Thesis

PHARMACOLOGICAL TARGETING OF ADENOSINE KINASE FOR SUSTAINED CARDIOPROTECTION

submitted by

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Graz, 29.09.2023

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 29.09.2023 *Eva Teschl m.p.*

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Glossar und Abkürzungen – List of Abbreviations

$2Na^{+}$-Ca^{2+} exchanger ..... *Sodium-calcium exchanger*
$3Na^{+}$-$2K^{+}$-ATPase ..... *Sodium potassium ATPase*
ABT-702 ..... *ABT-702 (Adenosine kinase inhibitor)*
ADA ..... *Adenosine deaminase*
ADK ..... *Adenosine kinase*
ADP ..... *Adenosine diphosphate*
AK1 ..... *Adenylate kinase1*
AMI ..... *acute myocardial infarction*
AMP ..... *Adenosine monophosphate*
ATP ..... *Adenosine triphosphate*
BCA ..... *Bicinchoninic acid assay*
Ca^{2+} ..... Calcium
CAD ..... *Coronary artery disease*
cAMP ..... *Cyclic messenger adenosine monophosphate*
CNTs ..... *Concentrative nucleoside transporters*
cTn ..... *Cardiac troponin*
CVD ..... *Cardiovascular Disease*
DNA ..... *Deoxyribonucleic acid*
ENTs ..... *Equilibrative nucleoside transporters*
IPC ..... *Ischaemic preconditioning*
IR ..... *Ischaemia-reperfusion*
LAD ..... *Anterior descending artery*
MI ..... *Myocardial infarction*
MPTP ..... *Mitochondrial permeability transition pore*
Na^{+} ..... *Sodium*
Na^{+}-H^{+} ion exchanger ..... *Sodium-proton exchanger*
NO ..... *Nitric oxide*
PC ..... *Preconditioning*
PKA ..... *Protein kinase A*

PKC ..... *Protein kinase C*
RCA ..... *Right coronary artery*
RCX ..... *Ramus circumflexus*
ROS ..... *Reactive oxygen species*
SAH ..... *S-adenosylhomocysteine*
SDS ..... *Sodium dodecyl sulfate*
TBST ..... *Tris-buffered saline with Tween20*
TEA ..... *Triethanolamine hydrochloride*
TTX ..... *Triton X 100*
WBTB ..... *Western blot transfer buffer*

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Zusammenfassung

Herz-Kreislauf-Erkrankungen haben weltweit erhebliche Auswirkungen auf die Gesellschaft und die Gesundheitssysteme und waren in den letzten Jahren für die Mehrzahl der Todesfälle in Österreich verantwortlich. Die koronare Herzkrankheit ist eine der häufigsten Formen von Herz-Kreislauf-Erkrankungen und erhöht das Risiko eines akuten Myokardinfarkts (AMI) erheblich. Eine Steigerung der myokardialen Adenosinrezeptor-Signalisierung vor einem AMI oder bei der Reperfusion kann die Myokardschädigung verringern. Der potenzielle therapeutische Nutzen von Adenosinrezeptor-Agonisten wird jedoch durch die Adenosinrezeptor Präsens in unterschiedlichsten Gewebearten, starke physiologische Effekte der Adenosinsignalisierung in Nicht-Zielgeweben und aber vorallem durch die Tatsache, dass akute AMI leider nicht vorhersehbar sind, geschmälert. Die pharmakologische Hemmung des wichtigsten Enzyms, welches für den myokardialen Adenosin-Stoffwechsel verantwortlich ist, Adenosin-Kinase (ADK), erhöht die Adenosinfreisetzung aus den Kardiomyozyten und kann so eine akute Kardioprotektion im Rahmen einer ischämischen Schädigung bewirken. Ob diese Fähigkeiten auch für eine anhaltende Kardioprotektion anwendbar sind, war bisher unbekannt. Im Rahmen dieser Diplomarbeit wurde ein neuartiger Mechanismus der anhaltenden Kardioprotektion identifiziert, der durch die Hemmung der Adenosinkinase ausgelöst wird und zu neuen Ansätzen für die Auslösung der kardioprotektiven Eigenschaften von Adenosin im Herzen führen könnte.

Die Verabreichung von ABT-702 kann die durch Adenosinrezeptoren vermittelten physiologischen Wirkungen und die Kardioprotektion 24 Stunden nach der Verabreichung erhöhen, obwohl die Substanz zu diesem Zeitpunkt bereits abgebaut war. Die Behandlung mit ABT-702 verändert dabei die Expression der kardialen Adenosinrezeptoren nicht, aber es lässt sich überraschenderweise eine Verringerung der ADK-Proteinexpression beobachten, die zu einer anhaltenden Adenosinfreisetzung in den Kardiomyozyten führt. Dosis-Wirkungs-Studien zeigten, dass die orale Verabreichung von ABT-702 (3mg/kg) ebenfalls einen ADK-Abbau bewirkt, der vor ischämischen Schäden schützt. Wichtig ist, dass die anhaltende Kardioprotektion durch ABT-702 von einer erhöhten Adenosinrezeptor-Signalisierung unmittelbar vor der ischämischen Schädigung abhing, was auf einen neuartigen Mechanismus der verzögerten Vorkonditionierung hindeutet. Das Verständnis

des Mechanismus, durch den ABT-702 die kardiale ADK-Expression verringert, könnte zu neuen Therapien führen, die eine anhaltende kardioprotektive Wirkung haben.

Abstract

Cardiovascular diseases have a significant global impact on society and healthcare systems and accounted for the majority of Austrian deaths in recent years. Coronary artery disease (CAD) is one of the most prevalent forms of cardiovascular diseases and significantly raises the risk of acute myocardial infarction (AMI). Increasing myocardial adenosine receptor signaling prior to AMI or at reperfusion can diminish myocardial injury. However, the therapeutic utility of adenosine receptor agonists for this issue is limited by the wide tissue distribution of adenosine receptors, strong physiological effects of adenosine signalling in non-target tissues and, above all, the fact that acute AMI are unfortunately unpredictable. The pharmacological inhibition of the major enzyme responsible for myocardial adenosine metabolism, adenosine kinase (ADK), increases cardiomyocyte adenosine release and can thus provide acute cardioprotection against ischaemia-reperfusion injury. Whether these abilities are also applicable for sustained cardioprotection is not known. A novel mechanism of sustained cardioprotection may be induced by adenosine kinase inhibition, which may lead to novel approaches for eliciting the cardioprotective properties of adenosine in the heart. ABT-702 administration increases adenosine receptor-mediated physiological effects and cardioprotection 24 hours after administration, even though the drug had been eliminated by that time. ABT-702 treatment does not alter expression of cardiac adenosine receptors, but unexpectedly, causes a reduction in ADK protein expression that results in sustained cardiomyocyte adenosine release. Dose response studies demonstrated that oral administration of ABT-702 (3mg/kg) also caused ADK degradation that was protective against ischemia-reperfusion injury. Importantly, sustained cardioprotection by ABT-702 was dependent upon increased adenosine receptor signaling just prior to IR, suggesting a novel mechanism of delayed preconditioning. Understanding the mechanism(s) by which ABT-702 decreases cardiac ADK expression could lead to new therapies that provide sustained cardioprotection.

Information on previous publications

I had the honour of being part of the research group of the paper "Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection" by Wölkart et al. under the great supervision of John Fassett and as a result the data I generated for the thesis and now used has already been published recently.

I informed all co-authors about the publication of this diploma thesis. All co-authors have agreed to the inclusion of their published data in the diploma thesis and permission to reproduce illustrations and figures from the publication has been granted.

There were two aspects I worked on for the paper, firstly to find out whether intraperitoneal injection of 10mg/kg ABT-702 in mice induces an effect on cardiac adenosine receptor mRNA expression 24h after treatment.
Second, to perform a dose-response analysis of oral administration of ABT-702 (3, 1 or 0.3 mg/kg) by Western blot to investigate cardiac adenosine kinase expression.

1 Introduction

1.1 Cardiovascular diseases and economic impact

Cardiovascular diseases (CVD) have a notable economic impact on societies, healthcare systems and individuals worldwide. In Austria the direct and indirect costs of CVD are estimated to be at around 4.7 billion euros in 2015. Compared to this, the total cost in the United States are projected to be twice as high in 2035 as in 2015 (from $555 billion to $1.1 trillion) (1) (2). In addition to economic factors, this disease represents the highest proportion of all Austrian deaths in 2020 (3).

Approximately 17 million people worldwide die of CVD, particularly from heart attacks and strokes (4). Therefore, it is understandable that cardiovascular research has already been of great relevance in the past as well as in the future.

1.2 The acute myocardial infarction (MI

1.2.1 Etiology and pathophysiology of MI

The most common complication of coronary artery disease (CAD) is acute myocardial infarction (5). CAD results from exposure of the vascular wall to cholesterol and other risk factors that leads to damage of the vascular endothelium (= endothelial dysfunction) and triggers a chronic local inflammatory process. This is characterised by the invasion of leukocytes. Lipid phagocytising monocytes, which differentiate into lipid-laden macrophages or foam cells, that form so-called "fatty streak" lesions. Proinflammatory mediators secreted by foam cells increase the presence of macrophages and T-cells and increase proliferation and migration of vascular smooth muscle cells. An arteriosclerotic plaque develops due to the release of radicals and death of immigrated cells (6).

A myocardial infarction is said to occur when, in addition to myocardial damage and the laboratory values defined for it, at least one of the following criteria is met:

Symptoms of myocardial ischaemia, new ischaemic electrocardiogram changes, occurrence of pathological Q waves, imaging evidence of new loss of viable myocardium or new

regional wall motion abnormality in a pattern consistent with an ischaemic aetiology, or by identification of a coronary thrombus by angiography or autopsy (7).

Myocardial infarction can present with atypical symptoms or even no symptoms and can only be detected by cardiac imaging, electrocardiogram or elevated biomarkers (8).The preferred biomarker for myocardial necrosis is cardiac troponin (I or T), which has near absolute specificity for myocardial tissue as well as high clinical sensitivity, reflecting even microscopic areas of myocardial necrosis (9). There are several possibilities that can lead to myocardial injury (= detection of an elevated cardiac troponin (cTn) value above the 99th percentile of the upper reference limit) that may eventually end in infarction, e.g. anaemia, ventricular tachyarrhythmia, heart failure, renal disease, hypotension/shock or hypoxaemia (7).

Therefore, MI is classified into 5 types based on the aetiology and circumstances. When MI is caused by rupture or erosion of an atherosclerotic plaque in patients with pre-existing atherothrombotic coronary artery disease, it is referred to as type 1 MI (7). The intraluminal thrombosis can be triggered by plaque rupture but also by bleeding into the plaque through the damaged surface (10, 11) and leads to myocardial ischaemia and finally MI. If a patient with CAD is exposed to an acute stressor (like mentioned above) the increased myocardial oxygen demand combined with the already existing inadequate blood flow can lead to myocardial damage and type 2 MI. Therefore, plaque rupture or thrombus is not a feature of this type, but rather a mismatch between oxygen supply and demand (7).
The remaining types are not relevant in this case and are therefore only briefly listed:

Type 3: sudden, unexpected presumed myocardial infarction, but not objectifiable due to exitus letalis
Type 4: associated with percutaneous coronary intervention or documented stent thrombosis
Type 5: associated with coronary artery bypass surgery (7)

1.2.2 The acute coronary syndrome

The main symptom of myocardial ischemia is angina pectoris. Acute angina pectoris at rest lasting for more than 20 minutes is commonly called acute coronary syndrome. However, it is impossible to diagnose an MI without using diagnostic tools like a 12-lead electrocardiogram or biomarkers.

ST-elevation myocardial infarction (STEMI) is usually caused by a thrombotic occlusion of a coronary artery that extends to all wall layers of the myocardium from the epicardium to the endocardium. This is referred to as transmural infarct. Urgent reperfusion is the most important therapy in this case. It is diagnosed by a typical pattern seen in a 12-lead electrocardiogram.

If however, the electrocardiogram does not show such a typical pattern, the symptoms may still be related to an MI. The non-ST-elevation myocardial infarction (NSTEMI), which is usually associated with a non-occlusive thrombus, only affects to the inner third of the myocardium (non-transmural/subendocardial infarct) and is diagnosed by an elevation of cTn in a blood analysis. (12) (13)

From an anatomical view, myocardial infarction can also be divided into anterior wall and posterior wall infarction. If the left anterior descending artery (LAD) is occluded, it is called an anterior wall infarction. This means that large parts of left ventricle are affected. The right ventricle may also be involved, depending on the extend of the infarction. (14)

If the right coronary artery (RCA) or the ramus circumflexus (RCX) of the left coronary artery are obstructed, mostly smaller parts of the left ventricle are affected and the involvement of the right ventricule is more common (15). Of note, individual variations of the coronary arteries do not allow to clearly predict which part of the heart will be affected by the occlusion of one coronary artery. Figure 1 demonstrates the coronary arteries in relation to the heart.

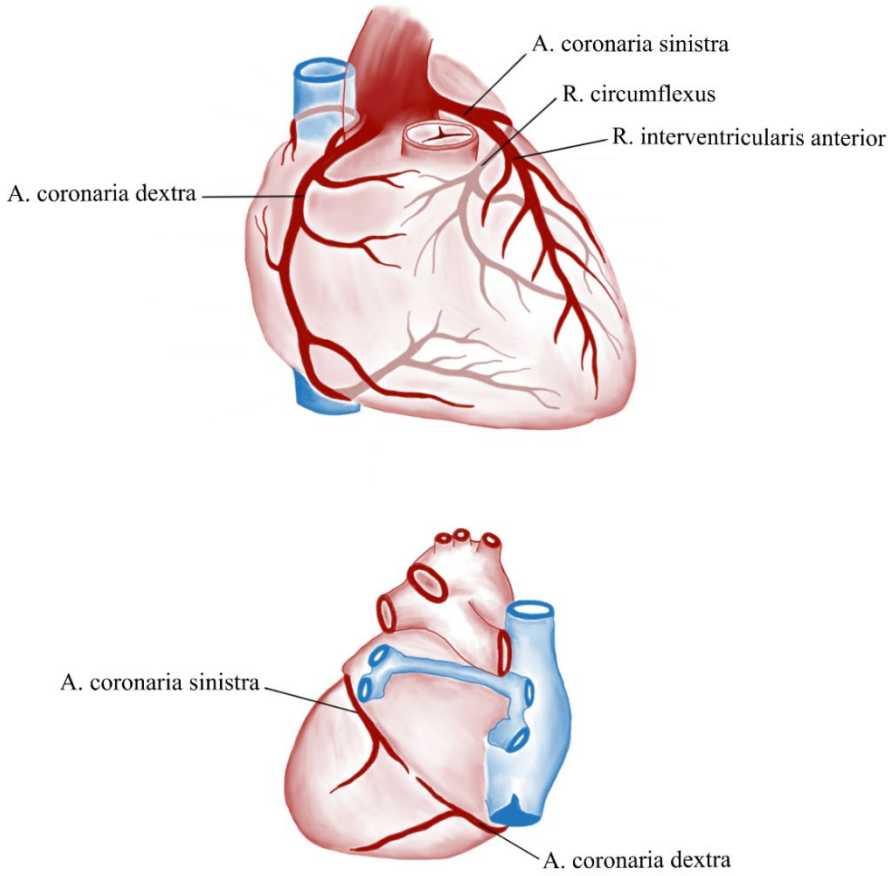

Figure 1: Ventral and dorsal view of the coronary arteries. (Reference: From Sobotta Atlas der Anatomie des Menschen, Innere Organe, p. 26)

1.2.3 Myocardial ischaemia on the cellular level

Due to the occlusion of the coronary artery, the affected myocardial tissue receives too little oxygen and nutrients. The loss of oxidative phosphorylation causes depolarisation of the mitochondrial membrane, adenosine triphosphate (ATP) depletion and inhibition of cardiac muscle contractile function. In absence of sufficient oxygen, increased anaerobic glycolysis lowers intracellular pH (to <7.0) due to accumulation of lactate. This activates the sodium-proton exchanger (Na^+-H^+ ion exchanger), which excretes protons from the cell and lets sodium (Na^+) in. The ischaemia-induced ATP deficiency also incapacitates the sodium potassium ATPase ($3Na^+$-$2K^+$-ATPase) leading to an increase of the already elevated intracellular Na^+ concentration. Consequently, there is an intracellular calcium (Ca^{2+})

overload as the cell tries to excrete Na^+ via a reverse activation of the sodium-calcium exchanger ($2Na^+$-Ca^{2+} exchanger) (16).
An important cause of ischaemic damage that occurs in myocardial infarction and stroke is opening of the mitochondrial permeability transition pore (MPTP) (17).
While MPTP opening and hypercontraction of cardiomyocytes is prevented by the low pH during the ischaemia (16), the restoration of blood flow and oxygen availability after thrombolysis causes opening of the MPTP, which causes mitochondrial death and contributes to reperfusion injury (18).

1.2.4 Reperfusion injury

The death of cardiomyocytes upon myocardial reperfusion is called myocardial reperfusion injury (19-21). During reperfusion, reactive oxygen species (ROS) generated by the reactivated electron transport chain contribute to myocardial damage by inducing MPTP opening, acting as a chemoattractant for neutrophils and causing sarcoplasmic reticulum dysfunction (16).
At the same time that acidosis is clearing, adenine nucleotides are depleted, intracellular Magnesium falls, intracellular Calcium and Phosphate are elevated. All of these factors during reperfusion promote MPTP opening (17).
In addition, free radicals from dysfunctional mitochondria cause damage to the cell membrane occurs through lipid peroxidation, which in turn results in the denaturation of enzymes and causes direct oxidative damage to deoxyribonucleic acid (DNA) (16).
Lipid peroxidation and damage to intracellular membranes also impairs Ca^{2+} handling, which contributes MPTP and promotes necrosis. (22).

Upon reperfusion, cell necrosis causes further damage by inducing an inflammatory response characterized by increased cytokine release, activated complement, and neutrophil accumulation in the infarct zone.
Therefore, we can identify and summarise important mediators of myocardial reperfusion injury: oxidative stress, intracellular Ca^{2+} overload, the rapid restoration of physiological pH at the time of reperfusion, opening the MPTP and inflammation (16).

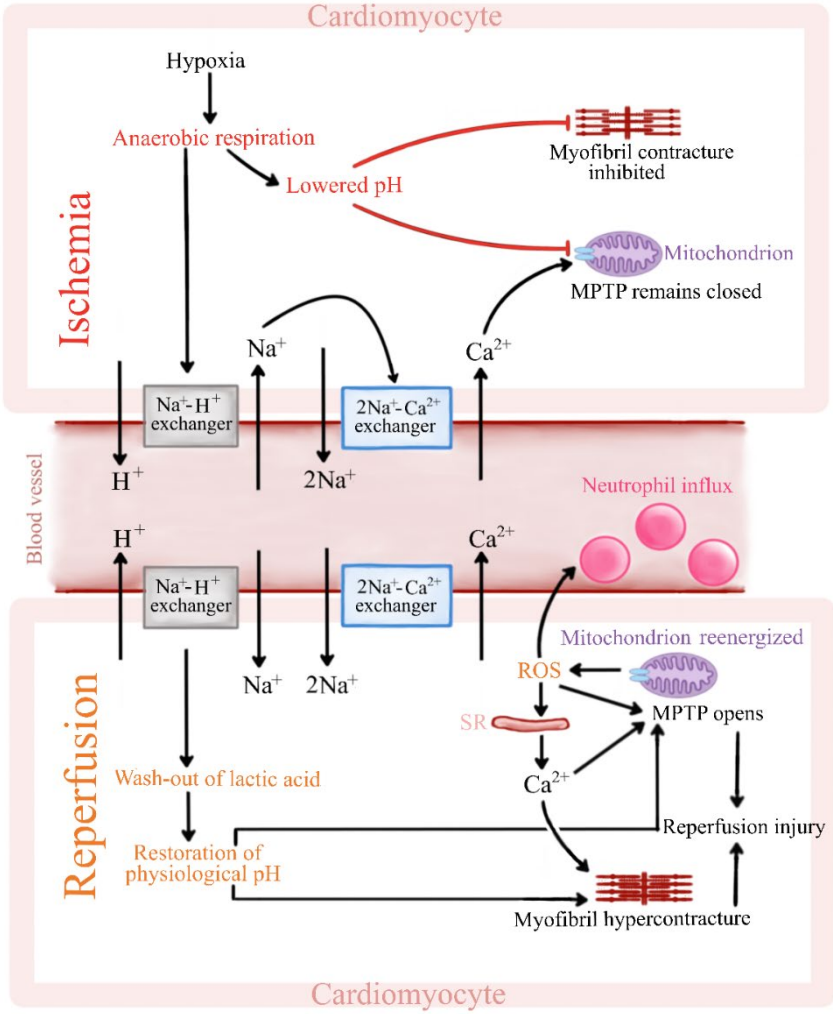

Figure 2: The main proponents of acute myocardial ischaemic reperfusion injury. (Reference: From Myocardial ischemia-reperfusion injury: a neglected therapeutic target, Hausenloy D. J. and Yellon D. M.)

1.2.5 Cardiac remodeling

Besides acute damage, myocardial infarction can also drive cardiac remodeling, which over time, can lead to contractile dysfunction and heart failure. The replacement of dead myocytes with collagen-producing fibroblasts results in formation of a stiff extracellular matrix that diminishes contractility. As remaining healthy cardiomyocytes attempt to compensate for the loss of contractility, the extra strain on these cells induces cardiomyocyte hypertrophy. Continued mechanical stress on these cells, as well as a compensatory increase in adrenergic

signaling causes cardiomyocytes to enlarge and elongate, while intracellular remodelling of microtubule and intermediate filament cytoskeleton begin to impair contractile function. The newly localised collagen fibres of the infarct region determine the characteristics of the tissue that was originally determined by the orientation and nature of the myocytes (23, 24).
Thus, fundamental permanent changes occur not only in the immediate area of the MI, but also in the non-ischemic area. Here, the healthy myocardium tries to compensate for the myocardial scar tissue, which is mechanically a non-linear anisotropic material, and thereby influences cardiac function (23, 25, 26). Together, this maladaptive remodeling, characterized by cardiomyocyte hypertrophy and fibrosis, eventually can progress into heart failure (23, 27).

1.3 Adenosine

In 1927, the story of adenosine began when extracts from cardiac tissue were found to slow heart rate (28). It is now known that adenosine is a ubiquitous endogenous molecule that has numerous actions on cardiovascular physiology and pathology.
Adenosine is a purine nucleoside consisting of adenine and ribose, which exerts most of its effects via four G-protein-coupled receptors: A1, A2A, A2B and A3. Adenosine receptor subtypes are expressed in all tissues and organs and affect numerous physiological and pathological processes. Adenosine receptor activity plays particularly important roles in the central nervous system, the cardiovascular system, the peripheral system and the immune system (29).

While adenosine does play a role in basal cardiovascular physiology, it's effects are most evident under conditions of increased ATP consumption (29) or other cellular stress, such as hypoxia, cell damage and platelet aggregation. During ischemia, for instance, the interstitial concentration of adenosine, which is normally in the low nanomolar range under basal conditions, increases rapidly up to 10-30 μM (29). Adenosine is produced both inside cells from ATP degradation, or outside of cells, mostly through the degradation of extracellular adenine nucleotides by cell membrane ectonucleotidases (30, 31).
The increased formation of adenosine in response to stress conditions has earned it the name "retaliatory metabolite"(32).

Other factors besides ATP depletion may also affect adenosine levels and therefore activate adenosine receptors, including various regulatory molecules such as nitric oxide (NO), histamine, catecholamines, production and degradation of cAMP and cell signalling pathways such as protein kinase A (PKA) and protein kinase C (PKC) signalling (33). Thus, adenosine and adenosine receptor signaling can indirectly influence the response to a variety of physiological and pathological stimuli.

1.3.1 Adenosine metabolism

Adenosine can be generated both intracellularly and extracellularly. Under basal conditions, Adenylate kinases (AK1), which convert 2ADP into ATP + 5'AMP (AMP), provide a constant source of 5'AMP, which is converted into adenosine by endo-5′-nucleotidase (5' cytosolic nucleotidase). Adenosine can also be produced intracellularly from S-adenosylhomocysteine
(SAH), which is cleaved by SAH hydrolase into adenosine and homocyteine (34) (35).

Of the adenosine produced intracellularly, a small part is degraded by adenosine deaminase (ADA) or leaves the cell by diffusion, but the large amount of about 90% is rephosphorylated by adenosine kinase (ADK) (36).
Extracellular generation takes place under stress conditions (29).
This is because damage to cell membranes, in the course of trauma, causes a massive increase in the extracellular content of ATP (30).
The energy carrier forms of adenosine, ATP, ADP and AMP are then dephosphorylated (29) by sequential hydrolysis of the interstitial adenine nucleotides by specific hydrolysing enzymes, the ectonucleotidases CD39 and CD73 (37). Interstital adenosine levels are also controlled by concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs).
These channels allow the passage of nucleosides through the cell membrane, so that adenosine is released cells when intracellular levels exceed interstitial levels. Thus, the difference in concentration determines the direction of adenosine uptake or release from the cells (29). In the heart, constant recycling of adenosine by cardiomyocyte ADK results in a

higher interstitial/intracellular adenosine gradient that generally drives adenosine uptake under basal conditions.

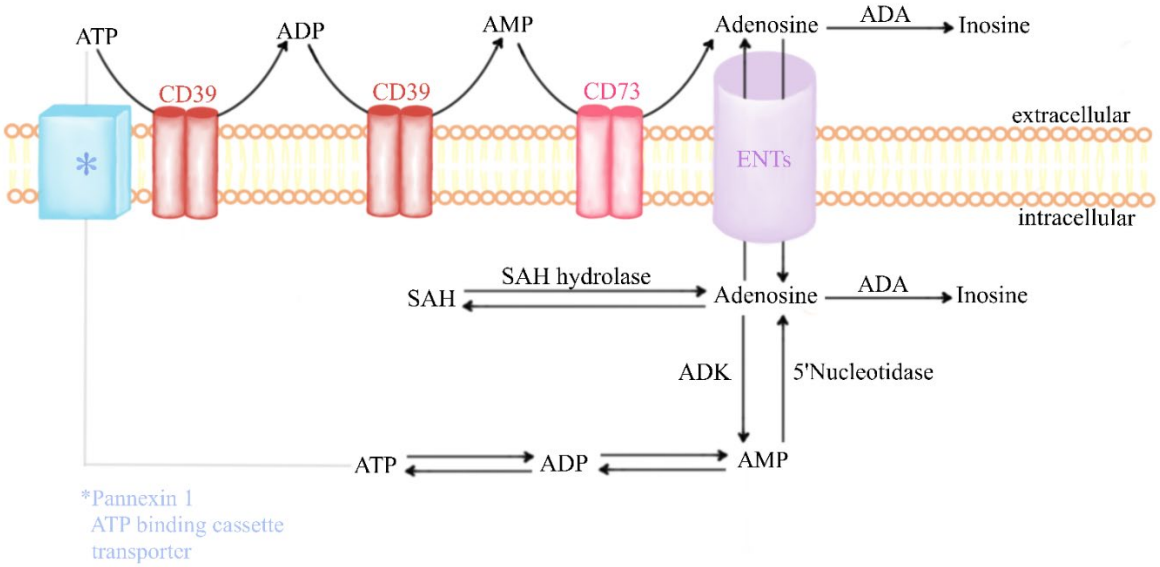

Figure 3: Adenosine metabolism. (Reference: From Pharmacology of Adenosine Receptors: The State of the Art, Pier et al. mixed with Adenosin, Adenosinrezeptoren und adenosinrezeptoraktivierte Signalwege; Schulte G. mixed with Adenosine and adenosine rezeptor- mediated action in coronary microcirculation, Zhang Y et al.)

1.3.2 Cardiovascular adenosine and adenosine receptor effects

Adenosine has many regulatory roles in the body. In the heart, adenosine receptor signaling is especially recognized for several important cardioprotective effects.

1.3.2.1 Adrenergic signalling

One example of this is the attenuation of the adrenergic signalling by adenosine A1 receptor signaling (38). As a result, adrenergic receptors are not activated and thus the usual production of the cyclic messenger AMP (cAMP) (cAMP) does not occur (38, 39). A1

receptor signaling can also attenuate pressure overload-induced cardiac hypertrophy and left ventricular- dysfunction, but whether this is due to anti-adrenergic effects is unclear (40).

1.3.2.2 Anti-inflammation

A2As have great potential for anti-inflammatory effects. Their stimulation leads to a cardioprotective effect by lowering neutrophil accumulation (41). In tissue injury and inflammation, adenosine regulates the transport of lymphocytes within the vascular system and has an inhibitory effect on their migration. This occurs through activated A2A receptors, which cause a reduction in the release of chemotactic substances (adhesion molecules, interferon-γ production) and thus a decrease in the extravasation of lymphocytes. In addition, A2B receptors influence the barrier function of the vascular endothelium (42).
Another important receptor related to inflammation is the A3 receptor. It probably mediates anti-inflammatory effects during reperfusion and also limit injury processes within myocardial tissue (33). This suggests that A3 receptor agonists could be protective of post-ischaemic neutrophil-mediated injury and may be associated with the cell regulation of the bone marrow (43).
On the other hand, A3 receptor knockout mice were protected against pressure overload-induced heart failure (44), suggesting A3R signaling may also have detrimental effects in this setting.

1.3.2.3 Angiogenesis

Adenosine, released from hypoxic tissue, plays an important role in angiogenesis. It is known to alter vascular tone but it also has the ability to affect the growth of vascular cells, leading to angiogenesis and vasculogenesis (45).

1.3.2.4 Reduction of cardiac fibrosis

Previous studies have suggested that endogenous adenosine may protect against cardiac fibrosis (46). Later, adenosine was demonstrated to inhibit cardiac fibroblast proliferation and cardiomyocyte hypertrophy. After myocardial infarction, long-term stimulation of

adenosine A2B receptors attenuates cardiac fibrosis in non-infarcted myocardium, thereby diminishing maladaptive cardiac remodeling and deterioration of cardiac function (47).

1.3.2.5 Vasodilation

Adenosine has especially important roles in the heart during energetic stress conditions. Adenosine activation of vascular adenosine A2 receptors, especially A2A receptors, cause vasodilation, which is associated with an increase in the supply of nutrients and oxygen (29, 39). Adenosine also influences the inotropic (29) as well as chronotropic effects of the heart. A1 receptors activation exerts negative chronotropic effects through inhibition of K+ and Ca2+ currents and pacemaker current (48). Thus, adenosine protects the heart during energetic stress conditions by increasing blood supply via A2A receptor-dependent vasodilation and by lowering ATP use via A1R-dependent decreases in heart rate and inotropy (29).

1.3.2.6 Effects on myocardial metabolism

Since adenosine also regulates glucose metabolism and fatty acid supply (33), it has a significant impact on myocardial metabolism and consequently also affects the response to stress such as hypoxic or ischaemic. Thus, according to the literature, A2B adenosine receptors may reduce vascular injury, as knockdown of these receptors in an apolipoprotein E-deficient mouse model exacerbated diet-induced atherosclerosis (33, 49).

1.3.2.7 Ischaemic preconditioning

Adenosine also plays an important role in a phenomenon called ischaemic preconditioning (IPC), which diminishes myocardial cell damage from ischemia and reperfusion. In IPC, the heart is exposed to a short-term local sublethal ischaemia under control (for example through coronary occlusion). This treatment can provide robust protection against myocardial damage from a second, prolonged lethal episode of ischaemia (50, 51).

Interestingly, preconditioning with adenosine, one of the substances naturally released during myocardial ischemia, can provide equal protection against myocardial damage as IPC without inducing ischaemia (51).

Preconditioning induces two phases of cardioprotection against IPC; an acute phase, that lasts from 15 min to 2 hours after induction, and a delayed phase, which starts at 12-24 hours and lasts up to 72h (52).

1.3.2.7.1 Mechanism of acute IPC

Although the precise mechanism of IPC is not yet understood, it can be divided into three components. The first component is a trigger, which is the initial stimuli released by ischaemic tissue that stimulate the system. Various chemical stimuli including neurotransmitters, hormones, cytokines and autacoids, such as bradykinin, opioids, and adenosine can trigger preconditioning (31). Evidence of a critical role for adenosine in IPC is supported by the finding that G-protein-coupled A1 and A3 adenosine receptors are indispensable for IPC (53).

The triggers of preconditioning initiate an intracellular mediator cascade. Interestingly, unlike bradykinin and opioids, adenosine does not depend on the opening of mitochondrial K(ATP) channels, but directly activates phospholipase C and/or PKC (31). PKC is thus an important mediator of preconditioning triggered by adenosine.

The third component of the preconditioning response is the end effector. Generally, most mediators of preconditioning appear to converge upon mitochondria. This cell organelle is directly linked with the life or death of the cell, which occurs when the MPTP opens. Therefore, its inhibition is important for the cardiomyocyte to enable cardioprotection (54). This opening leads to a change in the inner membrane potential and a swelling of the matrix. Finally, the outer membrane collapses (55). Thus, inhibition of MPTP opening appears to be a major end effector of preconditioning. The precise mechanism MPTP opening is prevented, however, remains unclear.

Thus, adenosine has four major cardioprotective properties, as summarized in figure 4: anti-inflammatory effect, stimulation of angiogenesis, improvement of the oxygen supply/demand ratio and preconditioning (56).

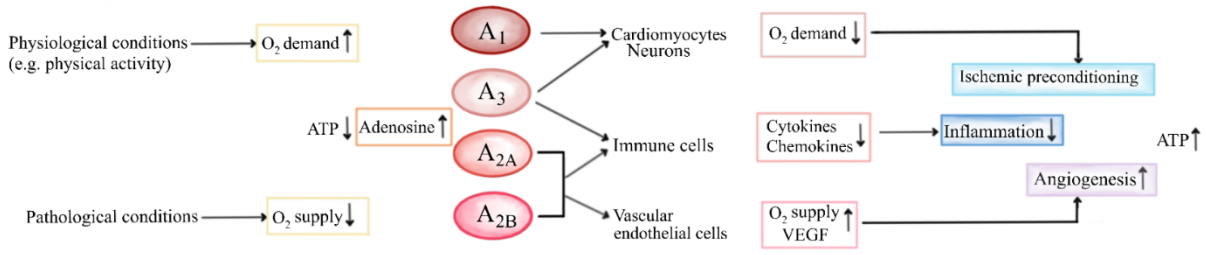

Figure 4: Adenosine mediated effects. (Reference: From Pharmacology of Adenosine Receptors: The State of the Art, Pier et al.)

1.3.3 Delayed preconditioning

The delayed phase, so-called second window of protection, begins 12-24 hours after preconditioning and lasts up to 72 hours (51, 52). The difference between the phases is not only reflected in time but also in the spectrum of effects.

Although the later phase is less robust and lags behind the acute phase in limiting infarct size, it is still able to protect against reversible post-ischemic contractile dysfunction, unlike the acute phase.

It is therefore only logical to assume that the mechanisms also differ. While the early phase is based on the post-translational modification of already existing proteins, the late phase focuses primarily on the new synthesis of cardioprotective proteins and mediators. This fact could also be seen as a potential source for pharmacologically targeted therapies (57).

Up to now, the first 24 hours after Preconditioning (PC) have been the main focus of investigation, and the common explanation for the delayed preconditioning is that the protein kinase signaling cascades get temporary activated by adenosine or/and among others during PC, which has already known play a role in acute cardioprotection (58). Also, this process activates different transcription factors, which lead to de novo transcription of distal mediators. Inducible nitric oxide synthase (59), cyclooxygenase-2 (60), manganese-dependent superoxide dismutase (61), and heat shock proteins (62) are some of the multiple

distal essential mediators for late cardioprotection. Through an interplay of this interrelated factors, ischaemia- reperfusion (IR) tolerance is increased in the late phase to prevent cell death.

1.3.4 Adenosinekinase

Adenosine kinase is a cytosolic enzyme that is the rate-limiting factor for the extracellular adenosine concentration (63). It re-phosphorylates most of the myocardial adenosine, as mentioned earlier (36). In this process, the energy carriers ADP and AMP are formed from adenosine and ATP.
Consequently, it has already been proven that minor changes in ADK activity immediately lead to major changes in the concentration of adenosine (64). Increased adenosine release through ADK inhibition would lead to cardioprotection (52) due to the resulting activation of all adenosine receptor types found in different cell types of the heart, which may increase resistance to ischaemia and reperfusion injury (65).

There are two isoforms of ADK. ADK-S, the short isoform, is located in the cytoplasm, while the longer isoform ADK-L is specific to the nucleus (66). These different isoforms are produced by alternative transcriptional start sites. The only difference between the L and S isoforms is at the N-terminus, where the L isoform contains a nuclear localization signal that is lacking in the S isoform.

The therapeutic potential of adenosine kinase inhibitors has been examined in several experimental models of pain, inflammation, and seizure activity. It is also interesting to note that systemically administered adenosine kinase inhibitors, compared to direct-acting agonists, require a lower dosage to exert the therapeutic effect (i.e. anti-hyperalgesia) (63). But as Jarvis 2019 mentioned, the development of ADK inhibitors for clinical use fails due to the numerous side effects that affect the whole organism (63), which result from chronic, systemic ADK inhibition.
Thus, while pharmacological decrease of ADK activity in the heart might be cardioprotective, undesirable side-effects have prevented development of ADK inhibitors into clinically useful drugs (52).

1.3.5 ABT-702

The non-nucleoside, orally available ADK inhibitor, 4-amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2, 3-d]pyrimidine known as ABT-702, causes an interstitial adenosine increase by inhibiting intracellular adenosine degradation (67). The half-life of ABT-703 is about 0.9hr (54min), which is many times longer than that of adenosine, so this ADK inhibitor can cause prolonged increases in interstitial adenosine (52).

Aims of this thesis

In a rat model with preserved ejection fraction, regular treatment with ABT-702 has already been shown to have several beneficial effects, including improved diastolic function, improved vasodilation and reduced cardiac fibrosis (68).
Acute cardioprotection against myocardial infarction by adenosine inhibition has also been demonstrated in mice (69). however, it was unknown whether ADK inhibition could also induce delayed cardioprotection. In this thesis, the effects of intraperitoneal ABT-702 administration on cardiac expression of adenosine receptors and also oral ABT-702 administration on cardiac expression of ADK in the late phase of preconditioning were examined. This data was included in a recent publication “Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection” Wölkart et al. (52).

2 Material and methods

Name	Manufacturer
ABT-702	Tocris
Albumin Standard (2mg/ml)	Thermo Scientific
Ammonium persulfate (APS)	Sigma
Bromphenol Blue	Sigma
COmplete tablets EDTA-free, EASYpack	Roche
DL-Dithiothreitol (DTT)	Sigma-Aldrich
Ethylene-1,2-tetraacetic acid (EDTA)	Sigma
Glycerol	Roth
Glycine	Roth
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Hybond-ECL Nitrocellulose membrane	VWR
Maleimide	Sigma-Aldrich
Methanol	Roth
N,N,N',N'-tetramethylethylenediamine (TEMED)	Roth
PageRulerTM Prestained Protein Ladder	Thermo Scientific
PhosSTOP EASYpack	Roche
PierceTM BCA Protein Assay Reagent A	Thermo Scientific
PierceTM BCA Protein Assay Reagent B	Thermo Scientific
Ponceau S	Sigma
Powdered milk (Blotting Grade, low in fat)	Roth
Restore™ Western Blot Stripping Buffer	Thermo Scientific
Rotiphorese® Gel 30 (37,5:1)	Roth
Sodium azide	Sigma-Aldrich
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS) pellets	Roth
SsoAdvanced Universal SYBR® Green Supermix	Bio-Rad
Sulfosalicylic acid	Sigma-Aldrich
TRI Reagent®	Sigma
Trichloroacetic acid	Fluka
Triethanolamine hydrochlorid (TEA)	Sigma
Tris(hydroxymethyl)aminomethane (TRIS Pufferan®)	Roth
Triton® X- 100	Merck
Tween® 20	Sigma-Aldrich
WesternBright™ ECL	Advansta
WesternBright™ Peroxide	Advansta
WesternBright™ Quantum	Advansta

Table 1: List of all used materials

Antibody	Manufacturer & product number
α- Tubulin	Cell Signaling 2125S
ADK (H-1)	Santa Cruz sc-514588
Anti- mouse	Cell Signaling 7076
Anti- rabbit	Cell Signaling 7074
GAPDH	Sigma G8795
Mouse lgG$_K$-BP HRP	Santa Cruz sc-516102
Sarcomeric Actin	Sigma-Aldrich A9357
Vinculin	Santa Cruz sc-25336

Table 2: List of antibodies

2.1 Tissue Preparation

2.1.1 Animals

Whole hearts from mice orally administered ABT-702 (ABT-702(Adenosine kinase inhibitor)) or vehicle controls were used for this project. Animal care was in accordance with the Austrian law and also conformed to the local ethical committee (BMWFW-66.007/0012-WF/V/3b/2016 and BMBWF-66.0030-V/3b/2019). Mice were on a 12 hour day and night cycle and had the possibility to eat and drink at will.

2.1.2 Tissue extraction

For the experiments the mice were orally administered 0.3, 1 or 3mg/kg ABT-702 by gavage. The mice were anaesthetized 24 hours after the treatment with ketamine/xylazine and the organs were harvested. The required tissues were immediately frozen in liquid nitrogen und stored at -80°C until they were needed.

2.1.2.1 Pulverization in liquid nitrogen

The heart tissues were pulverized under liquid nitrogen with a pestle and a mortar. The powder was divided up to similar amount in liquid nitrogen -cooled Eppendorf tubes and saved at -80°C until processing.

2.1.2.2 Homogenizing

400 microliters of TEA (Triethanolamine hydrochloride) / Triton X 100 (TTX) preparation buffer was added to each of the pulverized hearts per Eppendorf tube. To ideally homogenize the tissue, the UtraTurrax (IKA T10 standard, level 5) was used for about 10 seconds. The total homogenate was then stored for solubilization on ice for 5 minutes and afterwards centrifuged for 5 minutes at 20 000 g with a temperature of 4 ° Celsius. As a result, two layers arised: a clearly visible pellet, which consists of the cytoskeleton of the cells, and the supernatant, which is made up of cytosol, soluble membrane and nuclear proteins. The supernatant was transferred to a new Eppendorf tube, vortexed and further processed.

TEA/TTX buffer	
Triethanolamine hydrochlorid	928 mg
Distilled Water	90 ml
Triton X 100	1 ml
final pH 6,8	

Table 3: Composition of TEA/TTX buffer

Preparation buffer	
TEA/TTX buffer	2 ml
Complete (50x)	40 µl
Phosstop (50x)	40 µl
EDTA 200 mM	20 µl
Maleimid 1M	65 µl

Table 4: Composition of preparation buffer

2.1.3 Preparation for Western blot analysis

150 microliters of the supernatant were mixed with the same amount of 2x Laemmli buffer and DL-dithiothreitol was added for a final concentration 100 mM. The samples were heated for 3 minutes and 30 seconds at 95 ° C and finally stored at -20°C until use.

2x Laemmli buffer	
Tris base	3,02 g
SDS	8 g
Glycerol	40 ml
Bromphenol Blue	0,04 g
Distilled Water	ad 180 ml
final pH 6,8	

Table 5: Composition of 2x Laemmli buffer

2.2 BCA-Assay for quantitative Determination of Protein Content

The bicinchoninic acid assay (BCA) from PierceTM BCA Protein Assay Kit was used to determine the protein concentration. This quantification is based on two reactions. Firstly, Cu^{+2} is quantitatively reduced to Cu^{+} in an alkaline solution of proteins and secondly, one Cu^{+} ion can bind two bicinchoninic acid molecules to itself. This colour reaction is detected photometrically at a wavelength of 562 nm. For the determination, a standard series was prepared from the preparation buffer, which was diluted 1:20, and an albumin stock solution (2 mg / ml). The following concentrations were generated: 1000, 500, 250, 100, 50, 25 and 12.5 micrograms / ml. The diluted preparation buffer was used as a blank and also as basis for the homogenate's dilution, which were 1:20 and 1:40. Each sample were transferred twice on to a 96well plate and were added with 200µl of a mixture containing Pierce® BCA Protein Reagent A and B. After a 15-minute incubation at 55°C the total protein content was determined with the SpectroStar® Nano and were analysed with the SpectroStar Nano Data Analysis software.

2.3 Western Blot

To separate the proteins with regard to their molecular mass, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was used. First, 1.5mm thick gels were made with a 5% stacking layer and a subsequent separation layer of 10% resolving gel. 5µl of PageRulerTM Prestained Protein Ladder was added to the first slot and the following 14 slots were filled with equal amounts of protein (usually 20µg), according to BCA measurements. Electrophoresis was carried out in SDS buffer at 180 volts to separate the polypeptide chains in the polyacrylamide gel according to their relative size.

For the subsequent transfer of proteins onto the nitrocellulose membrane (0.45µm pore size), a blotting sandwich was used consisting of the following components in this order: Pad, filter paper, gel, nitrocellulose membrane, filter paper, and pad.
This layering was done under wet conditions with cold Western blot transfer buffer (WBTB) to prevent air bubbles that would hinder the process. The transfer box was also filled with this buffer and the blotting was carried out for 90 minutes at 0.24 ampere. After transfer, incubation with Ponceau S staining solution for 5 minutes allowed visual assessment of equal protein loading.

After washing the membrane with Tris-buffered saline with Tween20 (TBST) buffer, it was treated with 5% low-fat dry milk powder in TBST buffer at room temperature with gentle shaking for one hour to saturate the free binding sites of the membrane. This procedure prevents non-specific binding of the antibody. The membrane was then incubated with the primary antibody overnight at 4 °C on a bench rocker after the blocking buffer was removed. The next day, the membrane was washed 3 x 10 minutes with TBST buffer (at room temperature) and incubated for one hour with the second antibody conjugated to horseradish-peroxidase.
After repeating the washing steps to remove free secondary antibody, the protein bands were detected. This was done by chemiluminescence using 500 µl WesternBright™ ECL or 500 µl WesternBright™ Quantum and 500 µl WesternBright™ Peroxide. The membranes were incubated for 2 minutes and then evaluated with the densitometer (Fusion SL Vilber Lourmat, Peqlab).

	5% Stacking gel	**10% Resolving gel**
MilliQ H_2O	2100 µl	3000 µl
Polyacrylamide 30%	495 µl	2550 µl

Tris pH 6,8 (1,5 M)	375 µl	
Tris pH 8,8 (1,5M)		1950 µl
SDS 10%	30 µl	75 µl
APS 10%	30 µl	75 µl
TEMED	3 µl	5 µl

Table 6: Composition of stacking gel and resolving gel

SDS buffer	
Glycine	250 mM
Tris base	25 mM
SDS	0,1%
final pH 8,4	
Ponceau S – staining solution	
Ponceau S	0,2 g
Trichloroacetic acid	3 g
Sulfosalicylic acid	3 g
dH_2O	ad 100 ml
WBTB buffer	
Glycine	193mM
Tris base	48 mM
Methanol	20 %
final pH 8,4	
TBST buffer	
NaCl	137 mM
Tris base	20 mM
Tween® 20	0,1 %
final pH 7,6	

Table 7: Composition of SDS buffer, Ponceau S-staining solution, WBTB buffer and TBST buffer

Primary antibodies	Dilution in TBST buffer	Additives	Species
ADK (H-1)	1: 1000	1% MP, Sodium azide	mouse
GAPDH	1: 40000	1% MP, Sodium azide	mouse
Vinculin	1: 5000	1% MP, Sodium azide	mouse
Sarcomeric Actin	1: 20000	1% MP, Sodium azide	mouse
α- Tubulin	1: 1000	1% MP	rabbit

Table 8: List of primary antibodies, their dilution, additives and species

Secondary antibodies	Dilution in TBST buffer	Additives
Anti- mouse	1: 5000	1% MP
Anti- rabbit	1: 5000	1% MP
Mouse lgG_K-BP HRP	1: 5000	1% MP

Table 9: List of secondary antibodies, their dilution and additives

2.4 Quantitative Real Time -Polymerase Chain Reaction (qRT-PCR)

To isolate the mRNA of the powdered heart tissue, Trizol reagent (TRI Reagent®; Sigma) was applied according to the manufacturer's working instructions. The ratio of absorbance at 260 nm and 280 nm was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) to evaluate the purity and concentration of the isolated RNA. Working with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) cDNA was reverse transcribed from the mRNA using random primers as described by the manufacturer.

Amplification of cDNA was achieved using RT-qPCR. For this purpose, approximately 1 ng of cDNA, primer concentrations of 100 nM, including endogenous control (18S) and also SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) were used.
The green supermix contains a fluorescent substance that embeds itself in the DNA and enables the generation of a real-time curve per amplification cycle.

A pool of aliquots of all cDNA samples served as standard. These were serially diluted 1:5 to give a calibration line. Twenty-five µl of each, as well as 25µl of each sample, was pipetted into a 96-well MicroAmp plate (Applied Biosystems).
In addition, 10µl of mastermix was added to each and then the sealed plate was briefly centrifuged. Finally, samples were analysed in StepOnePlus (Applied Biosystems) using StepOne^{TM} software (Quantitation- Standard Curve) under following conditions:

Cycling conditions			
	Temperature	Time	Cycles
1. Heating up stage	50°C	2 minutes	1
2. Holding stage	95°C	10 minutes	1
3.Cycling stage	95°C	15 seconds	40
4.	60°C	1 minute	40
Melting curve conditions			
1. Heating up stage	95°C	15 seconds	1
2. Cooling stage	60°C	1 minute	1
3. 0.3 degree steps till 95°C			

Table 10: qPCR setup

Primer		
Target gene		**Sequence 5' – 3'**
Adora1	forward	5'-TGTGCCCGGAAATGTACTGG-3'
	reverse	5'-TCTGTGGCCCAATGTTGATAAG-3'
Adora2a	forward	5'-TCAACAGCAACCTGCAGAAC-3'
	reverse	5'-GGCTGAAGATGGAACTCGC-3'
Adora2b	forward	5'-GCATTACAGACCCCCACCAA-3'
	reverse	5'-TTTATACCTGAGCGGGACGC-3'
Adora3	forward	5'-GCTGTAGACCGATACCTGCG-3'
	reverse	5'-AAACTAGCCAGCAAAGGCCC-3'
18s	forward	5'-GTAACCCGTTGAACCCCATT-3'
	reverse	5'-CCATCCAATCGGTAGTAGCC-3'

Table 11: List of primers

3 Results

3.1 Adenosine receptor mRNA levels

Using the Langendorff model, we observed that intraperitoneal administration of 10mg/kg ABT-702 caused a late (24 hours afterwards), adenosine receptor-dependent increase in coronary flow (52). However, high pressure liquid chromatography (HPLC) analysis indicated ABT-702 was no longer present in the heart (52), so the reason for this increase in adenosine-receptor-dependent coronary flow was unclear. To find out if this increase in coronary flow might be related to a change in adenosine receptor expression, real-time quantitative polymerase chain reaction was used to examine heart homogenates from control mice as well as from mice administered ABT-702 24 hours prior to organ harvesting.

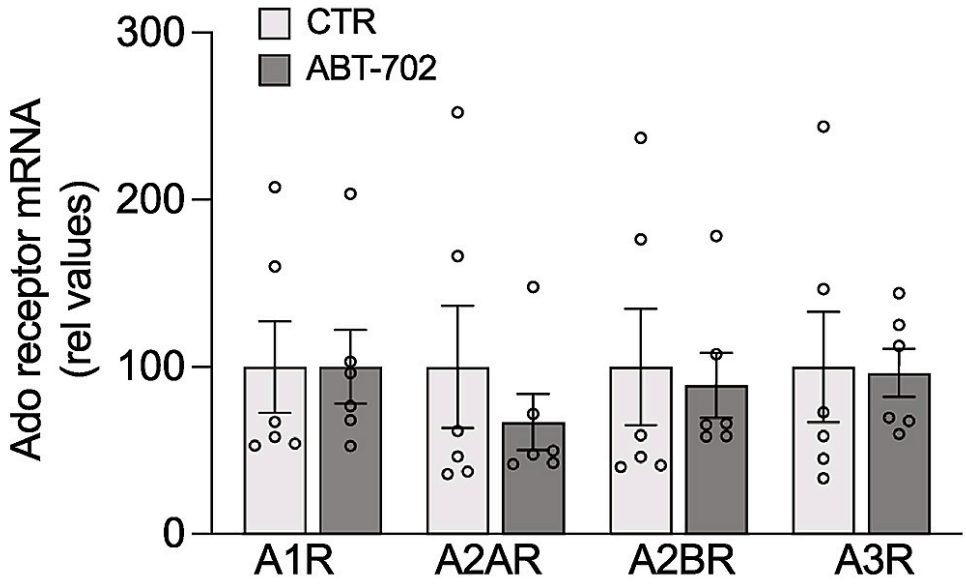

Figure 5: Adenosine receptor mRNA levels of intraperitoneal injection (10mg/kg) ABT-702 treated mice (dark grey bars) compaired to controls (bright grey bars; CTR), 24h after treatment; presented relative to 18s RNA (n= 5 ctr , 6 ABT-702,).(Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al. Eur J Pharmacol. 2022;927:175050)

There were no significant differences in cardiac adenosine receptor mRNA levels between vehicle- and ABT-702-treated mice. This finding suggests that the ABT-702-induced late increase in coronary flow, which were measured during Langendorff perfusion, is most likely not due to changes in adenosine receptor expression. Thus, administration of ABT-702, even after it has already been eliminated from cardiac tissue, results in a sustained increase in adenosine receptor-dependent coronary flow. Subsequently, it was determined that the increase in coronary flow arose from decreased expression of ADK 24 hours later and sustained increase in myocardial adenosine release.

3.1.1 3mg/kg ABT-702

Administration of 10mg/kg ABT-702 decreased mouse body temperature and diminished exploratory activity. Unlike many adenosine modulating drugs, ABT-702 is also orally available. Therefore, we wanted to find out whether the effects achieved with intraperitoneal injection (10mg/kg) of ABT-702 would also be possible with a lower oral dose. This would be advantageous because the treatment would be non-invasive, and a lower dose may have less side effects.

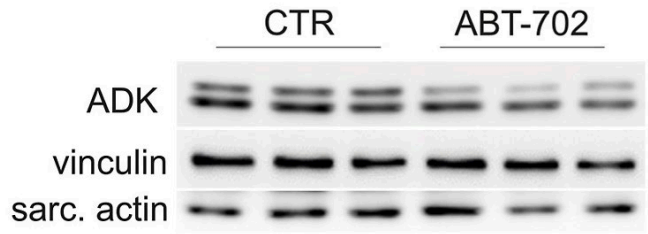

Figure 6: Representation of ADK, vinculin and sarcomeric actin expression in heart tissue. (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al. Eur J Pharmacol. 2022;927:175050)

Therefore, homogenates from cardiac tissue 24 h after oral ABT-702 (3 mg/kg) treatment were compared with controls.

This showed a significant decrease in the ADK-L isoform located at 48kDa and also a modest decrease in the ADK-S form at 46kDa in the ABT-702 treated mice.

Vinculin and sarcomeric actin were used to show equal loading of the proteins.

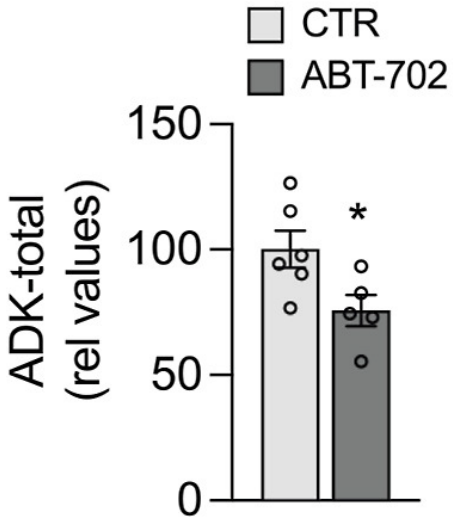

Figure 7: Representation of ADK protein levels normalized to sarcomeric actin (n= 6 ctr, 5 ABT-702), mean values relative to the loading control ± SEM, * indicates $p \leq 0.05$ comparing control to ABT-702. (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al. Eur J Pharmacol. 2022;927:175050)

Figure 7 shows a significant decrease in total ADK in cardiac tissue 24 hours after oral treatment with ABT-702 (3 mg/kg) compared to control. Importantly, this dose also did not decrease body temperature or cause other observable side effects (52).

3.1.2 0.3, 1, 3mg/kg ABT-702

Low dose oral treatment of 0.3 or 1 mg/kg ABT-702 showed 24h after administration a decreased cardiac adenosine kinase expression in some mice but had no effect in others, so that the difference from control mice did not reach significance.

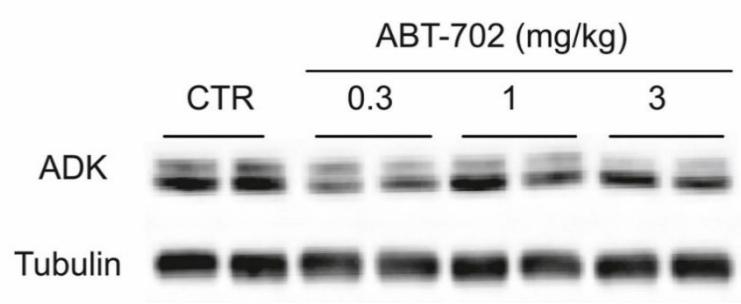

Figure 8: Representation of ADK and tubulin protein expression at different ABT-702 concentrations after 24h in heart tissue. (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al. Eur J Pharmacol. 2022;927:175050)

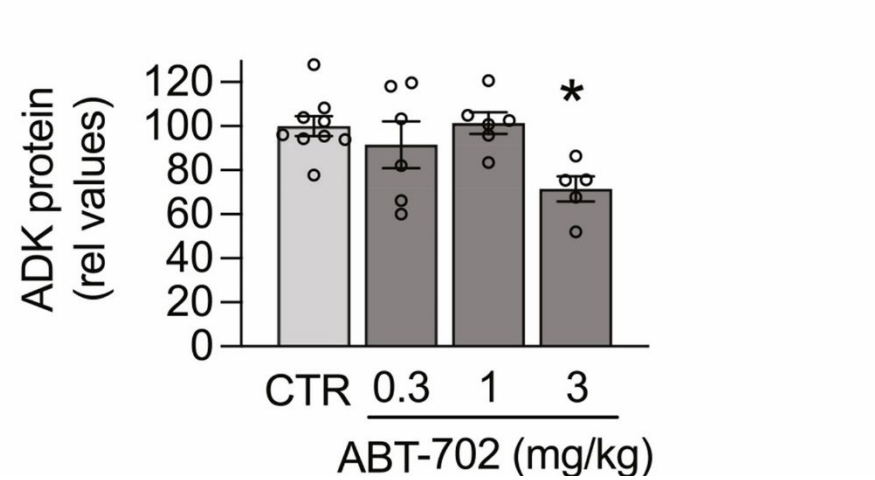

Figure 9: Representation of ADK protein levels normalized to tubulin (n= ctr (9), 0,3 mg/kg (6), 1mg/kg (6) and 3mg/kg ABT-702 (5)), mean values relative to the loading control ± SEM, * indicates $p \leq 0.05$ comparing control to ABT-702. (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al. Eur J Pharmacol. 2022;927:175050)

3.2 Effects of oral administration of ABT-702 on ischaemia-reperfusion induced infarct size

Experiments, performed later from Wölkart G. et al. in which I was not directly involved in, showed that orally administered ABT-702 also had a significant effect on ischaemia-reperfusion induced infarct size.

For this purpose, 24 hours after oral administration of ABT-702 (3 mg/kg) Langendorff-prepared hearts were subjected to 20 minutes of ischaemia and 2 hours of reperfusion.

The hearts were then stained with 2,3,5-triphenyltetrazolium chloride (TTC) and measured to describe the size of the infarct area.

Figure 10: Representation of the infarct size induced by ischaemia-reperfusion and the effect of oral administration of ABT-702 at 3mg/kg thereon. (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al Eur J Pharmacol. 2022;927:175050)

Visual differences can already be seen between the normal oxygenation state, the ischaemia-reperfusion state and the cardiac tissue protected by orally induced delayed ABT-702 preconditioning.

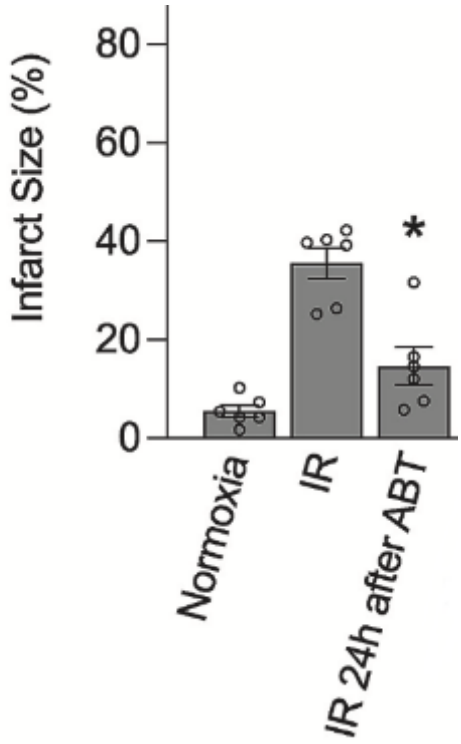

Figure 11: Representation of the measured infarct area (n = 6 normoxia, 6 ischaemia-reperfusion, 6 ischaemia-reperfusion 24h after ABT-702), * indicates $p \leq 0.05$ comparing IR and IR + ABT-702 (Reference: From Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection, Wölkart G. et al Eur J Pharmacol. 2022;927:175050)

The size of the infarct area in hearts of ABT-702 treated mice was reduced by more than 50 % as compared to control hearts.

4 Discussion

This thesis had two objectives, firstly to find out whether intraperitoneal injection of 10mg/kg ABT-702 in mice induces an effect on cardiac adenosine receptor mRNA expression 24h after treatment. Second, to perform a dose-response analysis of oral administration of ABT-702 (3, 1 or 0.3 mg/kg) by Western blot to investigate cardiac adenosine kinase expression.

Using qRT-PCR, it was found that there were no significant differences between control and ABT-702 treated mice on cardiac adenosine receptor mRNA expression. This means that the apparent changes in adenosine receptor-dependent effects, such as increased coronary flow, are not due to changes in adenosine receptor expression. Further examination of the mechanism of sustained cardioprotection Wölkart et al. revealed (by Western blot analysis), that there were no changes in several enzymes involved in adenosine metabolism, such as AK1, cd73 or ADA protein levels after 24 hours, but there was significant reduction in ADK expression after treatment with ABT-702.
Another key piece of information was how the NOS behaves under these conditions and whether the late increase in coronary flow is due to it. It was found that neither inducible and endothelial nitric oxide synthases (iNOS and eNOS) were significantly affected by ABT-702 administration. This suggests among other things that the mechanism of cardioprotection by ABT-702 was a reduction in ADK protein expression. Further experiments performed by other authors in Wölkart et al, demonstrated that ABT-702 caused proteasomal degradation of ADK through an adenosine receptor dependent mechanism (52).

Furthermore, a dose-response analysis of oral administration of ABT-702 was performed by Western blot, which showed that low doses (1 or 0.3 mg/kg) decreased cardiac adenosine kinase expression in some mice and had no effect in others, so that the difference from control mice did not reach significance. However, at a dose of 3 mg/kg, the adenosine kinase protein was found to be significantly reduced even after 24 hours. Later, it was found that the reduction in ADK expression lasts up to 72 hours, suggesting the potential for long term increase in adenosine-mediated protective effects.

As mentioned earlier, ADK consists of two isoforms, ADK-L, which is at 48 kDa and ADK-S, which is at 46 kDa. Treatment with ABT-702 has a strong reducing effect on the cardiac ADK-L form, whereas there was only a smaller effect on the short cardiac form, but the reduction in total ADK was about 40%. Further experiments indicated that treatment with ABT-702 causes the degradation of ADK-L rather than increasing the overall activity of the proteasome.
In addition, other tissues, such as liver and skeletal muscle treated with ABT-702 demonstrated no effect on ADK expression (52). To corroborate the suspicion of adenosine receptor-dependent proteasomal degradation of ADK, experiments were performed involving concomitant administration of bortezomib, a proteasome inhibitor. These induced conditions partially reversed the reduction in cardiac ADK protein. This fact reinforces the assumption of a post-transcriptional mechanism in the ABT-702-induced reduction of cardiac ADK protein. As a result, there is a sustained decrease in adenosine metabolism beyond the life-span of ABT-702 in vivo (52).

Additional data also show that oral administration of ABT-702 (3mg/kg) induces ADK degradation and protects against IR-induced myocardial injury via sustained adenosine release. This explains the increase basal coronary flow 24 hours after ABT-702 treatment.
The sustained cardioprotection by ABT-702 is due to increased adenosine receptor signalling just prior to IR. These results suggest an adenosine receptor-induced positive feedback loop that causes a sustained increase in myocardial adenosine and cardioprotection against IR. This adenosine-generating positive feedback loop might be exploited pharmacologically for long-term cardioprotection.

While these data indicate oral ABT-702 can exert protective actions against ischemia-reperfusion injury, there may also be potential risks in the use of ADK inhibitors. For instance, excessive adenosine release due to ADK inhibition can cause side effects, such as lowering heart rate, blood pressure and also body temperature (70).
Additionally, it was previously shown that cardiac-specific knockout of ADK increased stabilization of cardiomyocyte microtubules and exacerbates pressure overload-induced left ventricle dysfunction in mice (71).

In terms of side effects, studies for the paper Adenosine kinase (ADK) inhibition with ABT-702 induces ADK protein degradation and a distinct form of sustained cardioprotection by Wölkart et al. showed that intraperitoneal injection of 10 mg/kg reduced exploratory activity in mice and lowered body temperature by up to 10 % for up to three hours (unpublished observations). However, a lower dose of 3 mg/kg did not lower the body temperature after three hours, but reduced the ADK protein level (52). Thus, proper dosing may be able to induce a sustained decrease in ADK expression that is sufficient to achieve cardioprotection in vivo without undesirable side-effects.

Limitations and Strenghts

There are also limiting factors of this work. The number of samples could be increased, particularly with regard to the dose-response analysis, so that a more precise determination of the smallest possible, effective dose might be achieved.

Translation to humans is not possible with certainty, as this is hypothesis-generating work. However, the fact that the effect could be shown despite the small sample size and that the data generated are also in line with previous theories can be cited as a strength. The combination of different methods such as Langendorff and Western blot is also positive.

Conclusion

Oral treatment with ABT-702 can provide delayed cardioprotection through a novel mechanism that does not involve changes in adenosine receptor expression, but instead is dependent upon decreased ADK expression. Understanding the mechanism(s) by which ABT-702 decreases cardiac ADK expression could lead to new therapies that provide sustained cardioprotection.

5 References

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