SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

(PhD)

CELL-BASED HIGH THROUGHPUT SCREENING FOR THE IDENTIFICATION OF CYTOPROTECTIVE COMPOUNDS AND DOXORUBICIN-INDUCED DAMAGE

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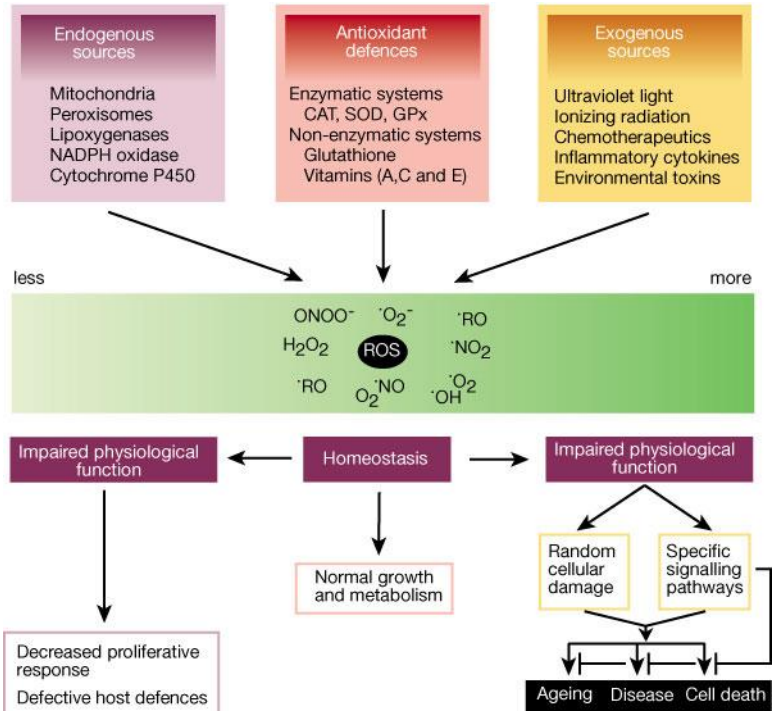
1. INTRODUCTION

Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low/moderate concentrations is the induction of a mitogenic response.

The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress. This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other.

Oxidative stress results from an imbalance between the production and elimination of reactive oxygen and nitrogen species (ROS and RNS, respectively). Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production. A sophisticated enzymatic and non-enzymatic antioxidant defence system counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defence. Similarly, increased ROS may also be detrimental and lead to cell death or to an acceleration in ageing and age-related diseases as shown below **(**Finkel, T. Holbrook, N.J. Nature *408*, 239-247, 2000).



Most disease states are accompanied by oxidative stress which contributes to tissue injury. Mechanism of oxidative stress-induced tissue injury includes oxidative damage to proteins, lipids and DNA culminating in cell dysfunction, apoptosis or necrosis depending on the intensity of oxidative stimuli. In addition to cumulative damage to macromolecules, oxidative stress also triggers tightly regulated signalling pathways mediating cell dysfunction, apoptosis and necrosis. These pathways include but are not limited to MAP kinases, apoptotic signal regulated kinases, and poly (ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a nuclear enzyme activated by DNA breakage. Activated PARP-1 tags the proteins at the site of DNA damage with an NAD^+-derived biopolymer, poly(ADP-ribose) (PAR) and thus facilitates DNA repair. However, during severe oxidative stress,

PARP-1 contributes to necrotic cell death via depletion of its substrate NAD+ and consequently ATP and in some cell types by activating the cell death pathway mediated by apoptosis-inducing factor.

Oxidative stress-induced cell death and the role of the above signalling pathways have been demonstrated in various life threatening conditions such as neurodegenerative disorders, myocardial infarction, stroke, different forms of shock and ischemia–reperfusion injury of different organs. A wide array of approaches has been devised to combat these diseases and many of these efforts aim at preserving cellular integrity. Cytoprotective compounds such as antioxidants, PARP inhibitors, JNK inhibitors, potassium channel openers have been shown to effectively protect tissues from oxidative tissue injury.

Nonetheless, successful treatment of oxidative stress-related diseases continues to pose challenges for physicians and requires the development of novel treatment modalities. The aim of the current study was to identify cytoprotective compounds with the potential to provide therapeutic benefit in conditions associated with tissue injury.

Doxorubicin is an anthracycline compound originally isolated from bacteria of the Streptomyces genus and used extensively for the treatment of various types of cancer. Acute leukaemia, Hodgkin and non-Hodgkin lymphomas, osteosarcoma, Ewing sarcoma, breast cancer, neuroblastoma, small cell lung cancer respond well to doxorubicin monotherapy or combination therapy. Even though doxorubicin and other anthracycline compounds such as daunorubicin have been used by oncologists for more than four decades, their mechanism of action is still not fully understood. Inhibition of topoisomerase II, generation of reactive oxygen species, DNA intercalation and triggering a signalling cascade that involves increased ceramide production, cleavage of the ER membrane protein CREB3L1, nuclear translocation of the N-terminal fragment of this protein and transcriptional

activation of genes that inhibit cell proliferation have been suggested to be responsible for the antitumor effect of anthracyclins.

The clinical use of doxorubicin is limited mainly by its severe cardiotoxic effect, which may lead to irreversible cardiomyopathy and heart failure. Incidence of heart failure shows close correlation with the cumulative dose of the drug. Cardiotoxicity is indicated by morphological alterations (myofibrillar disarray and vacuolization) as observed in cardiac biopsy specimens. Moreover, leakage of troponin can also be detected in the peripheral blood and shows positive correlation with the intensity of heart damage.

The mechanism of doxorubicin cardiotoxicity is complex and is closely linked to production of reactive oxygen species. These form in direct electron exchange between the oxygen molecule and the anthracycline's quinone moiety and can also be produced in redox cycling of doxorubicin-iron complexes. The significance of ROS signalling in doxorubicin-induced heart failure is supported by a number of animal experiments demonstrating the effectiveness of a ferroporphyrine antioxidant a vitamin E prodrug or a poly(ADP-ribose) polymerase (PARP) inhibitor in preventing or suppressing the cardiotoxic effect of doxorubicin. In recent years topoisomerase 2α has emerged as a central mediator of doxorubicin-induced cardiac injury. While topoisomerase 2β (expressed mostly in proliferating cells) is considered as the primary target of doxorubicin in tumour cells, topoisomerase 2α (expressed by quiescent cells) has been made responsible for suppression of antioxidant enzyme expression, inhibition of mitochondrial biogenesis, activation of p53 and p53-mediated apoptosis with all of these cellular events implicated in doxorubicin-induced heart failure.

Despite our increasing knowledge on the mechanism of doxorubicin-induced heart injury, it still represent an unsolved medical problem necessitating more mechanistic studies as well as the development of novel agents for the prevention of the side effect of anthracyclins.

2. OBJECTIVES

The principal aim of the thesis was to establish the high throughput screening (HTS) methodology in the setting of an academic laboratory in order to screen compound libraries and to identify novel cytoprotective agents

The specific aims were as follows:

1. To develop in vitro cell-based HTS assays for the screening of potentially cytoprotective and or antioxidant molecules.
2. To validate the HTS assays by well-established manual methods.
3. Screening the in-house library (about 2600 molecules) for novel cytoprotective compounds.
4. To develop a screening strategy for the identification of potentially cardioprotective compounds with the capacity to prevent doxorubicin-induced cardiomyocyte injury.

3. MATERIALS AND METHODS

Materials

The compound library of the University of Debrecen (UDM) was established and maintained by the Department of Organic Chemistry. The library contains about 2600 compounds of high (at least 95%) purity with the unique feature of the great number of oxygen heterocycles and their precursors. The compounds were obtained by traditional synthetic methods and show high diversity.

Chembridge compound library 10.000 (San Diego, CA, USA) and doxorubicin were purchased from Teva (Debrecen, Hungary). The sources of other chemicals, assay kits and antibodies are given in the papers published by us (see Appendix items 1 & 2).

Cell lines

H9C2 cells were cultured in DMEM (10% FBS and 2mM glutamine, 5g/L glucose). A549 cell line was cultured in RPMI 1640 medium supplemented with 10% FBS and 2mM glutamine. SAOS-2 cell line was cultured in DMEM (10% FBS and 2mM glutamine, 1g/L glucose).

Primary neonatal rat cardiomyocyte culture

Primary neonatal cardiomyocyte culture was prepared from 1-3 days old Wistar rats. Pups were killed by cervical dislocation, and then the hearts were harvested and rinsed in ice-cold PBS buffer. The ventricles were then chopped and digested in trypsin to increase the number of cardiomyoblasts in the cell suspension. Then cells were plated and maintained in a humidified incubator (37ºC, 95% O_2 and 5% CO_2). After 24 hours, the medium was changed to DMEM containing 1% FBS to help cardiomyoblast differentiation.

MTT viability assay (HTS version)

Cells ($7x10^3$/well) were plated to 96-well plates one day before the treatment. Compounds of the library were given to the plates with a Tecan Freedom EVO liquid handling robot (100 nL/well) to reach 10 μM final concentration. After 30 min incubation at 37°C, cells were further treated with the respective protocols. Finally MTT solution was added to the samples, supernatants were aspirated and the assay was performed with a Thermo Multiskan reader at 540nm.

Cytotoxicity assay

Cytotoxicity of the test compounds was determined with resazurin assay as described above. Cells were incubated with the compounds at different concentrations for 24 h and then the resazurin method was used to determine the viability of the cells.

Morphology-based cytotoxicity assay

Cells were treated with the hit compounds of the screening, in the final concentration of 12 μM. After 30 min incubation at 37°C, cells were treated with doxorubicin (300 ng/mL final concentration). Samples were incubated for 24 hours at 37°C. Cell culture medium was changed to Coomassie staining solution and incubated for 20 minutes. Samples were washed and dried. Photos were taken and image analysis performed with Tscratch software was used to determine the size of cell-covered areas.

ABTS decolourization assay

Preparation of ABTS was performed as described [10]. Assays were done in 96-well plates in triplicates. Samples were incubated at RT for 30 minutes. Absorbance was measured with Victor V^3 multi-label reader (405 nm). Antioxidant

activity was expressed as the percentage of control samples and was compared to the effect of Trolox.

Cupric ion reducing antioxidant capacity (CUPRAC) assay

Measures total antioxidant capacity based on reduction of copper(II) to copper(I). Trolox was used as positive control and the assays were performed in 96-well microplates.

Caspase-3 activity measurement

Apoptosis is mediated by a cascade of proteolytic enzymes known as cysteine proteases or caspases. We have used fluorescent substrate-based caspase-3 assay kit for microplates.

LDH release assay

Cells release LDH after tissue damage since LDH is a fairly stable enzyme, it has been widely used to evaluate the presence of damage and toxicity of cells. In the assay LDH reduces NAD to NADH, which is specifically detected by colorimetric (450 nm) assay.

PARP activity assay (DELFIA)

PARP inhibitory activity of the compounds was tested in DELFIA assay (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay). 96-well polystyrene plates were coated with 0.1% purified histone protein and were incubated overnight at 4^0C. PARP DELFIA assay was performed according to the manufacturer's instructions. PJ34 was used as positive control. Measurements were done with a Perkin Elmer Victor V3 multilabel reader.

Calcein-based viability assay

To further confirm the cardioprotective effect of the lead compound (EODB) against doxorubicin induced cellular damage, two days old primary neonatal cardiomyocyte cultures were pre-treated with 5-25 μM EODB for 30 minutes followed by 300 ng/mL doxorubicin treatment for 24 hr. At the end of the protocol viability was determined by calcein assay. In living cells, the cell-permeable calcein AM (nonfluorescent) is hydrolysed by intracellular esterases to calcein (green-fluorescent). Fluorescence intensity was measured using 490-nm excitation and 520-nm emission filters.

Detection of MAP kinase activation by Western blotting

MAP kinases and their phosphorylated active forms were detected by Western blotting using antibodies for the respective forms of proteins.

Statistical analysis

All experiments were performed three times on different days. Analysis of variance followed by Bonferroni's test was applied for statistical analysis and for the determination of significance with $p < 0.05$ considered as significant. Analysis of variance for Chembridge molecular library was performed by one way ANOVA followed by Tukey's test for statistical analysis and for the determination of significance with $P < 0.05$ considered as significant.

4. RESULTS AND DISCUSSION

HTS screening and validation

By screening the compound library of the University of Debrecen in a resazurin reduction-based cytotoxicity assay, out of 1863 molecules we identified 29 cytoprotective compounds protecting Jurkat cells from hydrogen peroxide-induced toxicity. In the antioxidant assay based on ABTS radical scavenging, 123 compounds were found to have antioxidant properties. To validate our HTS data on antioxidant effects, we also worked out a manual antioxidant assay with the cytoprotective compounds which confirmed the results of the screen. Out of the 29 cytoprotective compounds, 11 were found to possess antioxidant activity (ABTS radical scavenging).

Identifying PARP inhibitory cytoprotective compounds

In a cell-free enzyme assay two compounds of the UDM molecular library showed PARP inhibitory activity. In order to confirm this inhibitory effect in a cellular system we have pre-treated Jurkat and A549 cells with these two compounds and then treated the cells with hydrogen peroxide to stimulate PARP activation and PAR synthesis. Hydrogen peroxide treatment leads to significant PARP activation as indicated by the appearance of PAR polymer as detected in Western blots and immunocytochemistry.

Dibenzoylmethane (DBM) derivatives as novel cytoprotective agents

After retesting our hit compounds in MTT viability assay, and in two additional antioxidant assays (CUPRAC assay and Amplex red assay) DBM derivatives emerged as novel cytoprotective agents with no hydrogen peroxide-specific antioxidant effects. DBM derivatives did not prevent caspase-mediated-apoptotic death in hydrogen peroxide-treated cells, nor have they inhibited

hydrogen peroxide-induced mitochondrial depolarization as would be expected from an antioxidant molecule.

We have also investigated whether any of the MAP kinases [extracellular signal regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase, JNK] are targeted by the cytoprotective DBM compounds. We found that hydrogen peroxide activated ERK1/2 and p38 but not JNK as indicated by Western blot detection of the phosphorylated active form of these kinases. The DBM derivatives inhibited ERK1/2 activation but had no effect on the phosphorylation of p38. These data suggest that these DBM derivatives primarily target the necrotic cell death pathway via inhibition of ERK1/2 kinases.

DBM proved to be a promising chemopreventive agent for different types of cancer (e.g. colon, breast and skin cancers) by mediating the induction of phase II and phase I detoxification enzymes. In gastrointestinal cancers, dietary DBM administration decreased the levels of prostaglandin E2 or leukotriene B4 and inhibited cell survival and growth-related signalling pathways. An antiproliferative effect of DBM has also been reported. Moreover, antiinflammatory activity of a DBM analogue was found to be comparable to aspirin in TPA induced ear oedema.

Screening for cardioprotective compounds protecting from doxorubicin toxicity

We have screened the Chembridge Diverset Compound library consisting of 9680 compounds. For this we used H9C2 rat cardiomyocytes and determined cell viability 24h after doxorubicin treatment. Compounds showing at least 20% cardioprotection were considered potentially cardioprotective. Fifteen compounds met this criteria and were used in subsequent experiments. According to our experience a drawback of MTT-based or similar dehydrogenase activity-based viability assays is the frequent occurrence of false positive hits. Therefore we have analysed the morphology of cells after doxorubicin treatment and retested the 15

primary hit compounds. By determining the surface area occupied by living cells we have detected decreased viability in doxorubicin-treated samples. Out of the 15 compounds retested only compound #10 appeared to convincingly exert protective effect in doxorubicin-treated H9C2 cells. This compound is 3-[2-(4-ethylphenyl)-2-oxoethyl]-1,2-dimethyl-1H-3,1-benzimidazol-3-ium-bromide hereafter referred to as EODB.

Structural analysis of EODB may also give hints to explain both the protective effect of the compound and its side effects. EODB contains a benzimidazole moiety which may be linked to some of these effects. Benzimidazole derivatives represent a pharmacologically active family of agents with demonstrated antiviral, antimicrobial and antidiabetic effects. Moreover, compounds with a benzimidazole scaffold also have demonstrated antitumor effect via inhibition of topoisomerases.

On the one hand this may be important for explaining both the doxorubicin-protective effect and the toxic effect of EODB on tumour cell lines. EODB did not interfere with the antitumor effect of doxorubicin in the two tumour cell line we tested. In fact, it also proved cytotoxic in the absence of doxorubicin. We have not observed toxicity on H9C2 cells but in primary rat cardiomyocytes the toxicity may have contributed to the limited though significant protective effect of EODB.

EODB protects H9C2 cells both from apoptotic and from necrotic cell death.

We have further characterized the cytoprotective effect of EODB. It is well-known that doxorubicin induced cell death has both apoptotic and necrotic features. We have determined cellular caspases-3 activity and release of LDL to assess apoptotic and necrotic cell death, respectively. Pre-treatment of the cells with EODB inhibited both caspases-3 activation and LDH release indicating protection from both apoptotic and necrotic cell death.

EODB protects primary rat cardiomyocytes from doxorubicin-induced damage

Cell-based screening programs typically utilize immortalized cell lines due to relatively cheap culture, easy manipulation (e.g. gene silencing) and availability of high number of cells. However, the spontaneous or induced mutations that were required for immortalization, may alter the biological behaviour and responses of these cell. Therefore we have also investigated the effect of EODB on primary rat cardiomyocytes. We found that at 12 μM concentration EODB provided a significant (18 %) protection from doxorubicin-induced toxicity.

EODB lacks antioxidant activity

Since generation of ROS and RNS is considered as an important event in doxorubicin-induced cardiac damage and experimental compounds providing protection against doxorubicin-induced cardiotoxicity possess antioxidant effect we set out to determine whether or not EODB can scavenge radicals. First we tested the compound in parallel with Trolox (positive control) in ABTS decolourisation assay but it had no radical scavenging effect. In the CUPRAC assay which detects reducing activity the compound also showed no such effect. Thus it appears that the cytoprotective effect of EODB is not due to an antioxidant effect.

Thus it is quite likely that the cardioprotective effect of EODB is indirect and may interfere with damage-signalling pathways. In the doxorubicin-induced cardiotoxicity model, the lack of antioxidant effect is not incompatible with cardioprotection as indicated by the protective effect of inhibitors of poly(ADP-ribosyl)ation, topoisomerase or angiotensin type-1 receptor. EODB may also target a step in one of the many cell death pathways. Our data demonstrated that it inhibits both apoptotic and necrotic cell death suggesting that it more likely interferes with a proximal event of damage-signalling rather than specifically

targeting one particular cell death pathway. In fact such “indirect” effects may bear higher clinical relevance than direct radical scavenging, because, despite promising preclinical data, antioxidant approaches (e.g. N-acetyl cysteine or iron chelation) were not effective in humans.

EODB does not interfere with the antitumor effect of doxorubicin

For a potential drug candidate to be used to protect cardiomyocytes in doxorubicin-treated cancer patients, it is important not to compromise the antitumor effect of doxorubicin. Doxorubicin is used in the treatment of different kinds of tumours including cancers of lung and bone origin. Therefore we tested whether or not EODB affects the cytotoxic effect of doxorubicin in A549 lung epithelial carcinoma and SAOS-2 osteosarcoma cell lines. We found that EODB did not interfere with the tumour cell killing activity of doxorubicin. Interestingly, EODB alone (without doxorubicin) was toxic to these cancer cell lines; an effect we have not observed either in H9C2 cell cultures.

In summary, our experiments proved the viability of the cell-based HTS approach for the identification of doxorubicin protective compounds. EODB protected both H9C2 cells and rat primary cardiomyocytes from doxorubicin-induced toxicity without hampering the anti-tumour effect of doxorubicin. Through structure optimization EODB may serve as a template for the development of compounds protecting heart cells from doxorubicin-induced toxicity. Further investigations are needed to identify the exact molecular target of this promising drug candidate.

5. SUMMARY

Screening of a small in-house library (University of Debrecen) of 1863 compounds identified 29 compounds that protected Jurkat cells from hydrogen peroxide-induced cytotoxicity. From the cytoprotective compounds eleven proved to possess antioxidant activity (ABTS radical scavenger effect) and two were found to inhibit poly(ADPribosyl)-ation (PARylation), a cytotoxic pathway operating in severely injured cells. Four cytoprotective dibenzoyl-methane (DBM) derivatives were investigated in more detail as they did not scavenge hydrogen peroxide nor did they inhibit PARylation.

DBM compounds protected cells from necrotic cell death while caspase activation, a parameter of apoptotic cell death was not affected. Hydrogen peroxide activated extracellular signal regulated kinase (ERK1/2) and p38 MAP kinases but not c-Jun N-terminal kinase (JNK). The cytoprotective DBMs suppressed the activation of Erk1/2 but not that of p38.

Cytoprotection was confirmed in another cell type (A549 lung epithelial cells), indicating that the cytoprotective effect is not cell type specific. In conclusion we identified DBM analogues as a novel class of cytoprotective compounds inhibiting ERK1/2 kinase and protecting from necrotic cell death by a mechanism independent of poly(ADP-ribose) polymerase inhibition.

Anthracyclins are effective anti-tumour agents. One of the most commonly used anthracyclins is doxorubicin, which can be successfully used to treat a diverse spectrum of tumours. Application of these drugs is limited mainly by their cardiotoxic effect, which is determined by a lifetime cumulative dose. As the processes that are responsible for tumour chemotherapy and cardiotoxicity are different, this may give a hope for eliminating the side effect without affecting the anti-tumour effect.

In our recent work, 10 000 compounds of the Chembridge's Diverset compound library were screened to identify compounds that can protect H9C2 rat cardiomyocytes against doxorubicin-induced cell death. An MTT-based high throughput viability screening was performed followed by retesting of the hit compounds in a morphology-based assay. The most effective compound proved protective in DOX-treated primary rat cardiomyocytes and was further characterized to demonstrate that it significantly decreased doxorubicin-induced apoptotic and necrotic cell death without having a radical scavenging effect or interfering with the antitumor effect of DOX. This benzimidazole compound may lead – through further optimalization – to the development of a drug candidate protecting the heart from DOX-induced injury.

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6. APPENDIX

UNIVERSITY OF DEBRECEN
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Registry number: DEENK/123/2015.PL
Subject: Ph.D. List of Publications

Candidate: Szabolcs Gergely
Neptun ID: GTQG81
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Gergely, S.**, Hegedűs, C., Lakatos, P., Kovács, K., Gáspár, R., Csont, T., Virág, L.: High throughput screening identifies a novel compound protecting cardiomyocytes from doxorubicin-induced damage.
   Oxid. Med. Cell. Longev. "Accepted by Publisher" (2015)
   IF:3.363 (2013)

2. Hegedűs, C., Lakatos, P., Kiss-Szikszai, A., Patonay, T., **Gergely, S.**, Gregus, A., Bai, P., Haskó, G., Szabó, É., Virág, L.: Cytoprotective dibenzoylmethane derivatives protect cells from oxidative stress-induced necrotic cell death.
   Pharmacol. Res. 72, 25-34, 2013.
   DOI: http://dx.doi.org/10.1016/j.phrs.2013.03.002
   IF:3.976

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List of other publications

3. Brunyánszki, A., Hegedűs, C., Szántó, M., Erdélyi, K., Kovács, K., Schreiber, V., **Gergely, S.**, Kiss, B., Szabó, É., Virág, L., Bai, P.: Genetic ablation of PARP-1 protects against oxazolone-induced contact hypersensitivity by modulating oxidative stress.
J. Invest. Dermatol. 130 (11), 2629-2637, 2010.
DOI: http://dx.doi.org/10.1038/jid.2010.190
IF:6.27

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DOI: http://dx.doi.org/10.1016/j.febslet.2008.04.023
IF:3.264

6. Bai, P., Hegedűs, C., Erdélyi, K., Szabó, É., Bakondi, E., **Gergely, S.**, Szabó, C., Virág, L.: Protein tyrosine nitration and poly(ADP-ribose) polymerase activation in N-methyl-N-nitrosoguanidine-treated thymocytes: Implication for cytotoxicity.
Toxicol. Lett. 170 (3), 203-213, 2007.
DOI: http://dx.doi.org/10.1016/j.toxlet.2007.03.007
IF:2.826

Total IF of journals (all publications): 25,242
Total IF of journals (publications related to the dissertation): 7,339

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

05 June, 2015

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