The critical role of MAP-kinases and PI3K-Akt signaling pathways in inflammation and oxidative stress

PhD Thesis

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Abbreviations

AP-1: activator protein-1
Akt: Protein Kinase B (PKB)
Ask1: apoptotic signal regulating kinase
BAD: Bcl-2 associated death promoter
ERK: extracellular signal-regulated kinase
FKHR: forkhead homolog rhabdomyosarcoma transcription factor
GSK-3β: Glycogen synthase kinase 3 beta
IκB: inhibitors of NF-κB
IKK: inhibitor of NF-kappa B kinase
iNOS: inducible nitric oxide synthase
IRAK1: interleukin-1 receptor-associated kinase-1
IRAK4: interleukin-1 receptor-associated kinase-4
JNK: c-jun N-terminal kinase
LBP: LPS binding protein
LPS: lipopolysaccharide
MAPK: mitogen activated protein kinase
MKP-1: MAPK phosphatase -1
MyD88: myeloid differentiation primary response gene 88
NAC: N-acetyl cysteine
NADPH-oxidase: nicotinamide adenine dinucleotide phosphate-oxidase
NEMO: NF-κB essential modifier
NF-κB: nuclear factor-κB
P: phosphorylated
PARP: poly ADP-ribose polymerase
p38-MAPK: p38 mitogen activated protein kinase
p65: transcription factor p65 (RelA)
p50: NF-KappaB1
PI3K: phosphoinositide 3-kinase
PTEN: phosphatase and tension homolog deleted on chromosome 10
ROS: reactive oxygen species

TAB: TAK1-binding protein

TAK1: transforming growth factor- β-activated kinase 1

TBK1: TANK binding kinase 1

TIRAP: TIR domain-containing adaptor protein

TLRs: Toll-like receptors

TLR4: Toll-like receptor 4

TNFα: tumor necrosis factor-α

TRAF6: TNF receptor-associated factor 6

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor inducing IFN-β

Introduction

Inflammation and oxidative stress are main pathophysiological processes responsible for the cardiovascular morbidity and mortality. Both mechanisms activate and moderate same intracellular signal mechanisms. Recently, investigations focus to understand signal networks because they would become potential therapeutic target. On the other hand it may be consider to investigate the effect of new and "older" therapeutic drugs on signaling pathways.

Red wines are rich in different types of polyphenols and anthocyanins, and they have been reported to provide greater benefit in the prevention of cardiovascular diseases. Phenolic compounds have strong antioxidant, neuroprotective effect and their anticarcinogen and anti-inflammatory effects are also proven.

Malvidin and its glycosides are primary plant pigments playing an important role to protect plants from microbial infection and UV irradiation. Malvidin is responsible primarily for the color, and is included in the polyphenols of red wine together with other anthocyanidins, phenolic acids, flavonoids and trihydroxy stilbenes. Earlier studies have demonstrated that moderate red wine consumption reduce cardiovascular morbidity and mortality and have beneficial effects on chronic inflammatory diseases. These protective effects is contributed to the polyphenol contents of red wine.

The inflammatory response was extensively studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, which are very sensitive to LPS stimulation and respond by activation of the pro-inflammatory transcription factors; nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) resulting in tumor necrosis factor-alpha, interleukin-1beta (IL-1β, IL-6, IL-8) and nitric oxide production. These markers are associated with gram-negative sepsis and other inflammatory diseases. Furthermore, LPS also induces production of reactive oxygen species (ROS) and activation of the nuclear enzyme poly ADP-ribose polymerase (PARP). ROS are capable of eliciting a variety of pathological changes, including peroxidation of lipids, proteins, DNA damage, and elevated level of ROS activates mitogen activated protein kinase (MAPKs) and inflammatory transcription factors. Probably as compensatory mechanisms, LPS induces activation of the cytoprotective phosphytidylinositol 3-kinase (PI3K)-Akt pathway and expression of MAPK phosphates (MKP)-1. All these processes have significant role in innate immunity during the normal

immune response and in causing multiple organ failure and death during severe sepsis or septic shock.

The most investigated nutritional polyphenol, resveratrol was found to prolong lifespan, and was suggested as a potential anti-inflammatory, anti-aging, anti-cancer and protective cardiovascular agent. However, rather low bioavailability and abundance of resveratrol implies that other components may contribute substantially to the beneficial effects of red wine. A likely candidate is malvidin that exceeds resveratrol content at least 100 times in red wines. Recent data describe its beneficial effects in cardiovascular disease. On the other hand, only limited data are available about effect of malvidin on inflammatory processes and kinase signaling pathways. Therefore, in this study, we investigated the effect of malvidin on LPS induced processes in RAW 264.7 macrophages.

Another main goal of our study was to investigate oxidative stress which plays a crucial role of development and progression of cardiovascular diseases. Ischemia-reperfusion is one of the most common process is associated with enhanced formation of reactive oxygen species. Ischemia-reperfusion injury is a main pathophysiological process during acute cardiovascular events. Earlier studies widely investigated and searched potent drugs, which are able to promote cells against ischemia-reperfusion injury. Aim of these studies were to identify potential targets via can be modulated these mechanisms. Several studies have shown that ROS and proapoptotic signaling pathways play pivotal role in cell damage and death.

Reactive oxygen species (ROS) and elevated intracellular $Ca^{2+}$are implicated as important factors among the mechanisms leading to postischemic reperfusion injury. High levels of ROS can be generated during reperfusion from a variety of sources such as the xanthine oxidase system, the cyclo-oxygenase pathway of arachidonic acid metabolism, the respiratory burst of phagocyte cells and to a large extent, from the non-optimal operation of the mitochondrial electron transport chain. Elevated ROS levels can also induce oxidative DNA damage leading to single-stranded DNA breaks, which activate the nuclear protein-modifying and nucleotide-polymerizing enzyme poly (ADP-ribose) polymerase. PARP activation leads to NAD^+ and ATP depletion, which further destabilizes mitochondrial membrane system and eventually leads to apoptotic or necrotic cell death. Recently, inhibition of PARP was implicated in the therapy of myocardial infarction.

Among the kinase signaling pathways, PI-3K-Akt and mitogen activated protein kinase (MAPK) were implicated among the mechanisms of cardioprotection. Akt activation can be critical because Akt can phosphorylate a number of downstream targets leading to inactivation of GSK-3β, the pro-apoptotic Bcl-2 family member BAD, caspase-9, and

forkhead homolog rhabdomyosarcoma transcription factor (FKHR), as well as to activation of nuclear factor-κB (NF-κB), p70 ribosomal S6 kinase and endothelial nitric oxide synthase. Recently, we have proved that activation of Akt pathway significantly contributed to the cytoprotective effect of PARP inhibition under pathological conditions, however no such mechanism was previously indicated among the molecular effects of calcium channel- and β-adrenergic receptor blockers.

Aim of the study

ROS plays a key role in the pathogenesis and progression of septic shock and ischemia-reperfusion injury. To investigate direct effect of ROS and activated signaling pathways support development of new therapeutic targets. Our studies focus to identify crucial signaling pathways involved in inflammation and oxidative stress used two separate model (cell culture and Langendorff perfused heart). We investigate the effect of malvidin on inflammatory response and the effect of Metoprolol, Verapamil and PARP inhibitor on ischemia-reperfusion injury.

1. We intended to determine antioxidant and anti-inflammatory effectivity of malvidin.

2. We wanted to compare antioxidant and anti-inflammatory effects of malvidin and resveratrol on the molecular basis to find out which component is most likely to account for the positive effects of moderate red wine consumption.

3. We wanted to know whether activation of PI-3K-Akt and MAPK signaling pathways are involved in the molecular action of Ca^{2+} antagonist and a β-adrenergic receptor blocker cardioprotective drugs.

4. We intended to compare the cardioprotective potential of PARP inhibition, Ca^{2+} antagonism and blocking of the β-adrenergic receptor.

Discussion

Antioxidant and anti-inflammatory effects of malvidin

In response to LPS, nuclear localization signal of cytosolic NF-κB becomes unmasked resulting in nuclear translocation of the transcription factor. In the nucleus, NF-κB becomes phosphorylated and acetylated, thus activated to bind to its consensus promoter DNA sequences. This binding triggers the expression of its target genes (Fig.) including pro-inflammatory cytokines, chemokines, adhesion proteins, COX-2 and iNOS. These events are of pivotal importance in the development of inflammation-related chronic diseases. We demonstrated malvidin attenuates activating phosphorylation, nuclear translocation and binding to consensus DNA sequence of NF-κB. These data are completely in line with the results of other groups. Furthermore, we found malvidin antagonised NF-κB activation at much lower concentrations than trans-resveratrol. This indicates malvidin could account for the beneficial effects of red wine in inflammation-related chronic diseases. Furthermore, these results explain the finding of the 1999-2002 US National Health and Nutrition Examination Survey describing malvidin intake negatively correlates with serum C-reactive protein levels.

Binding of LPS to TLR4 receptor triggers activation of the MAPKs (Fig.) via various signaling pathways such as the myeloid differentiation primary response gene (MyD)88─interleukin-1 receptor-associated kinase (IRAK)─tumor necrosis factor (TRAF)-6─transforming growth factor-β activated kinase (TAK) pathway. In turn, MAPK pathways are involved in activation of the pro-inflammatory transcription factors; NF-κB and AP-1. In the present study, we observed malvidin attenuated LPS induced activation of all three MAPKs. However, this effect differed for the three kinases (JNK>p38>>ERK). By using specific kinase inhibitors, we aimed to establish the significance of these results. In agreement with others we found JNK and p38 inhibitors significantly reduce LPS induced nuclear translocation and DNA binding of NF-κB. However, ERK inhibition was ineffective. These data indicate early inflammatory response in RAW 264.7 macrophages is mediated, at least partially, via the aforementioned pathway. On the other hand, it is likely that malvidin decreased LPS evoked MAPK activation undirectly since in *in vitro* kinase assays malvidin

did not exert any effect. Most likely, it regulated MAPK activation by inhibiting other key mechanisms; ROS production.

MKP-1 is the major enzyme responsible for the dephosphorylation, thereby inactivation of all three MAPKs. It is critically involved in inflammatory signaling of macrophages, and is responsible for switching off pro-inflammatory cytokine production *in vitro* and *in vivo*. In agreement with others we found increased expression of MKP-1 in the LPS stimulated macrophages both at the mRNA and protein level. However, this was accompanied by an elevated activation of the MAPKs indicating MKP-1 induction was not sufficient to suppress LPS-induced MAPK activation. Malvidin enhanced MKP-1 expression both in the unstimulated and LPS treated cells, which was accompanied by decreased activation of the MAPKs. This suggests MKP-1 expression, when augmented by malvidin, could counteract the activating mechanisms induced by TLR4 signaling (Fig.). However, we found significant differences among the MAPKs regarding malvidin's effectivity in reduction of their LPS induced activation. Furthermore, these differences were reflected in the anti-inflammatory effect of MAPK inhibitors. All these data indicate, the network of MAPK activation and inhibition signaling is complex, and balance of the regulating processes differs for each MAPK.

Previous studies established *in vitro* antioxidant characteristics for malvidin. In agreement with these results, we found malvidin attenuates ROS production by LPS-treated RAW264.7 macrophages at an IC_{50} value comparable to that of trans-resveratrol. At the same time, this modulated a complicated network of processes produced and regulated by ROS (Fig.) including mitochondrial integrity and activation of MAPKs, Akt and PARP. It is feasible that LPS induced NF-κB activation in our experimental system was mediated partially via the TLR4-NADPH oxidases-ROS-PARP pathway. However, the complexity of the involved networks made it hard to distinguish between cause and consequence or identify upstream and downstream events. Nevertheless, it is likely that due to its antioxidant property, malvidin decreases ROS production, thereby reduces PARP and MAPK activation as well as oxidative damage to MKP-1. Reduced PARP activation leads to decreased NF-κB and MAPK activation, increased expression of MKP-1 and activation of the PI3K─Akt pathway that together with the decreased ROS results in maintained mitochondrial integrity (Fig.). Importance of the antioxidant mechanism in malvidin's anti-inflammatory effect is emphasized by us and others reporting NAC inhibits LPS induced NF-κB activation.

Recently it has been shown that Akt is a downstream target of TRIF/TANK-binding kinase 1 (TBK1), and there is an association between endogenous TBK1 and Akt in LPS

treated macrophages. TBK1 enhances phosphorylation of Akt on Ser(473), and siRNA-mediated silencing or knocking out of TKB1 compromises LPS induced Akt activation. On the other hand, elevated ROS also activates the PI-3K─Akt pathway via oxidative inactivation of the phosphatase and tensin homolog (PTEN) that inactivates the pathway by dephosphorylation. Akt activation may result in mitochondrial protection by phosphorylation, thereby inactivation of Bad, and indirect NF-kappaB activation. As we found, malvidin activated Akt both in the unstimulated and LPS-treated macrophages. Most likely, this effect of malvidin was also due to its antioxidant property. The augmented activation of Akt was most probably involved in malvidin's protective effect on LPS induced mitochondrial depolarization (Fig.). On the other hand, Akt was implicated in the phosphorylation thereby activation of NF-κB p65. Accordingly and in agreement with Zhao et al., we found that inhibition of the PI-3K-Akt pathway attenuated NF-κB activation suggesting a partial involvement of this pathway in mediating LPS's effect. All these data suggest Akt activating effect was unlikely to be involved in malvidin's anti-inflammatory effect.

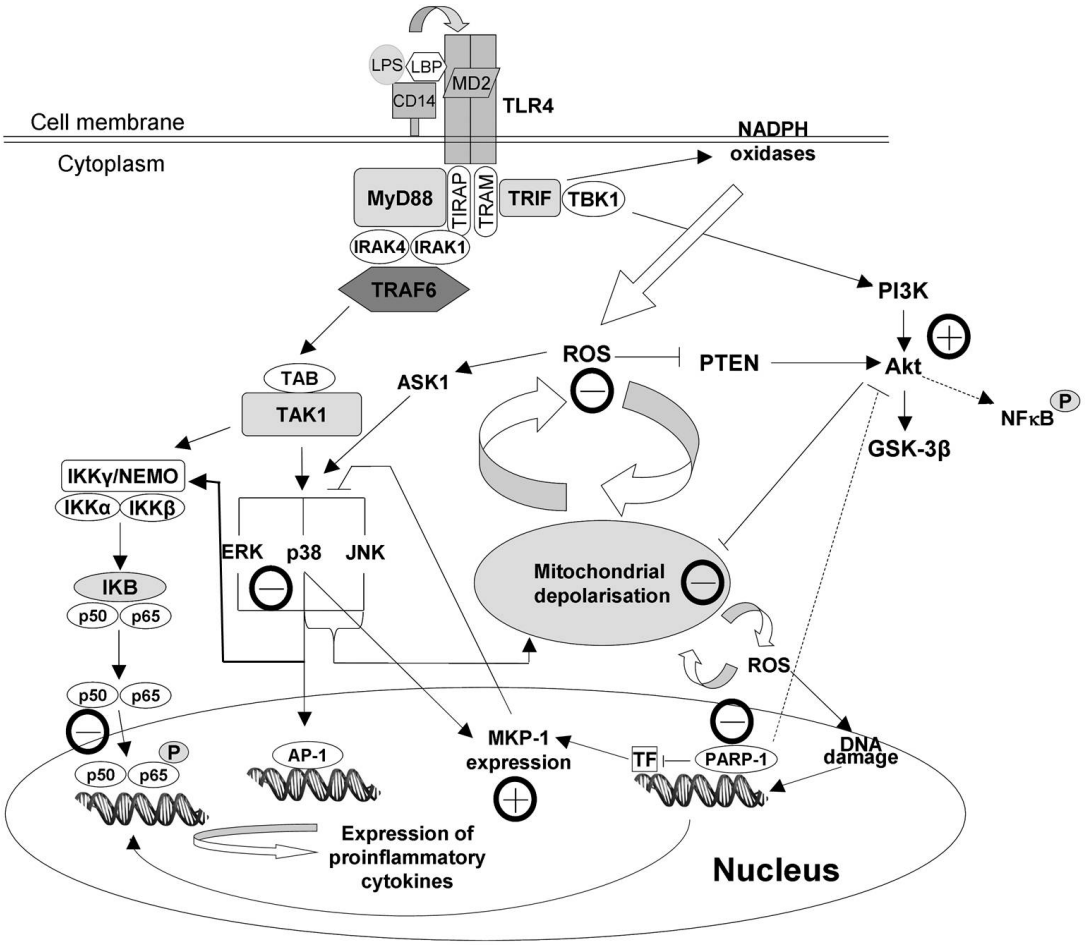

Figure. Effect of malvidin on LPS induced pathophysiological changes in RAW 264.7 macrophages. Well documented effects are indicated by solid lines, whereas effects involving yet unidentified mediator(s) or events are represented by dashed line. Lines with pointed end denote activation, whereas lines with a flat end indicate inhibition. Activating or inhibitory effect of malvidin is indicated by a circled + or ─ next to the line, respectively. LPS induces activating phosphorylation, nuclear translocation and DNA binding of NFκB, induction of ROS production, PARP activation, activation of MAPKs, MKP-1 expression, activation of the phosphatidylinositol-3 kinase-Akt pathway and destabilization of the mitochondrial membrane systems. Malvidin attenuates ROS production, mitochondrial destabilization, and activation of PARP and MAPKs. It also augments Akt activation and MKP-1 expression resulting in diminished activation of NFκB.

Comparison of cardioprotective effects of PARP inhibition, Ca^{2+} antagonism and β-adrenergic receptor blocking

In the present study, we assessed cardioprotective as well as Akt and ERK activating effects of two substances used in the clinical management of ischemic heart diseases, namely, Verapamil and Metoprolol in Langendorff perfused hearts during ischemia-reperfusion cycle, and compared them to that of HO-3089, a PARP inhibitor used as a positive control throughout the experiments.

Among other mechanisms, L-type Ca^{2+}-channel antagonists were reported to protect high-energy phosphates and cardiac functions as well as reduction of acidosis, infarct size in different ischemia models. Cardioprotecting mechanisms of β-adrenergic receptor blockers were reported to include improvement of cardiac functions, decrease of lipid peroxidation and cardiomyocyte apoptosis. Although it is very difficult to compare the results of different laboratories due to the different models and different ischemia and reperfusion protocols, our results on the different parameters of cardioprotection by Verapamil and Metoprolol were comparable with that of other laboratories.

In our experimental setup, we monitored the high-energy and inorganic phosphate levels during a 15 min equilibrating normoxic perfusion, and any significant deviation from the starting values in case of the untreated hearts resulted in the rejection of the given heart from the experiment. In the same way, we excluded those hearts treated or not, which did not regain spontaneous pulsing during the reperfusion period. We used a single concentration for Verapamil and Metoprolol since they were extensively studied in the past, and their effective concentrations in Langendorff-perfusion experiments were well established. The concentration (25μM) of HO-3089 was found to protect hearts against ischemia-induced damages in previous experiments.

According to our data, Verapamil and Metoprolol improved the recovery of creatine phosphate, ATP and pH, and the reutilization of inorganic phosphate in hearts subjected to ischemia-reperfusion significantly, although less effectively than HO-3089 did. The substances significantly attenuated oxidative myocardial damages, which were characterized by decreased lipid peroxidation and protein oxidation too. Furthermore, the favorable changes in cardiac energetics were accompanied by improved recovery of functional cardiac parameters and reduced infarct size.

We have previously demonstrated that activation of the cytoprotective PI-3 kinase/Akt pathway significantly contributed to cardioprotective and cytoprotective effects of PARP

inhibition. Activated Akt can phosphorylate several regulatory proteins, including GSK-3β, caspase-9, BAD, or FKHR. Phosphorylation and so inactivation of pro-apoptotic BAD protein contribute to the stabilization of mitochondrial membrane systems and may prevent the release of pro-apoptotic proteins, i.e. cytochrome c or apoptosis-inducing factor. Moreover, Akt can also phosphorylate and inactivate caspase-9, which can result in the blockade of cytochrome c/Apaf-1/caspase-9/caspase-3 pathway.

Although ERK 1/2 is considered to be mainly involved in growth factor-induced mitogen signaling and cellular differentiation, this kinase was reported to exhibit protective roles under circumstances of oxidative stress through the inhibition of apoptosis by downregulating c-jun N-terminal kinase (JNK) as well as caspase-3. ERK was also brought into connection with delayed cytoprotection, because its sustained activation seemed to mediate late cardiomyocyte protection after simulated ischemia-reoxygenation.

Because of their indicated involvement in cytoprotection, we studied effect of Verapamil and Metoprolol on Akt and ERK 1/2 phosphorylation during ischemia-reperfusion. To demonstrate activation of Akt, we determined phosphorylation of its downstream target, GSK-3β too. As we found, all the substances induced significantly elevated activation of Akt and ERK1/2 either in normoxia or after ischemia-reperfusion. We found even a strong correlation between extent of recoveries of creatine phosphate levels and Akt or ERK2 activation for the substances studied indicating a crucial role of Akt and ERK2 activation among the cardioprotective mechanisms of Verapamil and Metoprolol. An increasing number of evidence suggests that β-adrenergic receptor antagonists can induce β-arrestin-mediated trans-activation of epidermal growth factor receptor leading to activation of the down-stream target ERK, and a similar mechanism was reported for Akt activation that involved β-arrestin-1 and insulin-like growth factor 1. Although linking of L-type calcium channel blockers to Akt and ERK activation seems more elusive, calmodulin-dependent cyclic nucleotide phospho-diesterase that was indicated as a key regulator in the cross talk between cyclic nucleotide- and Ca^{2+}-dependent as well as Ca^{2+}-independent-signaling pathways might play role.

Regardless of the mechanism, for establishing physiological significance of the activation of these kinases in the cardioprotection by the β-adrenergic receptor blocker and the Ca^{2+} antagonist, we inactivated these kinases by inhibiting their respective upstream activator kinases during the ischemia-reperfusion cycle. The finding that inhibition of these kinases significantly decreased recoveries of high-energy phosphate levels for not just the PARP

inhibitor but for Verapamil and Metoprolol too clearly indicated that activation of Akt and ERK1/2 significantly contributed to the cardioprotective effects of these substances.

Conclusion

Ischemic heart disease and sepsis are the main causes of morbidity and mortality worldwide. Oxidative stress and inflammation play a pivotal role in both diseases. Searching for therapeutic targets we investigated the effects of naturally occurred polyphenol, malvidin and clinically used two drugs, Metoprolol and Verapamil. We investigated several signaling pathways which potentially could be therapeutic targets.

In conclusion, malvidin, the most abundant polyphenol ingredient of red wine, augments LPS-induced Akt activation and MKP-1 expression and attenuates mitochondrial destabilization, ROS production and activation of PARP as well as MAPKs resulting eventually in diminished activation of NFκB. All these data indicate malvidin significantly contributes to the antioxidant and anti-inflammatory effects of red wine, and could, at least partially, account for the positive effects of moderate red wine consumption on inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease.

We demonstrated at the first time that activation of PI-3K-Akt and ERK1/2 pathways significantly contributed to cardioprotective effect of a Ca^{2+} antagonist and a β-adrenergic receptor blocker. Furthermore, we found a strong correlation between cardioprotective and kinase activating potencies of the substances that indicate potentiality of these kinases as drug-targets in the therapy of ischemic heart disease.

Achievements

1. We demonstrated that malvidin, the most abundant polyphenol ingredient of red wine, augments LPS-induced Akt activation and MKP-1 expression and attenuates mitochondrial destabilization, ROS production and activation of PARP as well as MAPKs resulting eventually in diminished activation of NFκB.

2. We found that anti-inflammatory effect of malvidin exceeded that of resveratrol. Therefore, we provided experimental evidence at the first time that malvidin rather than resveratrol could account for the positive effects of moderate red wine consumption on inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease.

3. We demonstrated at the first time that activation of PI-3K-Akt and ERK1/2 pathways significantly contributed to cardioprotective effect of a Ca^{2+} antagonist and a β-adrenergic receptor blocker.

4. We found that cardioprotective effects of PARP inhibition much exceeded that of Ca^{2+} antagonism and blocking of the β-adrenergic receptor thereby confirmed the potentiality of PARP inhibitors in the management of cardiovascular diseases.

5. Furthermore, we established a strong correlation between cardioprotective and kinase activating potencies of the substances that indicate potentiality of these kinases as drug-targets in the therapy of ischemic heart disease.

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Publications/Publikációk

This work based on the following articles/Dolgozathoz kapcsolódó publikációk:

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