10 levels in their peritoneal inflammatory exudates when compared with wild-type mice and A2A knockout mice (Table 4). As expected, treatment with methotrexate stimulated IL-10 accumulation in the exudate by 56% in wild-type mice, but significantly decreased IL-10 levels in exudates of A2A-deficient mice. Although methotrexate increased IL-10 levels in the exudates of methotrexate-treated A3 knockout mice, this increase did not achieve statistical significance. Due to the high variability in the IL-10 levels we found in our experiments, it would
Adenosine is a ubiquitous autacoid present in all tissues and body fluids. Under basal conditions, the extracellular adenosine concentration is rather constant (30–300 nM), but its concentration can increase dramatically to 10 µM or even higher, as a result of ATP catabolism, when there is an imbalance between energy use and energy supply, such as in oxygen depletion, or when there is cell necrosis as a consequence of mechanical or inflammatory injury. Adenosine acts via four distinct adenosine receptor subtypes — the adenosine A1, A2A, A2B, and A3 receptors — that are all members of the large family of seven-transmembrane spanning, heterotrimeric G protein-associated receptors, coupling to classical second messenger pathways such as modulation of cAMP production or the phospholipase C pathway. In addition, they couple to mitogen-activated protein kinases, which could give them a role in cell growth, survival, death and differentiation (reviewed in [30]).

Adenosine is a potent endogenous anti-inflammatory agent, and all four adenosine receptor subtypes participate in this effect (reviewed in [14]). All cell subtypes involved in the inflammatory process differentially express functional adenosine receptors. It is well documented that microvascular endothelial cells, major players conducting the movement of leukocytes between tissue compartments, express adenosine A2A and A2B receptors [31,32]. Pharmacological and molecular approaches have shown that neutrophils, monocytes and macrophages express all four adenosine receptor subtypes. Although adenosine A1 receptor activation has been associated with proinflammatory properties in inflammatory cell types [33-35], the anti-inflammatory effect of selective A1 agonists acting in the central nervous system has been demonstrated in vivo [36-38]. Adenosine A2A receptor activation inhibits neutrophil and monocyte oxidative burst, degranulation and release of cytokines and chemokines [39-41]. Activation of A2B receptors selectively inhibits collagenase mRNA accumulation in synovial fibroblasts and mediates neutrophil-stimulated intestinal epithelial leakiness [42,43]. Adenosine A3 receptors have also been described as anti-inflammatory in human blood leukocytes and in murine models of inflammation [19,44-46].

The results of these reported studies confirm the anti-inflammatory effects of adenosine acting at A3 receptors because animals deficient in this receptor show an exacerbated response to the inflammatory insult. Moreover, we found that more polymorphonuclear leukocytes accumulate in the peritoneal exudates of A3 knockout mice in comparison with their wild-type littermates, consistent with the hypothesis that this receptor plays a greater role as an endogenous regulator of inflammation. Our data are in agreement with prior reports showing that adenosine A3 receptor agonists suppress the expression and production of macrophage inflammatory protein 1α, a chemokine that enhances neutrophil recruitment into inflammatory sites [45], and suppress the production of TNF-α by lipopolysaccharide-stimulated macrophages [19]. Adenosine A3 receptor agonists thus ameliorate joint inflammation in several murine models of arthritis [45,46].

Monocytes and macrophages synthesize and release into their environment a variety of cytokines and other proteins that play a central role in the development of acute and chronic inflammation. It has been firmly established that adenosine modulates the production of inflammatory cytokines, including TNF-α, IL-10, and IL-12 [23,25-27,47]. In addition to the regulatory effect of adenosine in cytokine secretion, we have further established that Th1 proinflammatory cytokine IL-1 and TNF-α treatment increases message and protein expression of A2A and A2B receptors by both microvascular endothelial cells and THP-1 monocytoid cells. IFN-γ treatment also increased the expression of A2B receptors, but decreased the expression of A2A receptors [25,32,48]. It is therefore probably at inflamed sites, where proinflammatory cytokines such as IL-1 and TNF-α are abundantly secreted, mostly by monocytes/macro-

### Table 2

|                      | Adenosine concentration in peritoneal exudates                                                                 |
|----------------------|---------------------------------------------------------------------------------------------------------------|
|                      | Wild-type mice (nM ± SEM) | A2A knockout mice (nM ± SEM) | A3 knockout mice (nM ± SEM) |
| Control              | 118 ± 6 (n = 19)           | 110 ± 6 (n = 14)            | 133 ± 6 (n = 12)            |
| Methotrexate (0.75 mg/kg/week) | 178 ± 12* (n = 15)         | 162 ± 7** (n = 7)          | 214 ± 10† (n = 9)           |

Wild-type mice, A2A receptor knockout mice or A3 receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected and the adenosine levels quantitated. Wild-type data are a combination from both mouse strains. *P < 0.0001 vs wild-type control mice, Student’s t test; **P < 0.0001 vs A2A knockout control mice, Student’s t test; † P < 0.0001 vs A3 knockout control mice, Student’s t test.
phages, that the subsequent upregulation of A$_{2A}$ and A$_{2B}$ receptors on endothelial cells and other inflammatory cells along with endogenous adenosine release constitutes a feedback loop to suppress further inflammation. The demonstration that adenosine receptors expressed in microvascular endothelial cells are modified during inflammation suggests an important role for these receptors in the increased angiogenesis and vascular permeability that characterize both acute and chronic inflammatory responses. Moreover, in previous studies, activation of both A$_{2A}$ and A$_{2B}$ receptors on either endothelial cells or macrophages has been reported to enhance the expression of vascular endothelial growth factor and to promote angiogenesis [21,49-51].

Methotrexate is an effective disease-modifying drug widely used in low doses at weekly intervals for the control of rheumatoid arthritis and psoriasis with a relatively safe profile compared with other therapies [1,2]. Since folate administration prevents many of the toxicities of methotrexate without affecting the therapeutic effects [52], there is little support for the hypothesis that inhibition of folate-dependent pathways (for example, cellular proliferation) is responsible for the therapeutic effects of the agent. Following administration, methotrexate is taken up by cells and undergoes polyglutamation, resulting in the intracellular accumulation of the long-lived polyglutamates of methotrexate. These metabolites, in addition to inhibiting folate metabolism, directly inhibit 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, resulting in an intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide, which is an intermediate metabolite in the de novo pathway of purine synthesis, and has been associated with increases in extracellular adenosine [9,13,53]. There is now increasing evidence that accumulation of adenosine at sites of inflammation plays a pivotal role in the anti-inflammatory effect of methotrexate. In vitro studies showed that methotrexate produces adenosine release by human fibroblasts and endothelial cells [53], and in vivo studies showed that methotrexate is ineffective in the presence of antagonists of adenosine or adenosine deaminase (the enzyme responsible for the deamination of adenosine to inosine) in animal models of acute and chronic inflammation [8]. Moreover, adenosine receptor antagonists and deletion of adenosine receptors eliminates the anti-inflammatory response to methotrexate in animal models of acute and chronic inflammation and patients with rheumatoid arthritis [13,16,54].

Although the contribution of adenosine to the mechanism of action of methotrexate is well accepted, it is still unclear which adenosine receptors participate in the effect of methotrexate. Results of early studies, using pharmacological tools, suggested that the adenosine A$_{2A}$ receptor was the main receptor subtype involved in suppressing inflammation [8]. In the model of adjuvant arthritis in rats, however, we found that only nonselective adenosine receptor antagonists could block the protective effect of methotrexate whereas selective antagonists of individual adenosine receptors did not alter the response to methotrexate [16], consistent with involvement of multiple adenosine receptors. Using knockout animals we observed that both A$_{2A}$ and A$_{3}$ adenosine receptors are involved in methotrexate-mediated suppression of air pouch inflammation [17] but, as reported here, only A$_{2A}$ receptors are involved in methotrexate-mediated suppression of peritoneal inflammation. Methotrexate exerted similar anti-inflammatory effects in wild-

| Table 3 |

| Tumor necrosis factor alpha concentration in peritoneal exudates |
|-----------------------------------------------|
| Wild-type mice (pg/ml ± SEM) | A$_{2A}$ knockout mice (pg/ml ± SEM) | A$_{3}$ knockout mice (pg/ml ± SEM) |
| Control | 42 ± 7 (n = 14) | 36 ± 8 (n = 10) | 75 ± 18* (n = 6) |
| Methotrexate (0.75 mg/kg/week) | 14 ± 4** (n = 15) | 25 ± 7 (n = 9) | 31 ± 11* (n = 8) |

Wild-type mice, A$_{2A}$ receptor knockout mice or A$_{3}$ receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected, centrifuged at 100 × g and frozen. Tumor necrosis factor alpha levels were later quantitated by ELISA. Wild-type data are a combination from both mouse strains. **P<0.001 vs wild-type control mice, Student’s t test; *P<0.05 vs wild-type control mice, Student’s t test; †P<0.05 vs A$_{2A}$ knockout control mice, Student’s t test.

| Table 4 |

| IL-10 concentration in peritoneal exudates |
|-----------------------------------------------|
| Wild-type mice (pg/ml ± SEM) | A$_{2A}$ knockout mice (pg/ml ± SEM) | A$_{3}$ knockout mice (pg/ml ± SEM) |
| Control | 62 ± 7 (n = 24) | 73 ± 9 (n = 12) | 115 ± 14** (n = 15) |
| Methotrexate (0.75 mg/kg/week) | 97 ± 18* (n = 12) | 41 ± 6† (n = 7) | 150 ± 31 (n = 7) |

Wild-type mice, A$_{2A}$ receptor knockout mice or A$_{3}$ receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected, centrifuged at 100 × g and frozen. IL-10 levels were later quantitated by ELISA. Wild-type data are a combination from both mouse strains. **P<0.001 vs wild-type control mice, Student’s t test; *P<0.05 vs wild-type control mice, Student’s t test; †P<0.05 vs A$_{2A}$ knockout control mice, Student’s t test.
type mice and A3 knockout mice, but failed to inhibit leukocyte and TNF-α accumulation in A3 knockout mice. Moreover, methotrexate treatment augmented the accumulation of IL-10, a known anti-inflammatory cytokine, in wild-type mice and A3 knockout mice, but actually decreased IL-10 levels in A2A knockout mice. We do not have a clear explanation for this other than to note it is probable that in the MTX-treated A2A knockout mice there is an imbalance in A1 adenosine receptor function in the absence of A2A, consistent with the previous observation of Hasko and colleagues that an A1 adenosine receptor agonist reduces IL-10 release by lipopolysaccharide-stimulated RAW macrophages [27]. IL-10 is therefore, as previously reported, a critical regulator of peritoneal inflammation stimulated RAW macrophages [27].

We infer from these results and previous reports that the involvement of different adenosine receptor subtypes depends upon the site of and stimulus for inflammation. We therefore conclude it is probable that the requirement for activation of multiple adenosine receptor subtypes in the pharmacologic control of chronic inflammation results from the involvement of different types of inflammatory cells and disease-specific differences in the inflammatory environment.

Conclusion

The studies reported here provide strong evidence that adenosine mediates the anti-inflammatory effects of methotrexate at doses relevant to those used to treat inflammatory arthritis. These results indicate that agents which interact with adenosine A2A receptors directly or promote adenosine release at inflamed sites may be useful for the treatment of inflammatory conditions, whereas occupancy of other adenosine receptors may be involved in suppression of inflammation in a site-specific fashion.

Competing interests

MCM and AD declare that they have no competing interests. BNC declares the following competing interests: consultant – King Pharmaceuticals, Tap Pharmaceuticals, Can-Fite Pharmaceuticals, Bristol-Myers Squibb, Regeneron, Centocor; grant support – NIH, King Pharmaceuticals; honoraria – Merck, Amgen; intellectual property – adenosine A2A receptors for wound healing, adenosine A2A receptor antagonists for fibrosis (both licensed to King Pharmaceuticals).

Authors’ contributions

MCM designed and coordinated the study, carried out the animal experimental procedures, performed the statistical analysis and drafted the manuscript. AD carried out the adenosine HPLC determinations and the immunoassays. BNC conceived of the study, participated in its design and corrected the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by grants to BNC from the National Institutes of Health (AR41911, GM56268, AA13336), King Pharmaceuticals, the General Clinical Research Center (M01RR00096) and by the Kaplan Cancer Center. MCM is beneficiary of the Ramón y Cajal program from the Spanish Government (Ministerio de Educación y Ciencia) and of a grant from the Valencian Government (Conselleria d’Empresa, Universitat i Ciència)(GV05/031).

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