Renal Effects of the Novel Selective Adenosine A\textsubscript{1} Receptor Blocker SLV329 in Experimental Liver Cirrhosis in Rats

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Abstract

Liver cirrhosis is often complicated by an impaired renal excretion of water and sodium. Diuretics tend to further deteriorate renal function. It is unknown whether chronic selective adenosine A\textsubscript{1} receptor blockade, via inhibition of the hepatorenal reflex and the tubuloglomerular feedback, might exert diuretic and natriuretic effects without a reduction of the glomerular filtration rate. In healthy animals intravenous treatment with the novel A\textsubscript{1} receptor antagonist SLV329 resulted in a strong dose-dependent diuretic (up to 3.4-fold) and natriuretic (up to 13.5-fold) effect without affecting creatinine clearance. Male Wistar rats with thioacetamide-induced liver cirrhosis received SLV329, vehicle or furosemide for 12 weeks. The creatinine clearance of cirrhotic animals decreased significantly (\textminus36.5\%, \textit{p}<0.05), especially in those receiving furosemide (\textminus41.9\%, \textit{p}<0.01). SLV329 was able to prevent this decline of creatinine clearance. Mortality was significantly lower in cirrhotic animals treated with SLV329 in comparison to animals treated with furosemide (17\% vs. 54\%, \textit{p}<0.05). SLV329 did not relevantly influence the degree of liver fibrosis, kidney histology or expression of hepatic or renal adenosine receptors. In conclusion, chronic treatment with SLV329 prevented the decrease of creatinine clearance in a rat model of liver cirrhosis. Further studies will have to establish whether adenosine A\textsubscript{1} receptor antagonists are clinically beneficial at different stages of liver cirrhosis.

Introduction

Liver cirrhosis is often complicated by an impaired renal capacity of maintaining water and sodium balance. Splanic arterial vasodilatation due to an increased release of endogenous vasodilators leads to compensatory activation of the endogenous vasoconstrictor systems: the sympathetic nervous system, the renin-angiotensin system and the non-osmotic release of vasopressin [1]. This causes renal sodium/water retention and renal vasoconstriction eventually leading to the hepatorenal syndrome. In many patients, water retention can be controlled by sodium restriction and diuretics. However, diuretic therapy often entails deterioration of renal function [1]. There is an urgent clinical need for alternative pharmacological approaches.

The adenosine system with its four receptors (A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B} and A\textsubscript{3}) is involved in several key functions of both liver and kidneys [2]. Decreasing the portal flow, as it is the case in cirrhosis, results in an activation of the hepatic adenosine system [3]. Adenosine, via A\textsubscript{1} receptors (A\textsubscript{1R}), serves as a mediator for triggering the hepatorenal reflex leading to renal water and sodium retention [4,5]. However, the exact localization of adenosine receptors within the liver remains unclear. In the kidney A\textsubscript{1R} are highly expressed in the preglomerular microcirculation, but also on the proximal tubules and other renal structures [6]. Short-term infusion of selective A\textsubscript{1}R antagonists inhibits the tubuloglomerular feedback and causes diuresis and natriuresis [7,8]. Blockade of hepatic and renal A\textsubscript{1}R could therefore provide a new therapeutic option in conditions with sodium and water retention such as liver cirrhosis [9]. Thioacetamide has been widely used to induce chronic liver injury in animal models since it mimics the human disease closely. Rats treated with thioacetamide present with typical cirrhotic liver...
damage and an impaired ability of excreting sodium and water [10].

The first part of the present study evaluated the receptor binding affinity of the novel adenosine A1R antagonist SLV329 - a pyrimidine derivative - and confirmed its in vivo action on diuresis and natriuresis in healthy animals. As main hypothesis of this study, we tested whether SLV329 might exert diuretic effects without impairing renal function in animals with thioacetamide-induced liver cirrhosis.

Results

In vitro selectivity profile of SLV329

In receptor binding experiments using cloned human receptors SLV329 behaved as a potent (pKᵢ 9.2) and selective A1R ligand. The affinity of SLV329 for the other adenosine receptors was at least 100-fold lower (Table 1). The receptor-binding affinities and enzyme inhibitory properties of SLV329 were evaluated in a series of 94 receptors and 6 phosphodiesterases including adenosine transporters and a wide range of adrenergic, muscarinic, nicotinic, dopaminergic, serotonergic, histaminergic, glutamatergic, opioi

Effects of SLV329 on renal function in healthy rats

Five different intravenous doses of SLV329 were tested in healthy anesthetized rats. Table 2 shows that SLV329 treatment resulted in a marked dose-dependent increase of diuresis by up to 3.4-fold and sodium excretion by up to 13.5-fold compared to the time-matched vehicle control group (both p<0.0001). The half-maximally effective concentration (EC₅₀) value of SLV329 is 3.2 µg/l.

Effects of SLV329 on renal function in thioacetamide-induced liver cirrhosis

Descriptive data, results from histological evaluation and Western blots are summarized in Table 3. Representative immunoblots for hepatic and renal adenosine receptors are shown in Figure 1. The results of plasma and urine analyses are shown in Table 4.

Table 1. Receptor binding affinities and enzyme inhibitory properties of SLV329.

| Assay                  | Cell/tissue | Ligand   | SLV329 Affinity |
|------------------------|-------------|----------|-----------------|
| Adenosine A₁           | CHO cells   | ³H-DPCPX | 9.2±0.2         |
| Adenosine A₂₃A         | HEK293 cells| ³H-CG521680| 7.2±0.2        |
| Adenosine A₃           | HEK293 cells| ³H-AB-MECA| 6.9±0.1         |
| Adenosine A₆₈         | HEK293 cells| ³H-DPCPX | 6.3±0.1         |
| Phosphodiesterase 4 rolipram binding | Total brain | ³H-Rolipram | 6.1±0.2         |
| Phosphodiesterase 4 enzyme | U-937 cells | ³H-cAMP | 5.4±0.1         |
| Phosphodiesterase 6 enzyme | Retina | ³H-cAMP | 4.3±0.1         |

Only significant affinities and enzyme inhibition of SLV329 are shown. Cells and tissues used to obtain receptors/enseymes and radioactive ligands of the respective assays are listed. Cells and tissues were provided by Cerep (Celle (Evreux), France).

Results are expressed as pKᵢ for radioligand affinity assays, and as pIC₅₀ for enzyme inhibition. Mean ± standard deviation of at least 3 determinations. doi:10.1371/journal.pone.0017891.t001
**Table 2. Effects of different doses of SLV329 in anesthetized rats.**

| SLV329 bolus (μg/kg) | Vehicle | 18 | 54 | 180 | 540 | 945 |
|----------------------|---------|----|----|-----|-----|-----|
| **Plasma SLV329 (g/l)** |         |    |    |     |     |     |
| infusion (μg/kg·min) | Vehicle | 0.37 | 1.1 | 3.7 | 11 | 19.3 |
| Diuresis (fold vs. vehicle) | 1±0.1 | 1.4±0.1* | 2.5±0.3*** | 2.3±0.3*** | 3.4±0.4**** | 2.8±0.4**** |
| Natriuresis (fold vs. vehicle) | 1±0.2 | 5.0±0.8*** | 7.6±0.9**** | 7.9±1.3**** | 13.5±1.3**** | 8.3±1.4**** |
| Natriuresis (μg/kg/h) | 65±16 | 324±52*** | 497±62**** | 518±84**** | 882±87**** | 544±94**** |
| Kaliuresis (fold vs. vehicle) | 1±0.1 | 1.6±0.2 | 1.4±0.1 | 1.7±1.0** | 1.3±0.1 | 1.6±0.2 |
| Kaliuresis (μg/kg/h) | 106±13 | 167±21 | 146±13 | 175±16** | 136±12 | 170±22 |
| Creatinine clearance (ml/min) | 1.9±0.3 | 3.2±1.1 | 2.0±0.3 | 1.6±0.2 | 2.6±0.8 | 2.4±0.7 |
| Adenosine excretion (nmol/h) | 2.4±0.7 | 3.1±0.4* | 4.9±0.8** | 5.9±1.5* | 10.2±2.3**** | 9.8±1.9*** |
| Body weight (g) | 363±3 | 360±2 | 356±3 | 362±2 | 364±2 | 357±2 |
| Systolic blood pressure (mmHg) | 107±6 | 108±2 | 104±3 | 110±5 | 113±7 | 116±6 |
| Heart rate (beats/min) | 313±19 | 317±23 | 328±21 | 350±10 | 294±23 | 301±30 |

A bolus of SLV329 followed by an intravenous infusion over 3 hours produces a strong dose-dependent stimulation of renal water, sodium and adenosine excretion without any effect on creatinine clearance. Diuresis and electrolyte excretion is expressed as fold stimulation vs. time-matched vehicle controls.

Data are means ± standard error of the mean.

n = 9-13 animals per dose.

*p < 0.05,

**p < 0.01,

***p < 0.001,

****p < 0.0001 vs. time-matched vehicle controls.

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Intravenous treatment of healthy rats with SLV329 resulted in a strong dose-dependent diuretic and natriuretic effect, whereas the effect on kaliuresis was relatively small and the creatinine clearance remained unchanged. This is in line with effects that have been reported in a variety of rat strains including Wistar rats [11–13].

The ability of diuretics to prevent sodium reabsorption results in an increased delivery of electrolytes to the distal tubule. This leads, in turn, to an augmented release of adenosine, which may activate A_1R in afferent arterioles [14]. By blocking those receptors, A_1R antagonists cause an uncoupling of the tubuloglomerular feedback which may at least partly explain the elevated concentrations of adenosine measured in the urine of animals treated with SLV329 [15,16]. However, urinary excretion of paracrine mediators do not necessarily reflect their local tissue concentrations. Alternatively, blockade of A_1R might result in elevated intracellular cyclic adenosine monophosphate levels and release in the kidney, which will eventually lead to increased extracellular adenosine concentrations due to cyclic adenosine monophosphate degradation [17–19]. However, in spite of the observed increase in urinary adenosine excretion, SLV329 did not decrease the rate of creatinine clearance, even at the highest dose.

Previous studies demonstrated that a single application of an A_1R antagonist causes an increase of renal sodium and water excretion in animals and patients with liver cirrhosis without affecting the glomerular filtration rate [20,21]. As a next step towards a possible clinical application, the present study investigated for the first time the effects of a chronic application of a selective A_1R antagonist on kidney function and mortality starting at an early stage of liver cirrhosis. This proof-of-concept experiment included a low-dose furosemide-treated group because loop diuretics are often applied in cirrhotic patients and tend to deteriorate renal function. However, monotherapy with a loop diuretic is of course not the typical clinical situation at an early stage of liver cirrhosis without severe water retention. In the present study the creatinine clearance, used as a surrogate for the glomerular filtration rate, was significantly reduced in cirrhotic animals, especially in those receiving furosemide. In contrast, the A_1R antagonist SLV329 was able to prevent this decline of creatinine clearance. The reduction of mortality in cirrhotic animals treated with SLV329 in comparison to vehicle treatment was not statistically significant. However, mortality was significantly lower in cirrhotic animals treated with SLV329 in comparison to animals treated with furosemide (17% vs. 54%). It is a limitation of this study that creatinine clearance was used instead of inulin clearance. Inulin clearance was not used because mortality would have increased further due to additional anesthesia. Creatinine clearance is influenced by muscle mass, liver function and tubular secretion of creatinine. However, there were no significant differences of neither body weight nor liver function between cirrhotic animals with and without SLV329 treatment. Tubular secretion of creatinine increases with declining glomerular filtration rate. Thus, the glomerular filtration rate of untreated cirrhotic animals and those treated with furosemide will be even lower than the creatinine clearance suggests, making the difference to the group treated with SLV329 even larger.

Mean SLV329 plasma concentrations of 49–54 μg/l were actually reached by chronic application in the cirrhosis model. This plasma concentration was able to cause strong short-term effects on diuresis and saluresis when administered intravenously as shown in Table 2. A steady-state is usually reached in the course of long-term diuretic therapy by means of compensatory mechanisms of tubular reabsorption [22]. This explains why the 24-hour urine volume and electrolyte excretion in week 16 is not different between the groups of the cirrhosis model. However, when looking at mean water intake over 8 weeks, as a putative surrogate of diuresis, the effects of furosemide and SLV329 can be detected. It would have been interesting to evaluate fractional sodium excretion and free water clearance. This was not done due to limited plasma quantities.

SLV329 does not affect the expression of hepatic or renal adenosine receptors in cirrhotic animals. The expression of A_2R

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**Adenosine Receptor Blockade in Liver Cirrhosis**
seems to be reduced in cirrhotic animals, independent of the treatment group. As yet, there are no reports on the expression of A1R in cirrhotic liver tissue. However, it is known, that hepatic A1R play an active role in the pathogenesis of hepatic fibrosis [23]. It is a limitation of this study that adenosine receptor expression was evaluated only in liver and kidney homogenates by Western blot. Immunohistochemistry or radioligand binding experiments might give further detailed information.

The beneficial effects of SLV329 in the cirrhosis model cannot be explained by morphological effects since SLV329 did not relevantly influence the degree of liver fibrosis, kidney histology or expression of hepatic or renal adenosine receptors. Animal studies suggest that liver cirrhosis activates the hepatorenal reflex via A1R, leading to renal water and sodium retention [24,25]. Animal and human studies suggest that a resetting of the tubuloglomerular feedback contributes to the pathophysiology of kidney impairment in liver cirrhosis [26]. Thus, inhibition of both the hepatorenal reflex and the tubuloglomerular feedback might explain the higher rate of creatinine clearance in the animals treated with SLV329. In addition to the effect on creatinine clearance, yet unknown effects of SLV329 might contribute to the reduction of mortality. As an addition to the present study it would be interesting to study liver cirrhosis in the established murine A1R knockout model.

The ability of A1R antagonists to induce diuresis and natriuresis while not compromising glomerular filtration rate has become an attractive therapeutic option for the treatment of other fluid retention disorders, e.g. in kidney disease and heart failure, especially in conditions associated with diuretic resistance [27]. The preexisting experience with this class of drugs for other indications (including a large phase 3 trial) might facilitate the future translation of the results of this study to clinical application [28].

In conclusion, this study described some pharmacodynamic characteristics of the novel adenosine A1R-specific antagonist SLV329 and demonstrated its long-term safety and efficacy in an animal model of liver cirrhosis. Chronic SLV329 treatment starting at an early stage of liver cirrhosis prevented the decrease of creatinine clearance. Further studies will have to evaluate, whether SLV329 or other A1R antagonists are clinically beneficial at different stages of liver cirrhosis, either as an add-on to aldosterone antagonists or in combination with loop diuretics.

### Materials and Methods

#### Receptor binding and enzyme assays

Receptor binding affinities as well as enzyme inhibitory properties of the new compound SLV329 were evaluated in a
Figure 1. Representative immunoblots for hepatic and renal adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors. Beta-actin was used for normalization before statistical analysis. Con, control; Cir, liver cirrhosis; Fur, furosemide; SLV, SLV329; kDa, kilodalton.
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Table 4. Effects of treatment with furosemide and SLV329 on plasma and urine parameters in rats with and without liver cirrhosis.

| Group                  | Con       | Con+Fur  | Con+SLV  | Cir       | Cir+Fur  | Cir+SLV  |
|------------------------|-----------|----------|----------|-----------|----------|----------|
| ALT week 8 (U/l)       | 26±3      | 36±5     | 29±4     | 41±6      | 27±3*    | 31±4     |
| ALT week 16 (U/l)      | 55±4      | 48±2     | 47±4     | 73±9      | 66±11    | 53±7     |
| Bilirubin week 8 (µmol/l) | 1.3±0.1   | 1.5±0.3  | 1.4±0.2  | 7.3±0.7*** | 6.0±0.8*** | 5.7±0.9*** |
| Bilirubin week 16 (µmol/l) | 1.2±0.3   | 1.5±0.4  | 1.3±0.3  | 16.1±1.8*** | 22±2.9*** | 12±2.0***    |
| Albumin week 8 (g/l)   | 31±0.4    | 32±0.4   | 31±0.2*  | 31±0.5    | 32±0.5   | 31±0.4   |
| Albumin week 16 (g/l)  | 29±0.5    | 29±0.7   | 28±0.3*  | 26±0.6**  | 25±1.0*  | 26±0.5*** |
| Creatinine week 8 (µmol/l) | 52±2      | 54±3     | 54±3     | 55±2      | 52±2     | 49±1*    |
| Creatinine week 16 (µmol/l) | 60±1      | 60±2     | 55.1±1.0*| 51±2*     | 47±2**   | 45±2*    |
| Urine volume week 8 (ml/d) | 51±6      | 59±4     | 52±8     | 58±3      | 61±3     | 65±5     |
| Urine volume week 16 (ml/d) | 71±8      | 75±3     | 67±9     | 67±8      | 58±6     | 69±7     |
| Na<sup>+</sup> excretion week 8 (mmol/d) | 3.2±0.5   | 3.1±0.2  | 2.9±0.5  | 3.6±0.5   | 3.7±0.4  | 2.9±0.3  |
| Na<sup>+</sup> excretion week 16 (mmol/d) | 3.7±0.6   | 5.5±0.5  | 4.4±0.6  | 4.7±0.6   | 3.8±0.9  | 4.1±0.3  |
| K<sup>+</sup> excretion week 8 (mmol/d) | 4.4±0.6   | 5.0±0.5  | 4.3±0.5  | 4.4±0.5   | 4.8±0.4  | 4.3±0.4  |
| K<sup>+</sup> excretion week 16 (mmol/d) | 7.6±0.6   | 7.7±0.8  | 6.7±0.7  | 6.2±0.6   | 5.0±0.7  | 7.0±0.4  |

Con, control; Cir, liver cirrhosis; Fur, furosemide; SLV, SLV329.

In week 0, there were no significant differences between the groups, except for plasma creatinine (Con+Fur 46±6 vs. Cir+Fur 53±5 µmol/l; p<0.05) and urine volume (Con+Fur 70±16 vs. Cir+Fur 58±6 ml/d; p<0.05).

Data are means ± standard error of the mean. n=8–14 per group.

*p<0.05,
**p<0.01,
***p<0.001 vs. Con group with same treatment,
*+p<0.05,
**+p<0.01 vs. same group without Fur or SLV treatment,
*+p<0.05 vs. same group with Fur treatment.
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series of 94 receptors and 6 phosphodiesterases as described previously [29]. All cells and tissues needed for the assays were provided by Cerep (Celle l’Evescault, France).

Receptor binding assays were conducted as follows: after incubation of SLV329 with a receptor preparation and its radioactive ligand, the receptor preparations were rapidly filtered under vacuum through glass fiber filters, the filters were washed extensively with an ice-cold buffer using a harvester. Bound radioactivity was measured by scintillation counting using a liquid scintillation cocktail. Enzyme assays were carried out as follows: after incubation of SLV329 with an enzyme preparation and its radioactive substrate, radioactivity of the enzyme product was measured by scintillation counting using a liquid scintillation cocktail. Testing was done at a 3-log concentration range around a predetermined half-maximally inhibitory concentration (IC_{50}) for the respective assay. The highest concentration tested for primes was 10 μM in receptor binding and 100 μM for enzyme assays. If no significant receptor binding or enzyme inhibition was detected at those concentrations SLV329 was considered to be inactive. Results were calculated as percentage of control values (enzyme assays) or for receptor binding assays as percentage of total ligand binding and that of nonspecific binding per concentration of SLV329. From the concentration-displacement curves IC_{50} values were determined by nonlinear regression analysis using Hill equation curve fitting. The inhibition constants (K_i) were calculated from the Cheng-Prusoff equation K_i = IC_{50}/(1+L/K_d), where L is the concentration of radioligand in the assay and K_d the affinity of the radioligand for the receptor. Results were expressed as mean pKi values ± standard deviation (SD) of at least three separate experiments.

Effects of SLV329 on renal function in healthy rats

All animal experiments of this study were conducted in strict accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS123) and the German law on animal welfare and all efforts were made to minimize suffering. The study protocol was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Bezirksregierung Hannover, approval number 509.6.42502-04/ 875).

Male Sprague-Dawley Crl:CD(SD)BR rats (150–170 g) were fasted overnight. The rats were anesthetized with 80 mg/kg thiobutabarbitral intraperitoneally; additional doses of 40 mg/kg were given 2.5 h and 5 hours later. Catheters were placed in one jugular vein (for SLV329 or vehicle administration), one carotid artery (for blood sampling and blood pressure measurements), and the bladder. The rats were kept on a heated table to maintain body temperature at 37°C. After an equilibration period of 30 min, urine was sampled for a period of 3 h, then the animals received vehicle or SLV329 as follows: a slowly applied (30–60 s) loading bolus of 18, 54, 180, 540, and 945 μg/kg SLV329 (in 0.45 ml/kg) followed by a continuous intravenous infusion at a rate of 0.37, 1.1, 3.7, 11, and 19.3 μg/kg per minute (in 9.3 ml/kg per minute) for three hours. Urine and blood samples were collected 2 min before the SLV329 bolus and after three hours of SLV329 infusion. 5-sulfosalicylic acid was added to the urine aliquots for adenosine measurements at a final concentration of 8 g/l before freezing. Electrolytes and creatinine in plasma and urine were measured by standard automated analyzer by Medizinisches Labor Hannover (Hannover, Germany). Urine adenosine concentrations were quantified by high-pressure liquid chromatography using a MS nucleosil C18 column (125×4 mm, 10 μm) with ultraviolet-detection (Immudagnostik, Bensheim, Germany). Quantification of SLV329 plasma concentrations was performed after solid phase extraction using a validated reversed phase high-pressure liquid chromatography method with MS/MS-detection (Sciex Api 3000, Perkin Elmer, Waltham, MA, USA). The data describing the concentration-dependence of the diuretic and natriuretic effects of SLV329 were fitted to estimate the half-
Effects of SLV329 in thioacetamide-induced liver cirrhosis

The study protocol was approved by the Landesamt für Gesundheit und Soziales, Berlin (approval number G0163/06). Male Wistar rats (250–300 g) were maintained under controlled conditions (20±2°C, 12 h light/dark cycle) and kept on a standard diet (0.2% sodium) with water ad libitum. Animals were divided into 6 groups:

1. Controls (Con; n = 8)
2. Controls with furosemide treatment (Con+Fur; n = 8)
3. Controls with SLV329 treatment (Con+SLV; n = 8)
4. Cirrhosis (Cir; n = 14)
5. Cirrhosis with furosemide treatment (Cir+Fur; n = 13)
6. Cirrhosis with SLV329 treatment (Cir+SLV; n = 12)

Liver cirrhosis was induced by oral administration of thioacetamide via drinking water for 18 weeks. The initial concentration was 0.03%. This concentration was modified weekly according to weight changes in response to thioacetamide. The concentration was increased/reduced by 0.015% (absolute) in case of weight gain/loss >25 g per week. Treatment with furosemide or SLV329 was started in week 8, since it is known that liver cirrhosis develops by then [30]. Thioacetamide concentrations remained unchanged from week 8 until week 12. In week 12 the concentration was increased by 0.015% in all animals receiving thioacetamide. The resulting concentration was given until week 18. Furosemide was injected intraperitoneally (7.5 mg/kg) thrice weekly (always between 8 and 10 a.m.) in the respective groups starting from week 8 until study end. Also starting from week 8 the respective groups received a standard rat chow formulated with SLV329. Chow was obtained from Altromin (Lage, Germany) in several conditions (202°C, 12 h light/dark cycle) and fed to the rats according to food intake. The target concentration was 5 mg/kg per day. Subsequently calculated mean intakes of the rats according to food intake. The target concentration was calculated according to a standard dilution series. The samples were diluted with lysis buffer (5 g/l) to assure equal loading. Samples (23 µg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (10%, 80 V for 30 min, then 110 V) and semi-dry-blotted (1.5 mA/cm², 60 min) onto nitrocellulose membranes. Ponceau staining of membranes confirmed equal loading of proteins. Membranes were blocked with 5% skim milk and incubated overnight with primary rabbit antibodies directed against A₁R (0.025%, Sigma-Aldrich, Munich, Germany), A₂AR (0.1%, Millipore, Schwalbach, Germany), A₃AR (0.1%, Millipore), A₂BR (0.2%, Millipore), and beta-actin for normalization (0.0025%, Sigma-Aldrich). The specificity of the antibodies has been documented by the manufacturers and was not tested again in this study. After extensive washing blots were incubated with a horseradish peroxidase-linked anti-rabbit IgG (60 min, 0.0001%, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected using an enhanced chemiluminescence system and were subsequently quantified with the AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). The values thus obtained were corrected for different conditions between single runs, using a standard dilution series that was run on each gel. Results of adenosine receptors were then normalized to beta-actin.

Statistical analysis

Data was analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The nonparametric Kruskal-Wallis and the Mann-Whitney-U test were used to detect significant differences between groups of interest. Mortality rates were estimated by the Kaplan-Meier method and compared by log-rank test.

Author Contributions

Conceived and designed the experiments: BH SH MA PK DZ YF TP. Performed the experiments: SH KVW AMA JR TP. Analyzed the data: BH SH TP. Contributed reagents/materials/analysis tools: BH AMA DZ YV. Wrote the paper: TP.

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