CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

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THE IMPACT OF SIRT1 INHIBITION ON ZEBRAFISH MORPHOLOGY AND BEHAVIOR

Diploma work

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Hradec Králové 2013 **Zuzana Faustová**

Declaration

„I declare that this thesis is my original work. All literature and other sources, that I used during my work are stated in the literature list and cited properly. This work has not been used to achieve same or another degree.“

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Abstrakt

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Název diplomové práce: Vliv inhibice SIRT1 na morfologii a chování Dánia pruhovaného

Po objevu spojitosti mezi Silent Information Regulator 2 (Sir2) a jeho schopností ovlivnit délku života v kvasinkách, se Sir2 a jeho sedm savčích ortologů stalo velice zajímavým cílem. Byly nazvány sirtuiny a patří do rodiny histon deacetyláz. Funkce sirtuinů je jedinečná, jako kofaktor mají nikotinamid adenin dinukleotid a jejich aktivita může být ovlivněna vnějšími faktory. Cílem této práce bylo zjistit více o Sirtuinu 1 (SIRT1), který by měl být ze všech savčích sirtuinů nejvíce podobný Sir2 kvasinek. Nejprve jsme testovali vliv inhibice SIRT1 na raný vývoj embrya a larvy Dánia pruhovaného (*Danio rerio*). Zjistili jsme, že SIRT1 je důležitý pro normální vývoj a že jeho nedostatek způsobí poruchy kardiovaskulárního charakteru, zpoždění ve vývoji až smrt. Dále jsme se snažili zjistit více o spojitosti SIRT1 s Parkinsonovou chorobou, tím že jsme kombinovali netoxické dávky EX527, inhibitoru SIRT1, a 1-methyl-4-phenylpyridinium (MPP^+), látkou využívanou k indukci symptomů Parkinsonovy choroby. Nicméně inhibice funkce SIRT1 neochránila embrya ani larvy Dánia pruhovaného před toxicitou MPP^+, ale dokonce právě naopak způsobila mnohem vážnější poškození. Toto naznačuje, že inhibice SIRT1 pravděpodobně není vhodný způsob léčby Parkinsonovy nemoci.

Abstract

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Title of diploma thesis: The impact of SIRT1 inhibition on zebrafish morphology and behavior

After discovery of connection between yeast Silent Information Regulator 2 (Sir2) and its ability to alter lifespan, Sir2 and its seven mammalian orthologs became very attractive therapeutic target. These so called sirtuins are members of a histone deacetylase family. They possess unique catalytic activity having nicotinamide adenine dinucleotide as a cofactor and their function can be influenced by environmental factors. The aim of this diploma thesis was to extend knowledge of Sirtuin 1 (SIRT1), which is from all mammalian sirtuins considered to have the closest relation to yeast Sir2. At first we tested the impact of SIRT1 inhibition on early developmental stages of zebrafish (*Danio rerio*) embryos and larvae, finding out that SIRT1 is important for normal development and SIRT1 inhibition or malfunction result in cardiovascular defects, delayed development, and death. Additionally, we tried to learn more about SIRT1 and its connection with Parkinson´s disease by combining nontoxic doses of SIRT1 inhibitor EX527 with 1-methyl-4-phenylpyridinium (MPP^+), drug known to induce Parkinson´s disease like symptoms. However, the SIRT1 down regulation not only failed to protect zebrafish embryos and larvae from MPP^+ toxicity, but even resulted in more severe phenotype, suggesting that SIRT1 inhibition might not be the right option of Parkinson´s disease therapy.

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1. LIST OF ABBREVIATIONS

AADPR	*O*-acetyl-ADP-ribose
AROS	active regulator of SIRT1
CpG	cytosine preceding guanine
DBC1	deleted in breast cancer 1
DMSO	dimethyl sulfoxide
dpf	days post fertilization
FOXO	forkhead transcription factors of class O
H	histone
hpf	hours post fertilization
HP1α	heterochromatin protein 1α
Hsp70	heat shock protein 70
K	lysine
KAT (HAT)	lysine (histone) acetyltrasferase
KDAC (HDAC)	lysine (histone) deacetylase
KDACi (HDACi)	lysine (histone) deacetylase inhibitor
LXR	liver X factor
MPP^+	1-methyl-4-phenylpyridinium
NAD^+	nicotinamide adenine dinucleotide
NAM	nicotinamide
Nampt	nicotinamide phosphoribosyltransferase
ND	not detected
NMN	nicotinamide mononucleotide
PD	Parkinson´s disease
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
PNC1	pyrazinamidase and nicotinamidase
PPARα	peroxisome proliferators-activated receptor α
PPARγ	peroxisome proliferators-activated receptor γ
SIRT	sirtuin
Sir2	Silent Information Regulator 2
UCP2	uncoupling protein

2. INTRODUCTION

Sir2 was discovered in 1979 but not until 1999 draw much attention. It was because in 1999 Kaeberlein et al. showed that Sir2 is capable of regulating length of life of Saccharomyces cerevisiae (Kaeberlein et al. 1999). Sir2 orthologs, which operate as lysine deacetylases, were called sirtuins and were identified in all eukaryotes up to seven sirtuins present in mammals including Homo sapiens. What is interesting about sirtuins is the fact that their activity can be influenced by external factors especially calorie restriction, because they operate by unique NAD^+ dependent mechanism thus reflecting the metabolic state of the cell (Kaeberlein et al. 1999, Imai and Guarente 2010, Haigis and Sinclar 2010).

There are seven mammalian sirtuins affecting a great number of biological processes (Haigis and Sinclair 2010). Indeed a great number of their targets was identified – such as p53, forkhead transcription factors of class O (FOXO), chaperons and many others including also histones making sirtuins significant epigenetic regulators. Sirtuins are linked to many cell events starting with embryo development up to disorders connected with ageing such as neurodegeneration and tumorgenesis (Haigis and Sinclair 2010).

From all mammalian sirtuins (SIRTs), SIRT1 has been given the biggest attention, because it is proposed to have the closest relation to the yeast Sir2. It is mainly located in the nucleus, but occasionally can be found in the cytoplasm. As a member of KDAC family it targets histones 3 and 4, but also other non-histone proteins such as peroxisome proliferators-activated receptor α (PPARα), heat shoch protein 70 (Hsp70), nuclear factor kappa-light-chain-enhancer of activated B cells, and above mentioned p53 and FOXO. SIRT1 is already active in early embryos and is involved in programming of embryo organ systems (Wang et al. 2008, McBurney et al. 2003, Cheng et al. 2003, Potente et al. 2007, Li et al. 2013).

Because neurodegeneration is connected with ageing and ageing with sirtuins, a link between SIRT1 and neurodegeneration became a question. However, this question is still under a debate, because studies involved in the

matter bring different often contradictory knowledge (Donmez and Outeiro 2013, Haigis and Sinclair 2010). SIRT1 overexpression might be protective against Parkinson´s disease (PD) as shown in animal models, because of chaperones activation (Donmez et al. 2012). Similarly, resveratrol, SIRT1 activator, protected mice from MPP^+ treatment (Blanchet et al. 2008). However, Park et al. (2011) showed that SIRT1 inhibition provides neuroprotection via apoptosis inhibition. This is consistent with findings by Kakefuda et al. (2009) who showed SIRT1 overexpression failed to save mice against MPP^+ toxicity.

The first aim of this diploma thesis was to elucidate SIRT1 and its biological role during embryo development by SIRT1 inhibition with EX527 in zebrafish embryos. The second goal was to find what role SIRT1 plays in *in vivo* zebrafish model during PD pathogenesis by combining SIRT1 inhibitor EX527 with 1-methyl-4-phenylpyridinium (MPP^+), drug known to induce PD like symptoms.

3. THEORETICAL PART

3.1. Epigenetics and its modulation

Epigenetics is the study of heritable changes in gene expression that occur without a change in DNA sequence (Wolffe and Matzke 1999). By this mechanism differentiated cells in multi-cellular organism though having full genetic information (with few exceptions) express only genes necessary for their own function (Reik 2007). Neurons, muscle cells, epithelium etc. all of them contain same amount of DNA with identical sequence, but thanks to epigenetic mechanisms, the difference in their function and appearance is obvious. When differentiated cells divide the daughter cells inherit both genetic information and epigenetic code (Reik 2007).

Epigenetic code can be influenced by external factors for instance smoking, diet and also pharmacologically (Csoka and Szyf 2009, Cooney et al. 2002, Pembrey et al. 2005). The evidence for that is epigenetic similarity in monozygotic twins in early years of life; however, as they get older they start to exhibit remarkable differences in their epigenome (Fraga et al. 2005).

When epigenetic changes are located in germ cells, they can be passed to following generations. Primordial germ cells are the only cells, where the DNA-methylation - one of epigenetic mechanisms - of imprinted genes can be erased. However, when there are requirements to retain the epigenetic mark, it can be passed to the next generation (Reik 2007). This way epigenetics can affect the evolution and that in much faster rate than genetic mutations (Rando and Verstrepen 2007).

Various epigenetics affecting processes exist. The most widely studied are (Csoka and Szyf 2009, Reik 2007):

- DNA methylation
- Histone modification

3.1.1. DNA methylation

DNA methylation has a critical role in the control of gene activity. It occurs in C-5 of cytosines that precede guanines, so called CpG dinucleotides. In

genome regions with accumulated CpGs can be found. These are called CpG islands and span the 5´end of the regulatory region of many genes (Weber et al. 2007).

DNA methylation is controlled by DNA methyltransferase family. Methylation of DNA is linked to long-term epigenetic silencing of particular sequences - transposons, imprinted genes and pluripotency-associated genes - in somatic cells. It is a mechanism to prevent chromosomal instability (Reik 2007, Esteller 2008).

DNA methylation is a very powerful tool affecting transcription. It operates either by preventing the binding of specific transcription factors or/and recruitment of methyl-CpG binding proteins, which in turn recruit additional chromatin modulators such as KDACs leading to increased level of gene silencing. This indicates a connection between DNA methylation and histone modification (Jones et al. 1998).

3.1.2. Histone modification

The basic unit of chromatin structure is the nucleosome, which contains an octamer of the four core histones (H2A, H2B, H3, H4) around which are wrapped 147base pairs of DNA. From this structure it is obvious that histones strongly influence DNA function. The influence can be caused by simple presence – for instance hiding or revealing transcription factor binding sites, or by their chemical modifications. These chemical modifications include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines and also ubiquitation, sumoylation, ADP ribosylation, deimination and proline isomeration. These modifications are located in amino-terminal tails of all eight core histones and these tails are exposed on the nucleosome surface where they can interact with DNA. Specific histone modifications also act as receptors and are recognized by specific binding proteins that afterwards affect the chromatin structure (Kouzarides 2007, Turner 2007).

Interactions between DNA and histone tails have both short term functions - regulating transcription, DNA replication and repair; and long term functions -

such as determinants of chromatin conformation and heritable markers.

Acetylation is a type of histone modification, a very important one. It is controlled by two conversely operating enzymatic groups – Histone lysine acetyltransferases (KATs, HATs) and Histone deacetylases (KDACs, HDACs). Acetylation of the ε-amino group of lysine histone residue neutralizes the positive charge it has in physiological pH, which decreases the affinity of lysine residue to negatively charged DNA backbone and leads to reduced DNA-histone binding. This contributes to chromatin opening which leads to activation of gene transcription (Fig. 1).

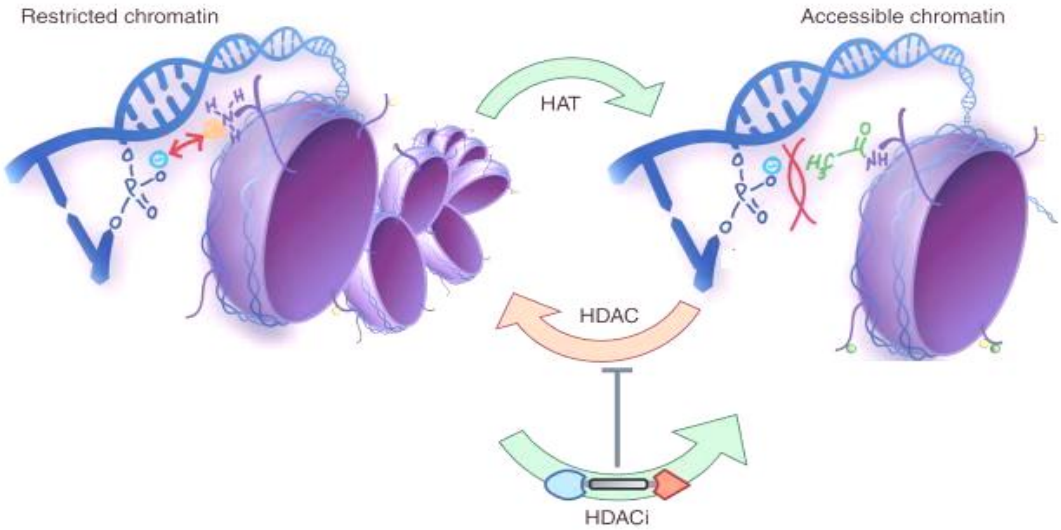

Fig. 1 The impact of Histone lysine acetyltransferase (HAT), Histone deacetylase (HDAC) and Histone deacetylase inhibitor (HDACi) on chromatin structure
Adapted from: Gryder et al. (2012)

There are eighteen human KDACs that are according to their catalytic mechanism and sequence homology divided into two families. First, classical KDAC family contains highly conserved catalytic domain with zinc cation. Second family are so called sirtuins and are unusual for their requirement of NAD^+ as a cofactor (Turner 2007, Michan and Sinclair 2007).

It is very important to note that KDACs deacetylate not only histones, but also other proteins such as p53, α-tubulin, FOXO, hypoxia inducible factor, chaperons and many others, which indicates their significance and also greatly widens means of epigenetic modulation (Ganesan et al. 2009).

3.2. Sirtuins

At the end of the millennium it was shown that yeast Saccharomyces cerevisiae SIR2 gene extends its life span by repressing genome instability. This attracted a lot of attention and led to a discovery of Sir2 orthologs, termed sirtuins, in flies, worms, and also mammals (Kaeberlein et al. 1999).

Sirtuins possess unusual catalytic activity working as NAD^+-dependent deacetylases or/and ADP-ribosyltransferases. The central NAD^+-binding and catalytic domain is highly conserved, but their N and C termini differ allowing us to distinguish seven mammalian sirtuins. This also suggests their highly divergent biological functions according to different enzymatic activities, unique binding partners and substrates, and distinct subcellular localization and expression pattern (Table 1). For better understanding of each sirtuin function and possible later pharmacological use of them a need to develop specific inhibitors and activators arose (Haigis and Guarante 2006, Liszt et al. 2005).

Table 1. Mass, activity and localization of mammalian sirtuins

	kDa	Enzymatic Activity	Sub-cellular Localization
SIRT1	81.7	Deacetylase	nuclear, cytoplasmic
SIRT2	43.2	Deacetylase	cytoplasmic, nuclear
SIRT3	43,6	Deacetylase	mitochondrial
SIRT4	35,2	ADP-ribosyltransferase	mitochondrial
SIRT5	33,9	Deacetylase, Demalonylase, Desuccinylase	mitochondrial
SIRT6	39,1	ADP-ribosyltransferase, Deacetylase	nuclear
SIRT7	44,8	Deacetylase	nuclear

Tab. 1 Mammals have seven sirtuins, SIRT1–7. All have an NAD^+-dependent catalytic core domain that may act preferentially as a mono-ADP-ribosyl transferase and/or NAD^+-dependent deacetylase. Additional N-terminal and/or C-terminal sequences of variable length may flank this core domain. The seven sirtuins show different cellular localization.
Adapted from: Donmez and Outeiro (2013)

Sirtuins perform deacetylation at modified lysine residues via a unique enzymatic mechanism that requires NAD^+ cleavage with each reaction cycle. The exact mechanism is shown at Fig. 2. Thus, unlike other KDACs, sirtuin activity is intimately tied to the metabolic state of the cell and can be influenced

by external factors (Haigis and Sinclair 2010).

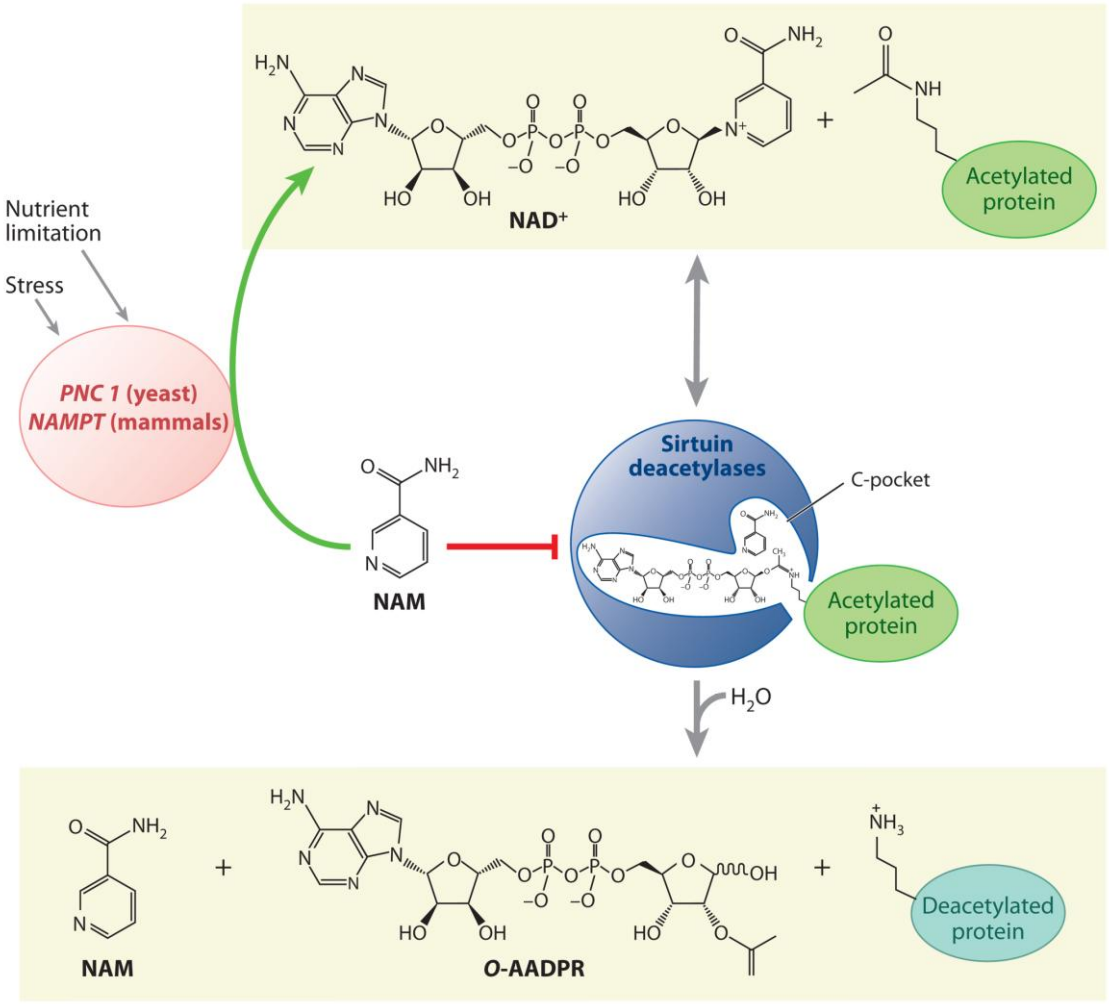

Fig. 2 The sirtuin deacetylation reaction and regulation by stress and nutrition. Unlike other deacetylases, which hydrolyze the acetyl group on a substrate, sirtuin deacetylases catalyze an unprecedented two-step biological reaction that consumes nicotinamide adenine dinucleotide (NAD^+) and releases nicotinamide (NAM), *O*-acetyl-ADP-ribose (AADPR), and the deacetylated substrate. Amide-to-ester acyltransfer is unfavorable, but hydrolysis of NAD^+ can provide a favorable driving force for the overall sirtuin reaction. Evidence favors a mechanism in which electrophilic capture of the acetyl oxygen in an ADP-ribosyltransfer reaction forms an ADP-ribose peptide-imidate complex. This intermediate may last for a few seconds, enough time for NAM to enter the C-pocket and catalyze a reverse reaction. Activation of sirtuins can be facilitated by the removal of NAM and its conversion to NAD+ by PNC1 (yeast and simple metazoans) or NAMPT (mammals and alpha proteobacteria), two genes upregulated by stress and nutrient limitation (Haigis and Sinclair 2010).

Adapted from: Haigis and Sinclair (2010)

From Fig. 2 it is apparent that NAD^+ presence is a condition for sirtuins´ activation. Reverse effect has nicotinamide (NAM), which is able to enter sirtuin´s C-pocket (NAM binding pocket) and react with the peptide-imidate intermediate of the reaction, thereby regenerating NAD^+. This type of reversible reaction is very rare and is thought to be a major mechanism of control for sirtuins. In mammals NAM is recycled back to NAD^+ by Nicotinamide phosphoribosyltransferase (Nampt) in two steps, intermediate is nicotinamide mononucleotide (NMN), indicating Nampt and NMN as sirtuin regulators. In yeast, flies and worms the recyclation takes four steps and is catalyzed by PNC1 (Sauve and Schramm 2003, Berger et al. 2005, Gallo et al. 2004). NMN and Nampt are present in human and mice serum, which proposes a theory that this might by the way of signaling from stressed or nutrient-deprived cells to other parts of the body (Imai 2009).

In zebrafish orthologs to all mammalian sirtuins were identified. There are eight sirtuin-related genes in zebrafish genome, because SIRT3 has two paralogs (Pereira et al. 2011).

3.2.1. Sirtuin1

From all sirtuins SIRT1 is the one that attracted most attention since it is proposed to have the closest relation to yeast Sir2 orthologue. SIRT1 activity is very complex, regulating many metabolic cascades and cooperating with a great number of binding partners. In mature organisms SIRT1 is linked to a lot of metabolic processes, some of which are shown in Fig. 3 (Haigis and Sinclair 2010).

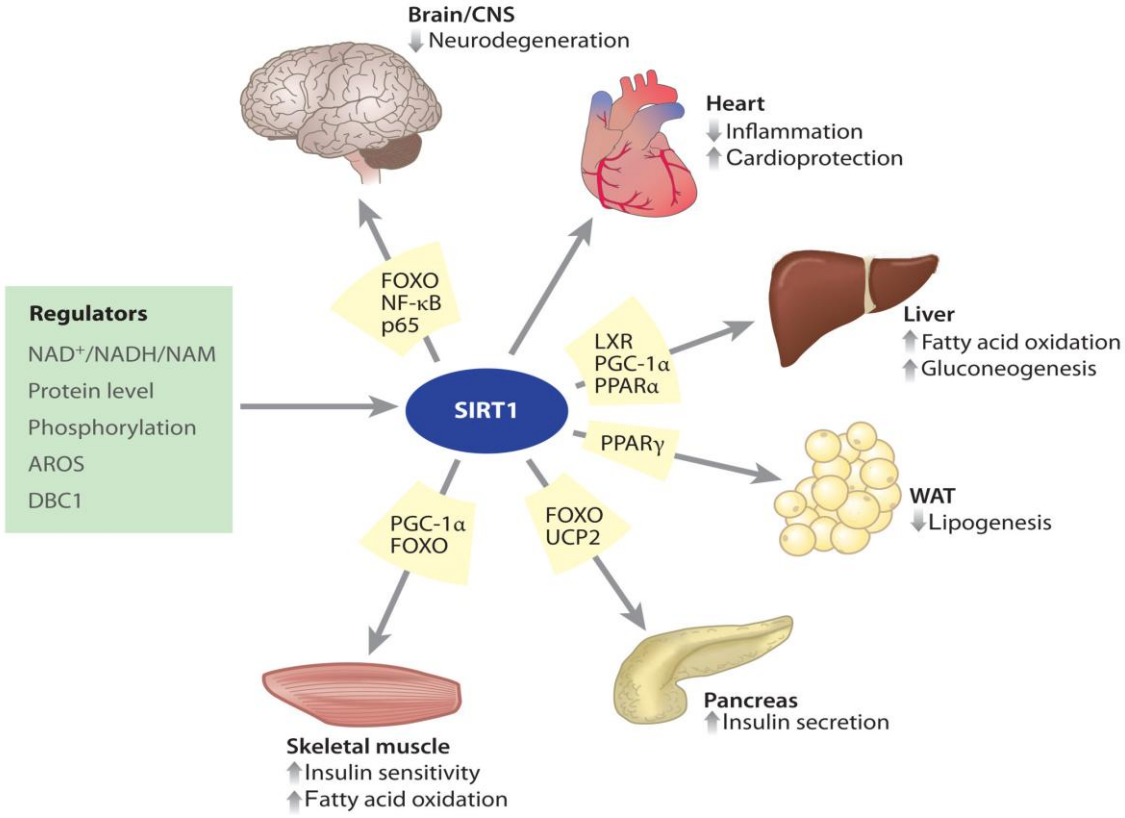

Fig. 3 SIRT1 regulation of age-related physiology. SIRT1 activity can be regulated through NAD^+ and NAM concentrations, by SIRT1 protein level, and by phosphorylation; SIRT1 can be activated by active regulator of SIRT1 (AROS) and inhibited by DBC1 (deleted in breast cancer 1). SIRT1 activation promotes survival of neurons and protects cardiomyocytes from death. In the liver, SIRT1 promotes fatty acid oxidation and gluconeogenesis during nutrient deprivation via LXR, PGC-1α, and PPARα. In white adipose tissue (WAT), SIRT1 decreases fat storage by repressing PPARγ. SIRT1 promotes insulin secretion and pancreatic beta cell survival by suppressing UCP2 and interacting with FOXO, respectively. In skeletal muscle, SIRT1 promotes mitochondrial biogenesis through the activation of PGC-1α (Haigis and Sinclair 2010).
Abbreviations: CNS - central nervous system; LXR - liver X receptor; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPARα - peroxisome proliferators-activated receptor alpha; PPARγ- peroxisome proliferators-activated receptor gama; UCP2 - uncoupling protein
Adapted from: Haigis and Sinclair (2010)

3.2.2. Sirtuin1 and embryo development

As KDAC family member SIRT1 target are also histones, in particular K9 in H3 (H3 K9) and K16 in H4. In early embryos SIRT1 seems to have very strong affinity to H3 K9. Deacetylated H3 K9 can be trimethylated. A function of

trimethylated H3 K9 is to recruit HP1α, which leads to closed chromatin configuration. Knock down of SIRT1 indeed causes abnormal chromosomal segmentation, thus nuclear fragmentation, which leads to aneuploidity and cell death (Wang et al. 2008).

Laboratories all over the world succeeded in producing SIRT1 mutant mice, using different strains. All of them confirm that SIRT1 knockdown has a great influence during embryo development, though the viability of mutants differed from 90% embryonic lethality and early postnatal death to abnormal small mutants surviving to adulthood. The different viability implies that the genetic background has profound effect on the phenotypes (Wang et al. 2008, McBurney et al. 2003, Cheng et al. 2003).

The mutant mice displayed several defects. In general they were smaller, though it might have been due to delayed development, because some of them thrived and reached the size of a wild-type animal. Most frequently identified were septic cardiac defects, smaller abnormally shaped eyes with defects in retina, encephaly, and abnormal head shape formation. SIRT1 also plays a role in primitive vascular network formation and hematopoesis regulating primitive erythroid progenitors and genes important for normal hemoglobin development (Ou et al. 2011). The organ histology seemed to be normal, but in general mutants´ organs were smaller compared with their wild-type littermates´. Mutants that managed to reach adulthood were sterile. McBurney´s team was the only one to describe abnormalities in pancreas and lungs (Wang et al. 2008, McBurney et al. 2003, Cheng et al. 2003).

There were other teams studying SIRT1 impact on vessel network formation. Two teams both studying the subject on zebrafish, brought different results. Potente et al. (2007) suggest that in zebrafish SIRT1 silencing with morpholino results in extra branching of intersomic vessels indicating that SIRT1 controls tip cell activity and is required for endothelial cell migration. The suggested mechanism is that SIRT1along with unidentified class I/II KDAC deactivate FOXO1, which is known to be crucial negative transcriptional regulator of blood vessel development. Zebrafish morphants also displayed hemorrhages and pericardial swelling (Potente et al. 2007). However, opposite results brought

Guarani et al. (2011) linking SIRT1 inhibition to Notch signaling resulting in reduced vascular branching.

SIRT1 plays a role in embryonic neuronal development and osteoblast differentiation (Li et al. 2013, Hasegawa and Yoshikama 2008, Srivastava et al. 2012).

SIRT1 is also linked to tumorgenesis, though the mechanisms are under a debate. The fact is that in many types of cancer SIRT1 function is altered in both directions, either inhibited or increased. Very important SIRT1 target is p53, which is deactivated by SIRT1, therefore SIRT1 increased activation could play its role in tumorgenesis. On the other hand, as mentioned earlier SIRT1 inhibition causes chromosomal instability which is carcinogenic risk factor (Wang et al. 2008, Huffman et al. 2007, Stunkel et al. 2007).

In zebrafish tissues SIRT1 has the highest expression among all sirtuins and is present in all organs, that were analyzed (Pereira et al. 2011).

3.2.3. Sirtuin1 and Parkinson´s disease

Since ageing is a major risk factor for the development of neurodegenerative diseases, the link between sirtuins and neurodegeneration came to scrutiny. PD is characterized by loss of dopaminergic neurons in substantia nigra pars compacta and pathological presence of intraneuronal cytoplasmic inclusions termed Lewy bodies. These are mainly composed of oxidatively modified hence misfolded α-synuclein protein. When α-synuclein is misfolded at first cells try to repair it with chaperons and if that fails, misfolded α-synuclein is polyubiquitized and degradated. With ageing cells ability for these protective mechanisms weakens, making old age and oxidative stress PD risk factors.

The role of cytoplasmic inclusions is still unclear. α-synuclein is named after its cellular localization in presynaptic nerve terminals, where it is expected to play crucial role in synaptic function, and also in nucleus. In mice it was described to translocate to the nucleus and interact with histones after injection with Paraquat, herbicide known to induce PD like symptoms (Goers et al. 2003). Both wild-type and mutant α-synuclein were shown to inhibit acetylation of histone H3 with equal affinity by yet unknown mechanism. However, the mutant

α-synuclein was demonstrated to have higher nuclear accumulation. This would support the theory that the cytoplasmic inclusions are a cellular mechanism to store mutant α-synuclein rather than a cause of toxicity and that the mutant α-synuclein toxicity takes place in nucleus (Tanaka et al. 2004, Kontopoulos et al. 2006).

The impact of SIRT1 in PD remains a question, though there seems to be a connection. In SIRT1 promoter region of PD patients were identified few sequences linked to the disease (Zhang et al. 2012). Donmez et al. (2012) demonstrated on mutant mice, that SIRT1 activates Hsp70, a chaperon family member, which decreases α-synuclein aggregation. Known SIRT1 activator resveratrol was shown to save mice from MPP^+ toxicity (Blanchet et al. 2007, Dore 2005, Alvira et al. 2007). Whether the rescue was caused by SIRT1 activation or other mechanism is not certain, because resveratrol is also strong antioxidant, but further studies show that the protective mechanism is rather independent on SIRT1 activation (Blanchet et al. 2007, Dore 2005, Alvira et al. 2007). On the other hand Park et al. (2011) proved on a cell culture that SIRT1 inhibition protected SH-SY5Y dopaminergic cells treated with MPP^+ against apoptosis. Kakefuda et al. (2009) showed that transgenic mice overexpressing in brain human SIRT1 were not protected from MPP^+ treatment.

From current results the role of SIRT1 in PD is very unclear; but seems to be an interesting target to further investigate in order to develop new options for yet very unsatisfactory PD therapy.

3.3. Sirtuin 1 Inhibition

3.3.1. EX527

EX527 is a selective inhibitor of SIRT1 that does not inhibit histone deacetylase (KDAC) or other sirtuin deacetylase family members. It is 200-500-fold more selective for SIRT1 than for SIRT2 or SIRT3 (Napper et al. 2005).

EX527 enhances p53 acetylation in response to DNA damaging agents. It shows mixed type of inhibitory activity. Firstly, it binds after the release of NAM from the sirtuin-ADP-ribose-peptide-imidate complex and prevents the release of deacetylated protein and O-acetyl-ADP-ribose; secondly, it blocks NAM

binding site of SIRT1 (Lawson et al. 2010, Napper et al. 2005). So far to our knowledge SIRT inhibition is the only mechanism of EX527 function and it has only been used as a selective SIRT1 inhibitor.

For molecular structure see Fig. 4.

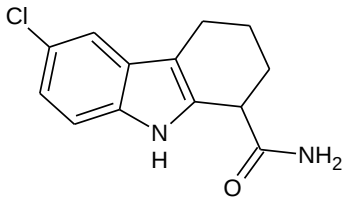

Fig. 4 EX527 molecule, chemical Name: 6-Chloro-2,3,4,9-tetrahydro-1*H*-carbazole-1-carboxamide

3.4. 1-methyl-4-phenylpyridinium

1-methyl-4-phenylpyridinium is an active form of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. MPP^+ is a widely used drug for studying PD, because in animal models its treatment reduces neuronal dopamine content and induces loss of dopaminergic neurons, which leads to Parkinson´s disease like syndrome characterized by bradykinesia, muscular rigidity and tremor. MPP^+ gets to dopaminergic neurons via dopamine transporters and then is transported to mitochondria, where it inhibits mitochondrial complex I which causes ATP deficit and mitochondrial dysfunction resulting in oxidative stress (Javitch et al. 1985, Minozo et al. 1987, Sallinen et al. 2009)

In zebrafish, because they do not develop midbrain dopaminergic system, the dopaminergic neurons analogous to mammalian nigrostriatal pathway are located in the posterior tuberculum of the diencephalon (Rink and Wullimann 2002). First dopaminergic neurons are detected 18hpf (Xi et al. 2011). Zebrafish larvae exposed to 1mM 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine at 4dpf start to develop PD like phenotype between first and second day of exposure (Sallinen et al. 2009). MPP^+ toxicity leads to reduction of dopaminergic neurons and decreased levels of dopamine, noradrenaline and histamine (Anichtik et al. 2004, Sallinen et al. 2009, Lam et al. 2005). It is associated with reduced motor activity, which can be measured by response to the tail or head cutaneous stimuli or as a length of a trajectory of spontaneous movements during a time

period (Lam et al. 2005, Farell et al. 2011).

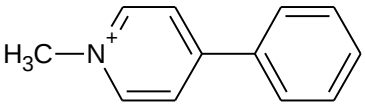

Fig. 5 MPP^+ molecule, chemical Name: 1-Methyl-4-phenylpyridinium ion

3.5. Zebrafish

Zebrafish (*Danio rerio*) is a small aquarium fish of southeastern Asia origin. It belongs to the order Cypriniformes, family Cyprinidae.

Zebrafish is a very popular and widely used model in the scientific area. Its ability to produce hundreds of eggs at interval of two to three days, undemanding and low cost life conditions provide us the advantages of invertebrate models (such as Drosophila melanogaster, Caenorhabditis elegans), but in combination with much closer anatomical and physiological relation to mammals (Fishman 2001).

Its genome is now fully sequenced. Most human genes studied so far have orthologs in the zebrafish (Fishman 2001). Zebrafish and its mutant variants seem to be suitable animal model to study human diseases (Flinn 2008) including the neurodegenative ones such as PD (Flinn 2008, Keller and Murtha 2004, Langheinrich 2003, Shin and Fishman 2002).

Another huge benefit of zebrafish as a model is the fact that after fertilization the embryos remain transparent which provides great conditions for *in vivo* developmental screening enabling us to see the development and function of organ systems (Pinho et al. 2013).

The water dependent way of life offers a simple way of water-soluble drugs administration via embryonic medium. Embryos tolerate higher concentration of DMSO up to 1%, which widens the scale of drugs possible to apply (Maes et al. 2012).

Zebrafish development since fertilization till early larval period was well studied and described in detail by Kimmel et al. (1995). In this research zebrafish in two stages of development were used and that were 6 hours post fertilization (hpf) and 3 days post fertilization (dpf) at the start of the treatment. The treatment in both cases took three days.

3.5.1. Zebrafish development 6hpf - 3dpf

Zebrafish development was well described by Kimmel et al. (1995). Briefly; 6hpf, when the drug exposure begins, is approximately an hour after the embryo entered gastrula period ($5^1/_4$ - 10hpf). From previous blastula period ($2^1/_4$- $5^1/_4$ hpf) we can distinguish two types of cells – yolk cells and blastoderm. At the end of blastula period embryo entered epiboly process.

During the gastrula period epiboly process continues, blastoderm is being differentiated into epiblast and hypoblast. When epiboly reaches 50% embryonic shield is being formed, so since this moment anterior-posterior and dorsal-ventricular axes are possible to define. At about this point - 6hpf - the embryos are being exposed to the drug treatment.

Epiboly continues, neural and prechordal plates are formed and cells that will later form the notochord, axial somite-derived muscles, and specific neurons in the hindbrain appear (Mendelson 1986). At about 10hpf epiboly reaches 100%, tail bud is formed, neural plate thickens and is regionalized and polster of postmitotic hatching gland cells are formed from part of prechordal plate cells. The segmentation period (10-24hpf) starts.

Segmentation period is named after somites that develop in anterior to posterior direction at the rate 3 per hour for the first six and 2 per hour thereafter. The final number of somites varies from 30 to 34. The embryonic body prolongs and straightens, the first cells differentiate morphologically, the primary organs are formed and the first body movements appear. Medial and lateral parts of the neural plate transform into a neural keel (6-10-somite, 13hpf) that later becomes a neural rod (14-somite, 16hpf) and after hollowing a neural tube (18-somite, 18hpf). During the 14-somite stage brain rudiment is subdivided into telencephalon, diencephalon, midbrain and hindbrain. At around 20-somite stage brain becomes hollow and ventricles are formed, cerebellum rudiment is visible and seven rhombomeres can be distinguished in hindbrain. At 26-somite stage (22hpf) rudiments of hypothalamus and epiphysis can be seen in diencephalon. Since 5-somite stage ($11^2/_3$hpf) optic vesicle is present, otic placode is formed in 9-somite stage and in 19-somite stage hollows into otic vesicle with two otoliths. Chevron shaped myotomes are formed in older

somites (14-somite stage) and around 17-somite stage, correlating with ingrowth of axons from developing primary motoneurons, first weak irregular contractions appear (Hanneman and Westerfield1989). At 8-somite stage rudiments of pronephros are visible. Since 14-somite stage notochord is recognizable and in 20-somite stage first vacuoles can be distinguished within. Embryonic development reached pharyngula period (24-48hpf).

Pharyngula period is named after 7 pharyngeal arches that develop during this period. The first two arches, mandibular and hyoid, later form jaws and operculum, the rest form gills. This period is divided into prim stages, which refers to the migration rate of the posterior lateral line primordium which moves along both sides of trunk and tail leaving behind cells that form the ganglion of the lateral nerve, and as it moves it deposits cells that form the neuromasts along the nerve (Kimmel et al. 1995). The stages are numbered according to the position of myotome that the migrating primordium has reached.

As the position of the head to the trunk straightens, the angle between them can be also used as a developmental index. Same way can serve the distance between the eye and otic vesicle since the later one moves closer to the eye until it reaches position less than one otic vesicle length from the eye.

The circulatory system undergoes great development in this period. The heart looks at first as a cone-shaped tube, but lately due to the changing position of the head to the trunk, it starts to relocate in the ventral direction until it reaches anteriormost position of the yolk, and takes the shape of a straight tube, that at about Prim-25 stage (36hpf) bends slightly. At the beginning of this period (Prim-5 stage, 24hpf) the heart starts beating and shortly afterwards blood circulation appears. First pair of aortic arches develops, but the five remaining arches will not appear until the end of the period. Blood-vessels are being formed. At the end of this period bend of the heart is prominent and indicates the division between atrium and ventricle. The frequency of heart beat is about 180 per minute. Segmental vessels are formed along the trunk and tail.

At around Prim-15 stage pigment cells starts to differentiate at retinal epithelium and also melanophores, which appear anteriorly on the head and also in dorsal, ventral and later also lateral stripe arrangement. Since Prim-15

stage tactile sensitivity can be noticed. At Prim-25 stage first iridophores are formed in the eye. At the end of the period (High-pec stage, 42hpf) first xantophores are present on the head, primordia of the liver and probably swim bladder are formed along the digestive tract. Fins are developed since 28hpf when first buds appear. At approximately Prim-15 stage, 30hpf first developmental reading is done.

Hatching period (48-72hpf) is named after the hatching that happens within the third dpf. The fact that some embryos hatch earlier than their siblings from the same clutch does not indicate their advanced development, because their development continues independently on hatching. Except for the digestive tract, morphogenesis of most organ rudiments is complete now. Gills develop from four brachial and aortic arches, jaw cartilages are formed and fins grow rapidly. Olfactory placodes are placed at the anterior line of the eyes. Xantophores are present on both head and dorsal part. At approximately 54hpf second developmental reading is done. 72hpf first bone, cleithrum, becomes visible.

After hatching period early larval period follows. Almost all morphogenesis is completed and larvae grow rapidly. Lot of swimming, eye, fin, and jaw movements can be noticed. The last developmental reading is done about 78hpf and behavioral tests follow.

3.5.2. Zebrafish development 3dpf - 6dpf

In this period most of the morphogenesis is completed and larvae grow rapidly. Prominent changes during the next day include the inflation of the swim bladder and the continued anterior-dorsal protruding of the mouth (Kimmel et al. 1995). The gut tube decreases ventrally. Yolk extension is almost empty.

Cartilages and muscles grow progressively and after 4dpf larvae are ready to start feeding themselves. Active swimming, lot of body movements and escape responses are observed (Schilling and Kimmel 1997).

After hatching larvae starts swimming, at first fast and sporadically, but after the fifth day, their velocity decreases and the movements get more mature pattern. Time spent by moving prolongs. Apart from spontaneous movements,

zebrafish larvae show reflex responses to visual, acoustic or cutaneous stimuli to the tail or head (Orger et al. 2004, Farell et al. 2012). Larval movements are modulated by dopaminergic function. Recent data suggest that dopamine is essential for movement initiation, but does not influence their velocity (Sallinen et al. 2008, Farell et al. 2012).

3.6. Comparison between human and zebrafish SIRT1 protein

Sirtuins belong phylogenetically to very conserved enzymatic class. In Fig. 6. is shown the similarity between zebrafish and human SIRT1 protein. For the comparison were used:

NCBI Protein database (http://www.ncbi.nlm.nih.gov/protein) to find the zebrafish and human SIRT1 protein sequences,

NCBI COBALT (http://www.ncbi.nlm.nih.gov/tools/cobalt/) for protein sequence comparison,

NCBI BLAST (http://blast.ncbi.nlm.nih.gov) for binding sites identification.

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From Fig. 6. it is clear that SIRT1 binding sites are phylogenetically unchanged between human and zebrafish.

Alignment	AAH12499.1 x XP_001334440.4
Anchor	XP_001334440.4 (130..650) PREDICTED: NAD-dependent deacetylase sirtuin-1 [*Danio rerio*]
Query	AAH12499.1 (1..466) SIRT1 protein [*Homo sapiens*]
Aligned residues	528
Segments	1
Coverage	82,70%
Identity	59,80%
Mismatches	143
Gaps	6

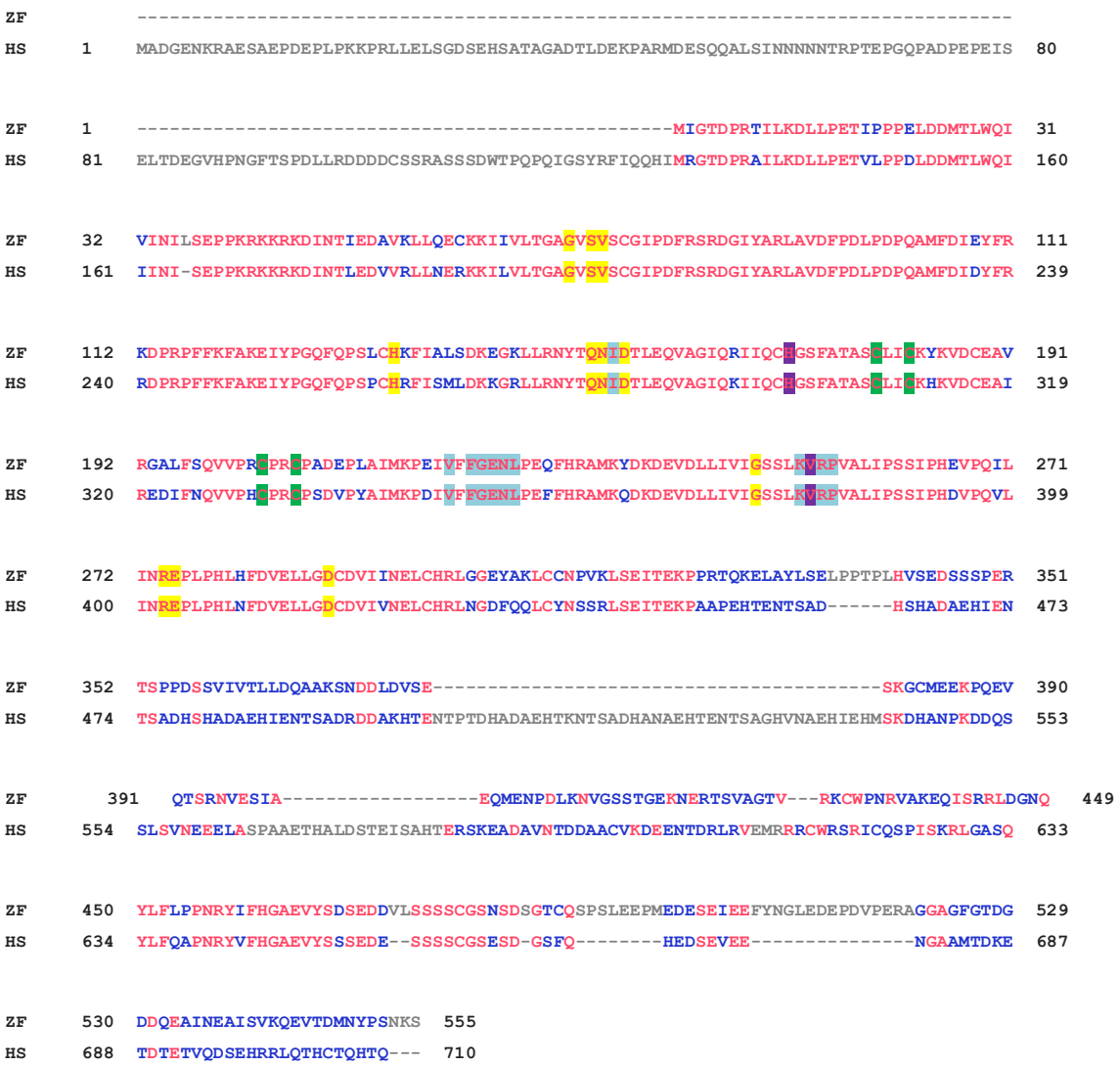


Fig. 6 Human (HS) and zebrafish (ZF) SIRT1 protein sequence comparison. Multiple sequence alignment columns are colored in red – marks conserved columns, and blue – marks less conserved columns. Gaps are marked in grey. By colored background are highlighted binding sites - • NAD^+ binding, • Both NAD^+ and substrate binding, • Zn binding, • Substrate binding
The protein sequence comparison was done by NCBI Cobalt database, binding sites were highlighted according to NCBI Blast database

4. AIM OF THE THESIS

This thesis had several main goals:

- To find out the role of SIRT1 in early embryo modulation by SIRT1 inhibition with EX527 during zebrafish development from early embryo stage till larvae period (6hpf-78hpf) and to test the impact on zebrafish embryo/larvae phenotype in relation to EX527 concentration.

- To find out the role of SIRT1 during zebrafish larvae period by SIRT1 inhibition with EX527 and to test the impact on resulting phenotype in relation to EX527.

- To find out whether SIRT1 inhibition is capable of providing neuroprotection against PD like syndrome caused by MPP^+ in zebrafish during developmental period 6-78hpf.

- To find out whether SIRT1 inhibition is capable of providing neuroprotection against PD like syndrome caused by MPP^+ in zebrafish during developmental period 3-6dpf.

5. EXPERIMENTAL PART

5.1. Drugs and solutions

EX527 was purchased from Tocris Bioscience, United Kingdom

http://www.tocris.com/

MPP^+ was purchased from SIGMA-ALDRICH, Portugal

http://www.sigmaaldrich.com

DMSO

Embryo medium

Water without chloride

5.2. Zebrafish

Adult wild-type male and female zebrafish were purchased from commercial supplier ORNI-EX (http://www.orni-ex.com/). They were kept as previously described (Lawrence and Mason 2012) in 80-liter aquarium in which water was maintained at 26°C by thermostat. They were fed twice a day with commercial fish food flakes TETRAMIN. Zebrafish were kept and treated in accordance with European Union directive 2010/63/EU.

5.3. Equipment

Surgical tweezers

24-well plates

Plastic pipettes

Automatic pipettes adjustable up to volume 10µl, 100µl, 200µl, or 1ml

Microtubes

Petri dishes

Water bath

Thermostat

An inverted epifluorescence microscope (Nikon, type Eclipse TE300)

Mini Incubator (VWR, type INCU-Line Digital)

Microscope (Zeiss, type Stemi DV4)

DinoEye camera AM-423U (Dino-Lite Digital microscope)

HD WebcamC525 (Logitech)

5.4. Programs

Excel, Microsoft office

ImageJ, by W. Rasband available at http://imagej.nih.gov/ij/

iWisoft free video converter available at http://www.iwisoft.com/videoconverter/

DinoCapture 2.0 software, AnMo Electronics Corporation available at http://www.dino-lite.com/

GraphPad Prism 5 for Windows

5.5. Experimental procedures

5.5.1. Breeding and embryo collection

Zebrafish were well fed and approximately 6 male and 8 female were put together into a breeding aquarium (water temperature 28°C). Embryos were collected next day at 8.30 in the morning. In order to prevent parasite growth embryos were cleaned properly. Embryos were stored in water without chloride in mini incubator, where the temperature was maintained at 28°C until the drug exposure.

5.5.2. Drug solution preparation

Tested concentrations for EX527 were 1, 10, 50 and 100μM and for MPP^+ 1mM. In combination was used MPP^+ 1mM and EX527 10μM. EX527 was dissolved in 1% DMSO, MPP^+ and MPP^+ combined with EX527 in embryo medium. After every developmental reading (except for the last) the drug solutions were being refreshed by solutions newly prepared from concentrates 10mM EX527 and 10mM MPP^+. For concentrated solutions drugs were diluted in 100% DMSO in case of EX527 and in distilled water in case of MPP^+. The tables according to which the solutions were prepared are shown in appendix.

5.5.3. Drug exposure

Embryos approximately 6hpf were placed by plastic pipette into 24-well plate,

5 embryos per well. The water was taken out carefully and replaced by 250µl of embryo medium per well. Embryos were checked under the microscope, damaged embryos were replaced. Afterwards the drug solutions were added in volume of 250 µl per well achieving the desired concentration. As control solutions of embryo medium and 1% DMSO were used. Experiment was performed in duplicate for each particular concentration. With larvae 3dpf the treatment procedure was the same as with embryos 6hpf.

After 24hours the drug solutions were renewed. 200µl of the drug solution were taken away from each well and 250µl of fresh solution were added instead. The difference in the volume of solutions was due to the evaporation.

5.5.4. Chorion permeability test

To test if the embryo chorion was permeable for the drug solutions and if the drug really reached the embryo, chorion permeability test was conducted. 24hpf half of the embryos were fully dechorionated and treated with solutions of 50, 100 µM EX527. As a control embryo medium was used. There were always 5 embryos per well and of each concentration two wells of dechorionated embryos were treated and two wells of embryos in chorion. Developmental readings under the microscope Nikon ECLIPSE TE300 were done 24 and 48 hours after drug application. Embryo condition was classified as normal – without any defect; abnormal – with present defect e.g. bradycardia, oedema, brain bleeding; and dead in comparison to the embryo medium control.

5.5.5. Developmental screening

Three following days after the first drug exposure embryos/larvae were being monitored under the microscope Nikon ECLIPSE TE300, magnifications used were 4, 10 and 20. Photos were taken by DinoEye camera AM-423U. Developmental abnormalities were marked in Excel tables. Based on these data graphs in GraphPad Prism 5 were made. After the first two readings followed solution refreshment. After the third reading larvae age 3dpf were used for touch response test, larvae age 6dpf were used for touch response test and locomotion monitoring.

5.5.6. Touch response

This test was done to evaluate whether larvae ability to respond to the touch stimuli was affected by the drug treatment. After the third developmental reading larvae that did not display any severe abnormalities were put separately into a Petri dish which was placed in water bath with thermostat to maintain water temperature 28°C.

After larva settled in stable position in the Petri dish center it was gently touched to the side of the tail with the pipette tip. The escape response was evaluated as positive/negative/reduced.

5.5.7. Locomotion assay

After absolving three days long drug treatment larvae age 6dpf were transferred into a 12-well plate. Always was placed one larva per one well and only larvae that did not display any abnormalities were used. Wells were filled with water without chloride in the amount to enable larvae to swim in the whole area of the well but not vertically (approximately 2 mm high water column). In one plate there were only larvae that received the same treatment (same drug and concentration). The plate was placed into water bath in which temperature 28°C was maintained by thermostat. After 10 minutes, which were provided for the fish to accommodate to the temperature and environment, a video started being made by Logitech HD Webcam C525.

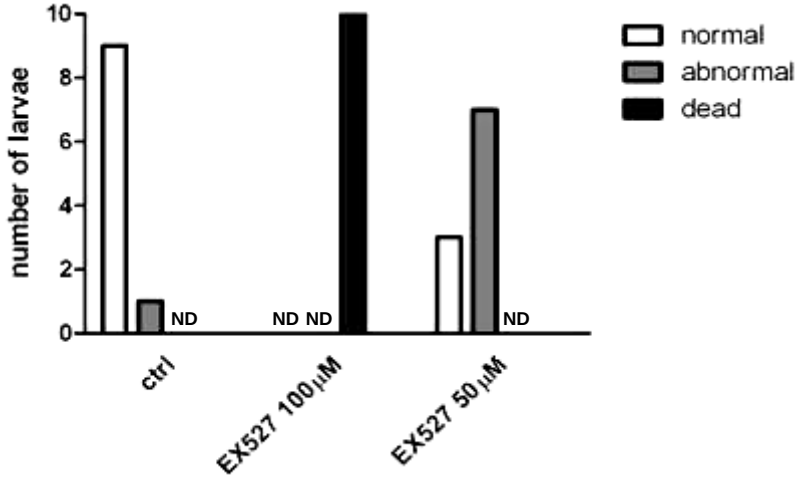
The video was then converted into image sequence by iWisoft free video converter and ImageJ program was used to analyze the image sequence. In the locomotion assay ten minute video was recorded and subsequently analyzed.

6. RESULTS

6.1. Chorion permeability test

From the drug permeability test results - see Fig. 7, it is obvious that zebrafish chorion is permeable for EX527. Developmental reading was done 24h (data not shown) and 48 hours after the drug application and toxicity of drug treatment was similar between corresponding wells of decoronated embryos and embryos in chorion. In Fig. 8 larvae displaying some typical abnormalities caused by EX527 treatment are depicted. These pictures were taken the last day of the test, when larvae were at the age of 3dpf and went through 48 hours of drug treatment. Test was done once.

Graph A: decoronated embryos treatment



Graph B: embryos in chorion treatment

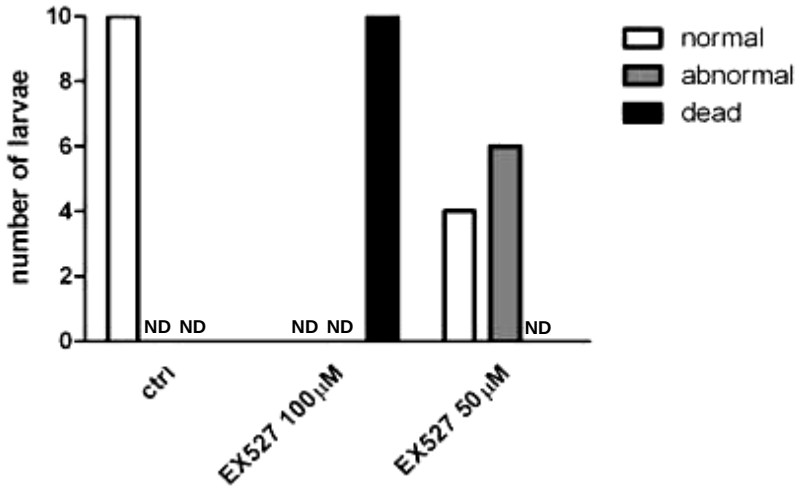
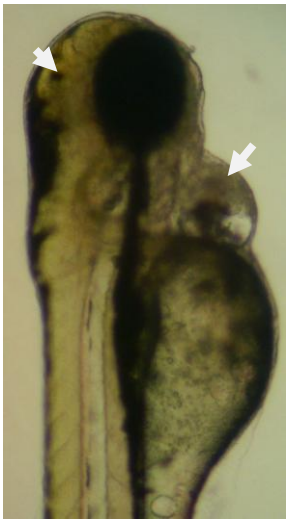


Fig. 7 Graphs showing the impact of chorion presence after 48 hours of drug treatment. Graph A – embryos that were treated without chorion presence; Graph B – embryos that were in chorion during the drug treatment; ctrl – control; ND – not detected

EX527 50µM

A



B

EX527 100µM

C

D

E

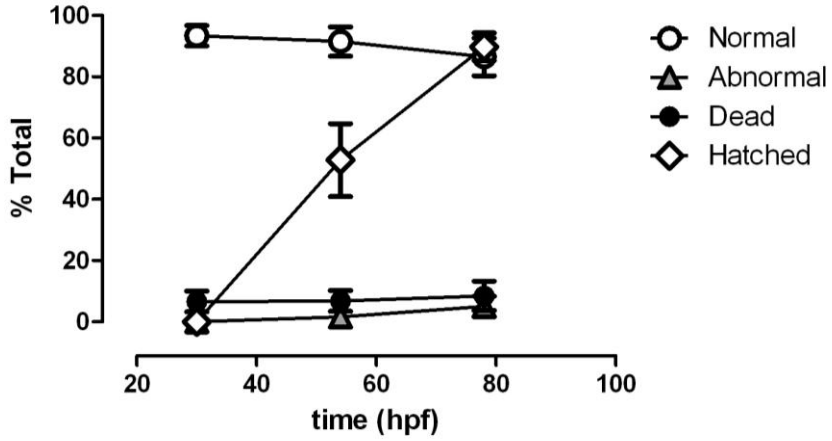
Fig. 8 Photos showing abnormalities of larvae 3dpf caused by 48 hours EX527 treatment; 50µM concentration - A white arrows mark cardiac oedema and brain necrosis and B detail of cardiac oedema. 100µM concentration – C arrows mark brain necrosis, cardiac edema and yolk necrosis; D shows tail lordosis; E tail detail with necrotic tissues

6.2. Developmental screening 6hpf – 3dpf

6.2.1. Control

Graphs from Fig. 9 show that both embryo medium and 1% DMSO have no impact on zebrafish development during the tested time period. The treatment was in case of embryo medium as well as 1% DMSO applied to 60 embryos/larvae in developmental period of 6-78hpf. Development of embryos/larvae was classified as normal – without any defect; abnormal – with present defect e.g. bradycardia, oedema, brain bleeding; and dead. Graphs also show the hatching curve.

Graph A: embryo medium control



Graph B: 1% DMSO control

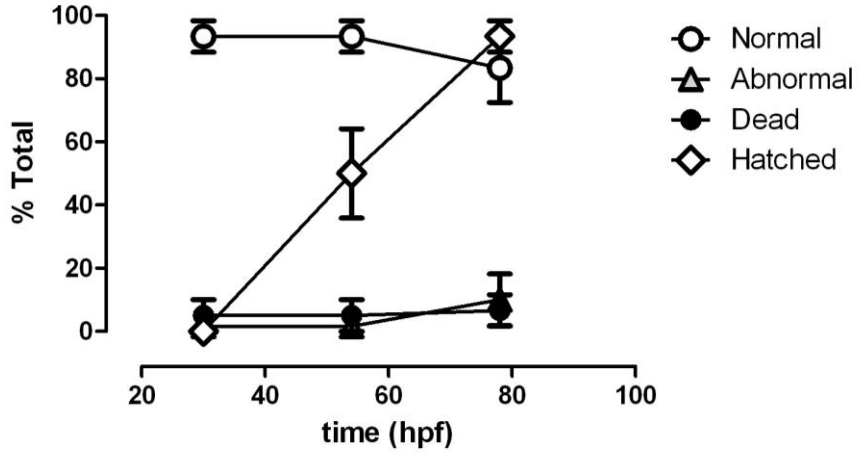
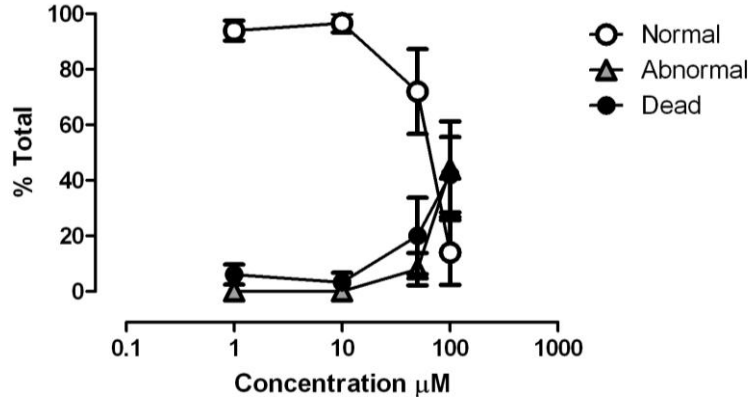


Fig. 9 Graphs showing viability of zebrafish embryos/larvae between 30-78hpf; Graph A – embryo medium control; Graph B – 1% DMSO control

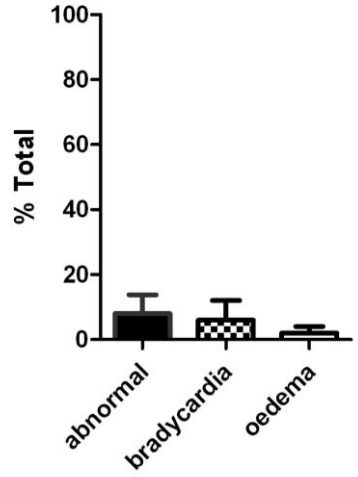
6.2.2. EX527

The number of embryos/larvae in period of 6-78hpf that received EX527 treatment was 40 for 1μM, 60 for 10μM, 50 for 50μM and 50 for 100μM concentration. 24 hours after the first drug application, when embryos were 30hpf, the first developmental reading was done. Concentrations 1μM and 10μM did not seem to cause any abnormalities; concentrations 50μM and 100μM were toxic, even lethal (Fig. 10 graph A). Toxicity results and abnormalities caused by 50μM and 100μM EX527 are shown in Fig. 10 (graphs B and C).

Graph A: EX527 toxicity 30hpf



Graph B: 50μM



Graph C: 100μM

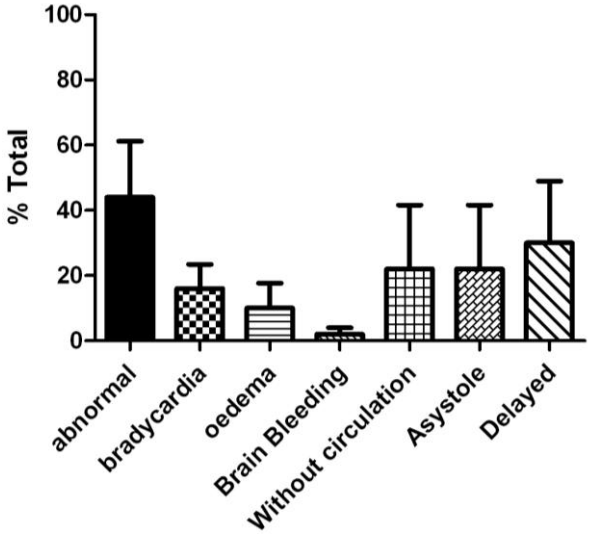
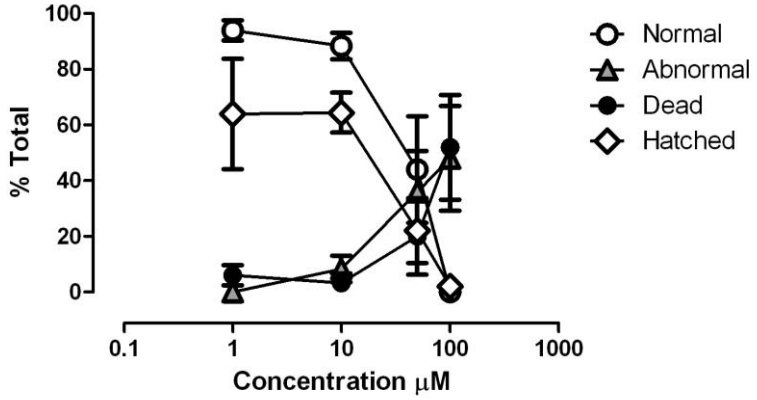


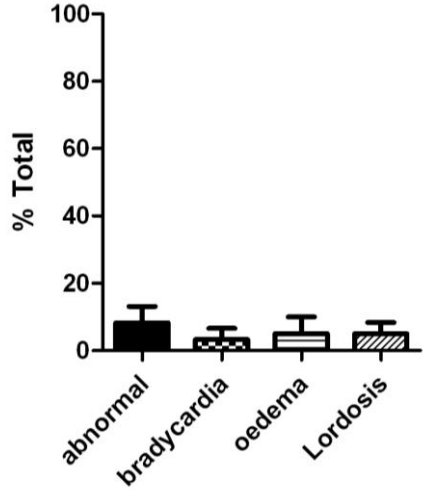
Fig. 10 Graphs showing impact of EX527 on embryos 30hpf; Graph A – curves showing percentage of normal, abnormal, dead embryos in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 50μM EX527; Graph C - percentage and type of abnormalities caused by 100μM EX527

Second developmental reading was done after next 24hours, when age of embryos was 54hpf. Third day post fertilization is a day when embryos get hatched and become larvae, therefore in graph A is also hatching curve marked to follow the rate of hatching. Fig.11 shows toxicity curves and abnormality occurrence.

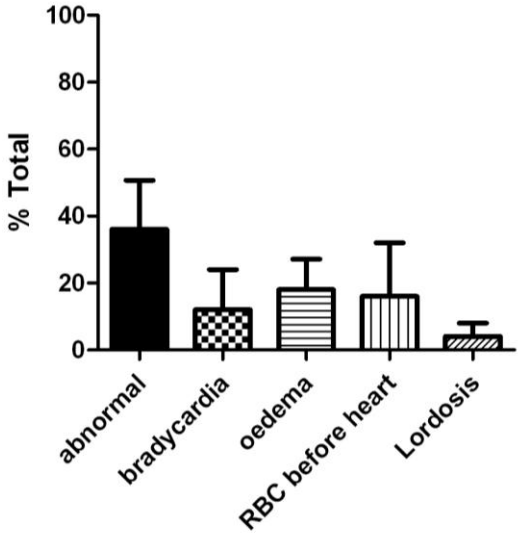
Graph A: EX527 toxicity 54hpf



Graph B: 10μM



Graph C: 50μM



Graph D: 100µM

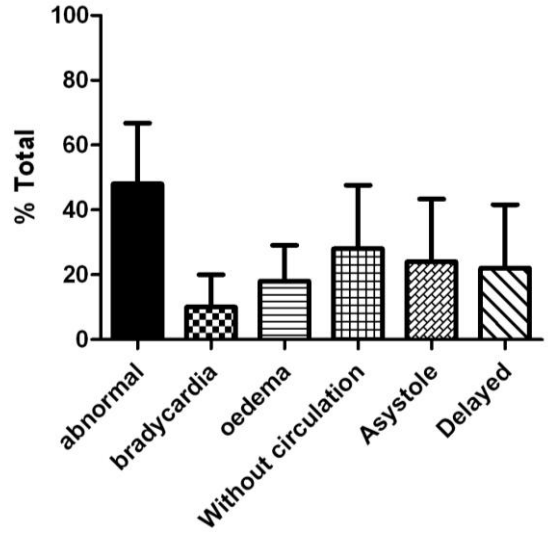


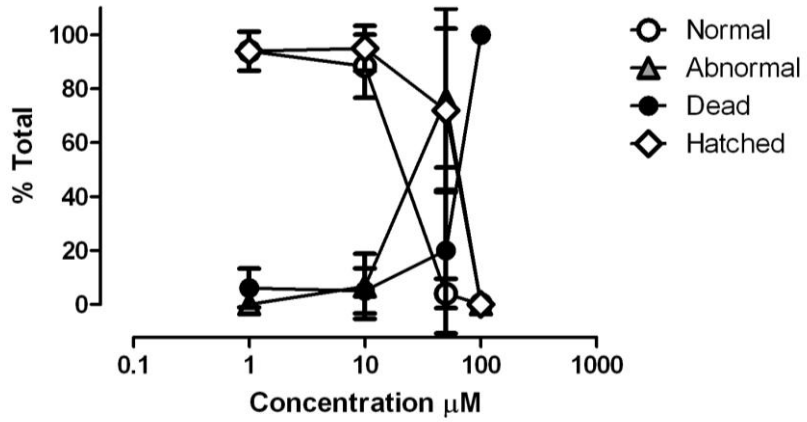
Fig. 11 Graphs showing impact of EX527 on embryos 54hpf; Graph A – curves showing percentage of normal, abnormal, dead and hatched embryos in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 10µM EX527; Graph C - percentage and type of abnormalities caused by 50µM EX527; Graph D - percentage and type of abnormalities caused by 100µM EX527

During the second developmental reading more severe toxicity was noticed. Abnormalities though infrequently were displayed also by embryos treated with 10µM EX527 and embryos treated with higher concentration in case of survival displayed much severe phenotype. Most frequent abnormalities were oedema, bradycardia or asystole, no circulation, and lordosis.

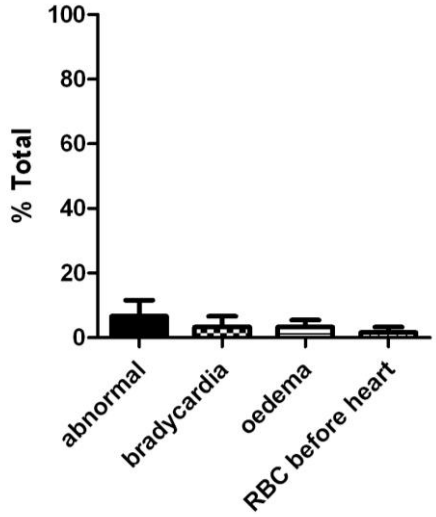
The last, third developmental reading was done 78hpf. By then embryos should have been hatched and started early larvae period. Toxicity results are shown in Fig. 12. Embryos that were treated with 100µM EX527 were dead; those treated with 50µM concentration displayed a great number of severe abnormalities.

The pictures of abnormalities caused by 50µM and 100µM EX527 concentrations during whole developmental screening with comparison to the embryo medium control are shown in Fig. 13.

Graph A: EX527 toxicity 78hpf



Graph B: 10μM



Graph C: 50μM

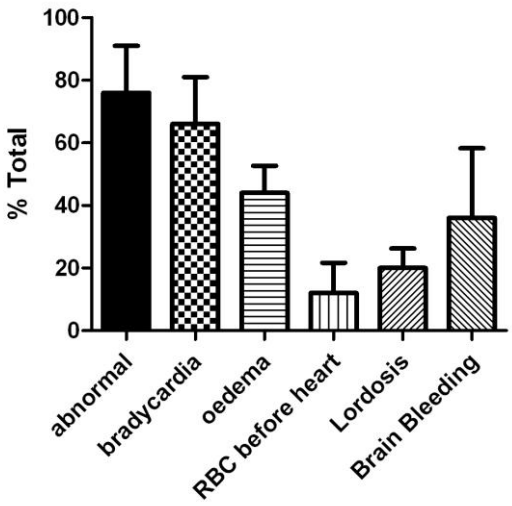


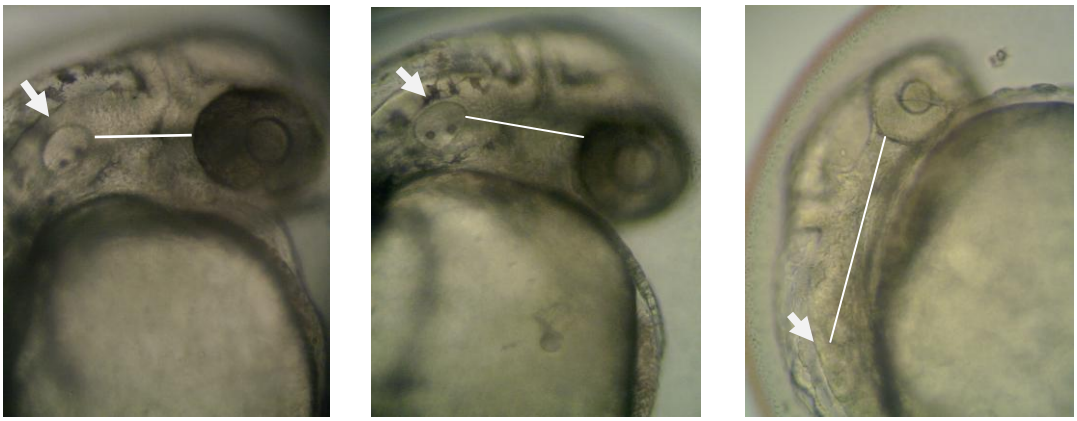
Fig. 12 Graphs showing impact of EX527 on embryos 78hpf; Graph A – curves showing percentage of normal, abnormal, dead and hatched embryos in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 10μM EX527; Graph C - percentage and type of abnormalities caused by 50μM EX527

Control EX527 50μM EX527 100μM

30hpf

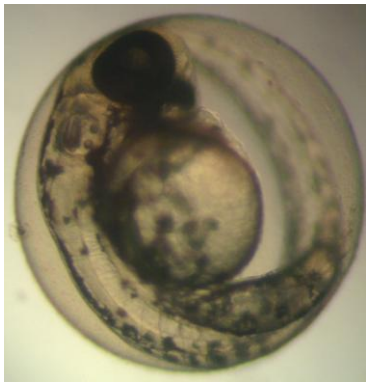
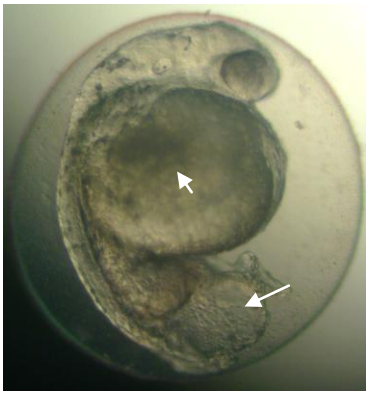
A: whole embryo

B: head detail

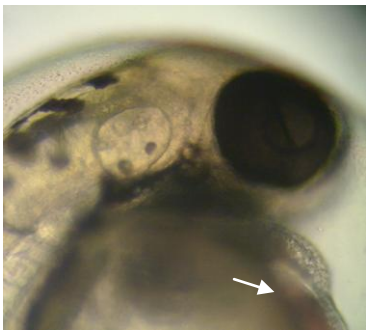
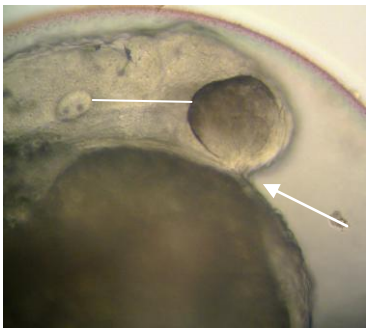


54hpf

C: whole embryo

D: head detail

78hpf

E:

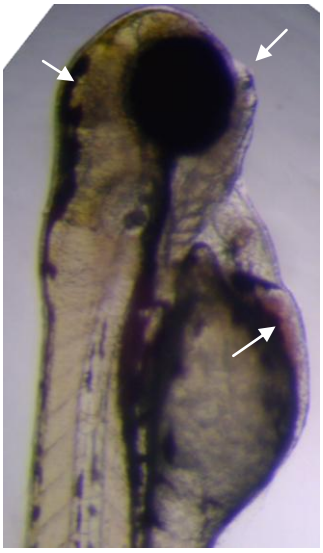
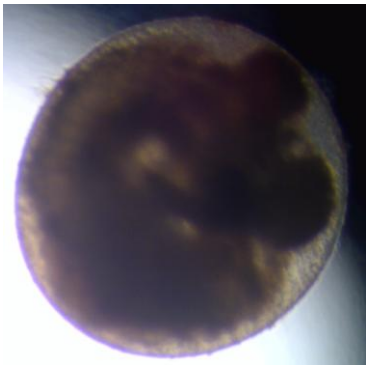




Fig. 13 Photos from zebrafish development 30-78hpf screening; First column: embryo medium control, second column: EX527 50μM, third column: EX527 100μM.
30hpf A: EX527 100μM – arrows mark abnormal shape of head, abnormal somite structure. Embryo has no melanophores nor eye pigmentation.
B: white line shows distance between otic and optic vesicle, in EX527 is clearly visible the delay of otic vesicle shape formation and its migration towards the eye.
54hpf C: EX527 100μM – embryos that survived till second developmental reading show developmental delay huge abnormalities, arrows mark abnormal tail formation, yolk necrosis and neoplasmatic structure. EX527 50μM - arrows mark brain bleeding, yolk necrosis, and accumulation of red blood cells before heart.
D: EX527 100μM – delay is shown by the position of heart (arrow), by this time it should be placed more ventrally. The distance between eye and otic vesicle shortened, but still shows delay. In some embryos eyes start to get pigmentation. EX527 50μM – arrow shows erythrocytes accumulation.
78hpf E: All EX527 100μM treated embryos are dead by now. EX527 50μM larvae show abnormalities – arrows mark brain bleeding, erythrocytes before heart and little delay in head shape formation. Yolk necrosis can be noticed.

6.2.3. 1-methyl-4-phenylpyridinium

The total number of embryos/larvae that were exposed to 1mM MPP^+ during their development in 6-78hpf was only 20. In 10% of embryos the treatment resulted in abnormal phenotype (oedema) and 10% were dead the last day of treatment not displaying any abnormal phenotype. Though the number of embryos that underwent the experiment is quite small, the results do not show any evident toxicity in comparison with embryo medium control – see Fig. 14 and Fig. 9.

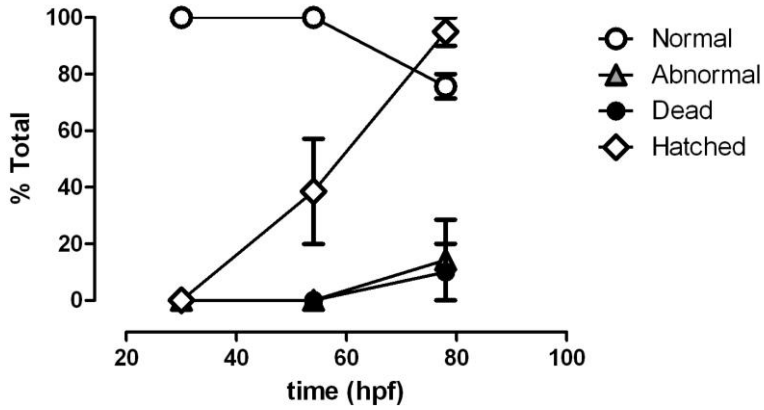


Fig. 14 Graph showing 1mM MPP^+ toxicity on zebrafish development in period of 30-78hpf

6.2.4. Combination of EX527 and 1-methyl-4-phenylpyridinium

The total number of embryos/larvae that were exposed to a combination of 10μM EX527 and 1mM MPP^+ during their development in 6-78hpf period was only 20. All of them survived the therapy without any evident developmental changes – see Fig. 15.

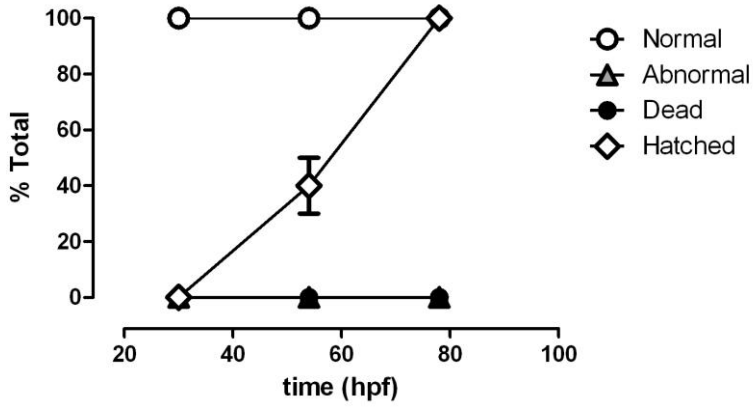


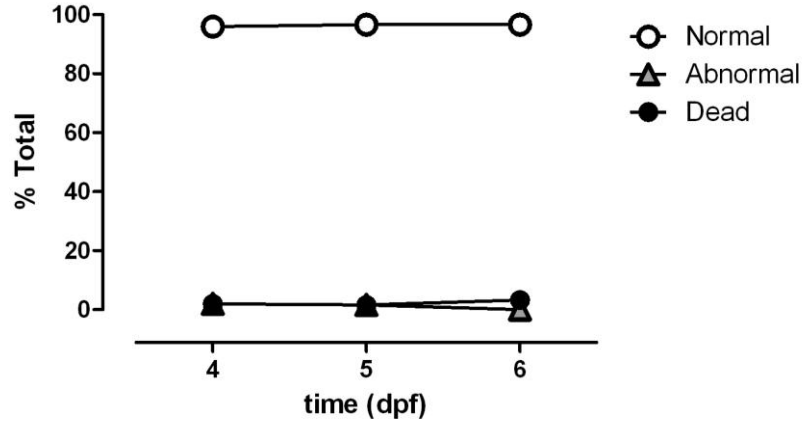
Fig. 15 Graph showing the viability curves of zebrafish treated with EX527 and MPP^+ combination in their 6-78 hpf development.

6.3. Developmental screening 3dpf – 6dpf

6.3.1. Control

Graphs from Fig. 16 show that both embryo medium and 1% DMSO have no impact on zebrafish larvae development between 3-6 dpf. The experiment included 59 larvae treated with embryo medium and 60 larvae treated with 1% DMSO. Development of larvae was classified as normal – without any defect; abnormal – with present defect e.g. bradycardia, oedema, brain bleeding; and dead.

Graph A: embryo medium control



Graph B: 1% DMSO control

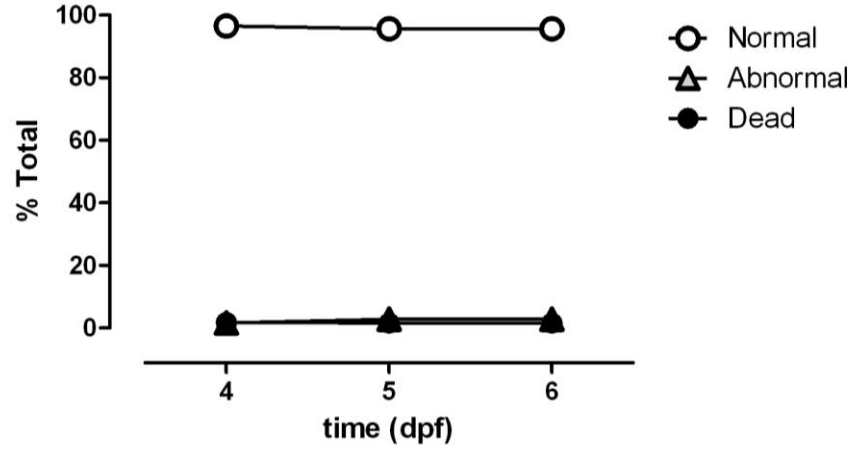


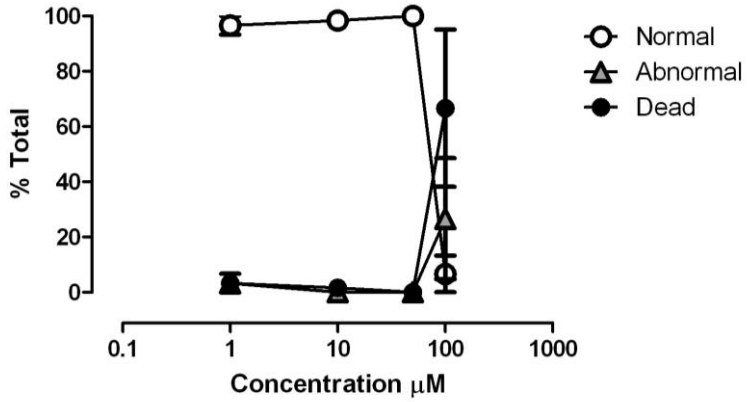
Fig. 16 Graphs showing viability of zebrafish larvae between 4-6dpf; Graph A – embryo medium control; Graph B – 1% DMSO control

6.3.2. EX527

The number of larvae in period of 3-6dpf that received EX527 treatment was 40 for 1µM, 50 for 10µM, 20 for 50µM and 50 for 100µM concentration.

24 hours after the first drug application, when larvae were 4dpf, the first developmental reading was done. Concentrations 1µM, 10µM and 50µM did not seem to cause any abnormalities, concentration 100µM caused abnormalities or death – see Fig. 17.

Graph A: EX527 toxicity 4dpf



Graph B: 100µM

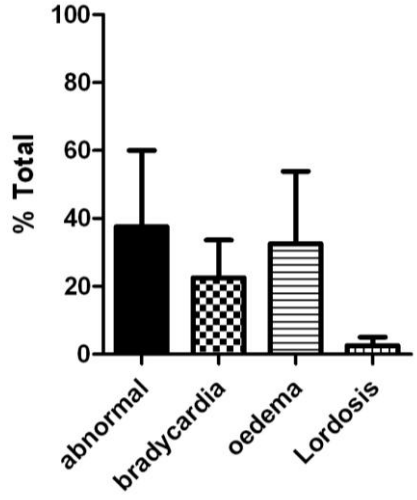
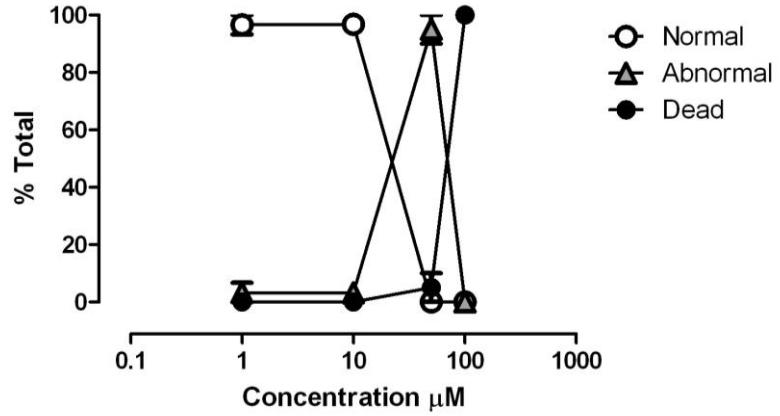


Fig. 17 Graphs showing impact of EX527 on embryos 4hpf; Graph A – curves showing percentage of normal, abnormal, dead larvae in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 100µM EX527

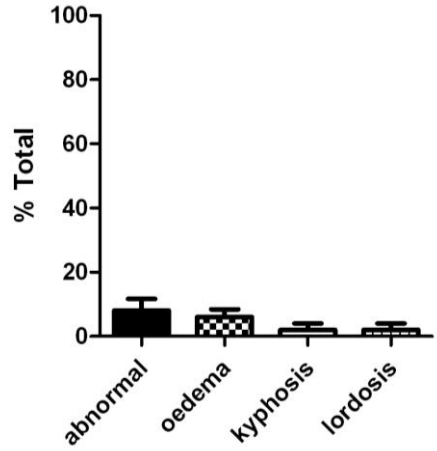
Second developmental reading was done after next 24hours, when age of larvae was 5dpf. Concentration 10µM did not seem to cause any significant

toxicity, but 50μM EX527 caused abnormal phenotype and 100μM caused death almost in all larvae treated – results are shown in Fig. 18.

Graph A: EX527 toxicity 5dpf



Graph B: 10μM



Graph C: 50μM

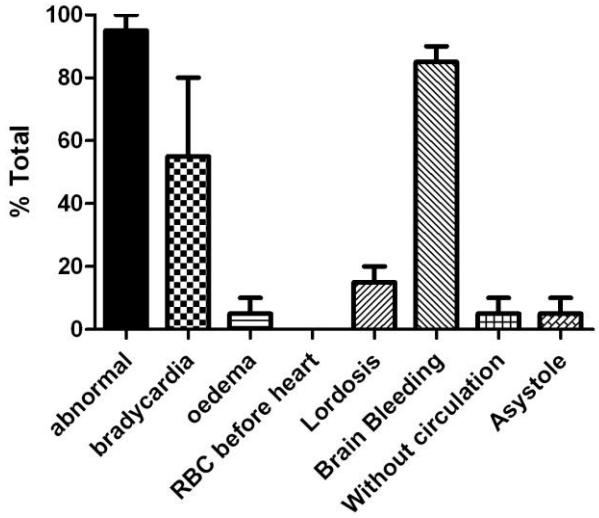
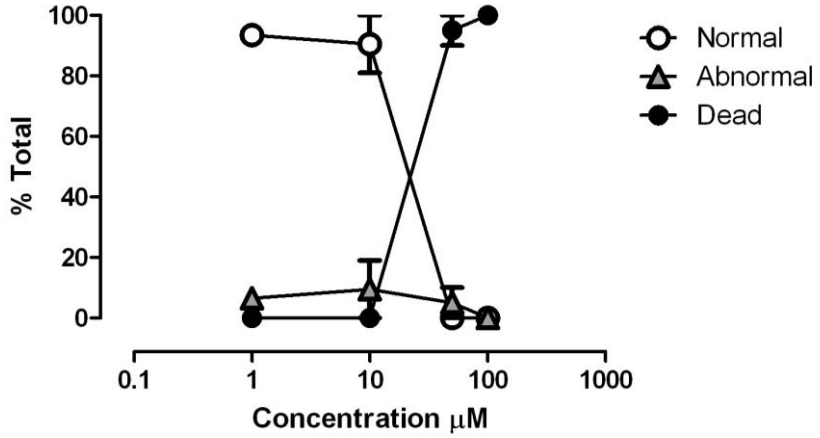


Fig. 18 Graphs showing impact of EX527 on larvae 5dpf; Graph A – curves showing percentage of normal, abnormal and dead larvae in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 10μM EX527; Graph C - percentage and type of abnormalities caused by 50μM EX527

The last, third developmental reading was done 6dpf. Toxicity results are shown in Fig. 19. Larvae treated with 100 μM EX527 died the day before, but also larvae that were treated with 50μM EX527 were dead by now and about 20% of larvae treated with 10μM concentration displayed abnormalities.

Graph A: EX527 toxicity 6dpf



Graph B: 10μM

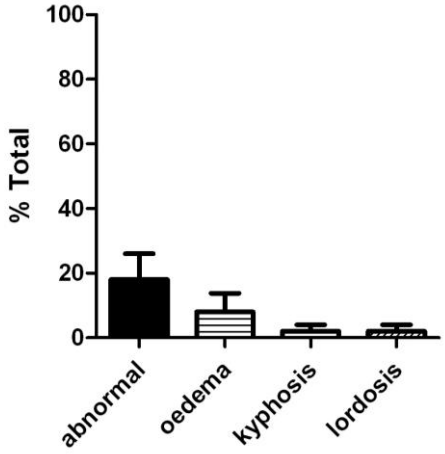


Fig. 19 Graphs showing impact of EX527 on larvae 6dpf; Graph A – curves showing percentage of normal, abnormal and dead larvae in relation to drug concentration; Graph B – percentage and type of abnormalities caused by 10μM EX527

6.3.3. 1-methyl-4-phenylpyridinium

The MPP^+ treatment in 1mM concentration absolved 30 larvae in period of 3-6dpf. At this stage of development MPP^+ seems to have much more severe impact than on development of embryos/larvae at 6-78hpf. On the 6dpf, the end of the treatment, MPP^+ resulted in 33% lethality. The larvae that survived did not display any abnormalities. The results are shown in Fig. 20.

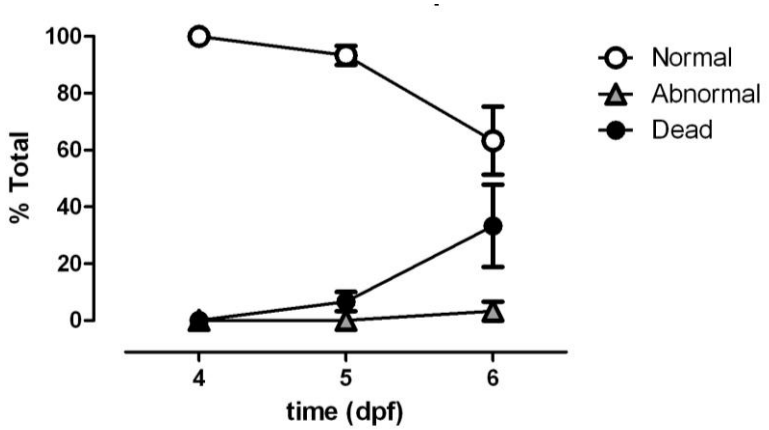


Fig. 20 Graph showing 1mM MPP^+ toxicity on zebrafish development in period of 4-6dpf

6.3.4. Combination of EX527 and 1-methyl-4-phenylpyridinium

To the combination of 10μM EX527 and 1mM MPP^+ was exposed 20 larvae during their development in 3-6dpf. The lethality of larvae was much higher compared to treatment with only MPP^+, since 25% of larvae were found dead in the second reading and 95% did not survive the whole therapy – see Fig.21.

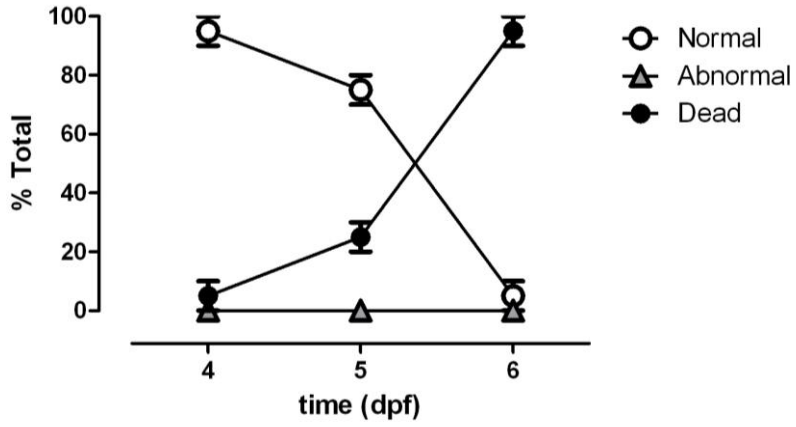


Fig. 21 Graph showing the viability curves of zebrafish treated with EX527 and MPP^+ combination in their 3-6 dpf development.

6.4. Touch response

The touch response test was always conducted with a different number of larvae, because some larvae died during the treatment and only larvae displaying none or only minor abnormalities were used. Therefore the values for the graph were calculated as a percentage to the respective control.

In Fig. 22 are shown the results of touch response test conducted on zebrafish larvae after receiving drug treatment in 6-78hpf period. The response was visually evaluated as positive – fast escape with expected distance of larvae with normal development; reduced – decreased distance and usually also speed of escape; negative – no response at all. The test was done with larvae that received 10μM EX527, 1mM MPP^+ or 10μM EX527 combined with 1mM MPP^+ treatment. As control were used larvae treated with embryo medium. The graph shows that EX527 has no effect on larvae reaction after touch response. 1mM MPP^+ reduces ability of larvae to respond to the touch and in combination with EX527 this ability is reduced even more.

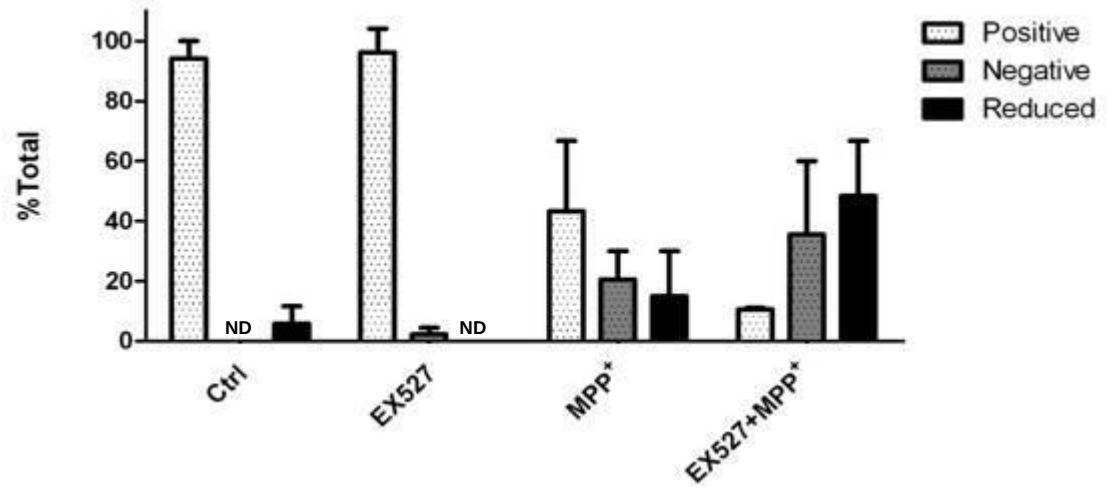


Fig. 22 Touch response of larvae 78hpf

In Fig. 23 are shown the results of touch response test conducted on zebrafish larvae after absolving drug treatment in 3-6dpf period. The larvae absolved 10μM EX527, 1mM MPP^+ or 10μM EX527combined with 1mM MPP^+ treatment. As control were used larvae treated with embryo medium. The graph shows that EX527 might influence larvae response to the touch at this point of development. 1mM MPP^+ has even more severe impact on larvae respond than

EX527. Combined MPP^+ and EX527 treatment had very severe influence and only one larva survived and had reduced response to the touch.

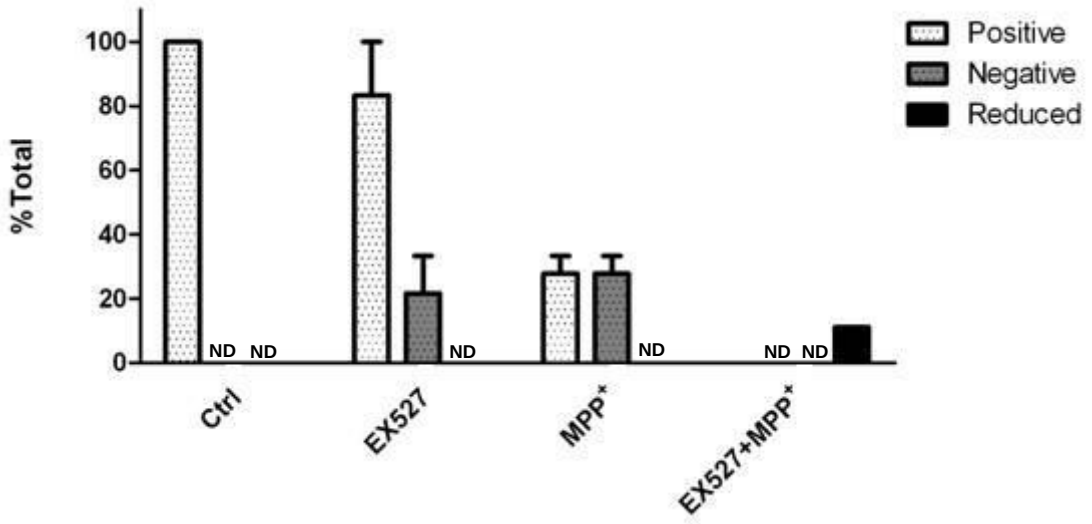


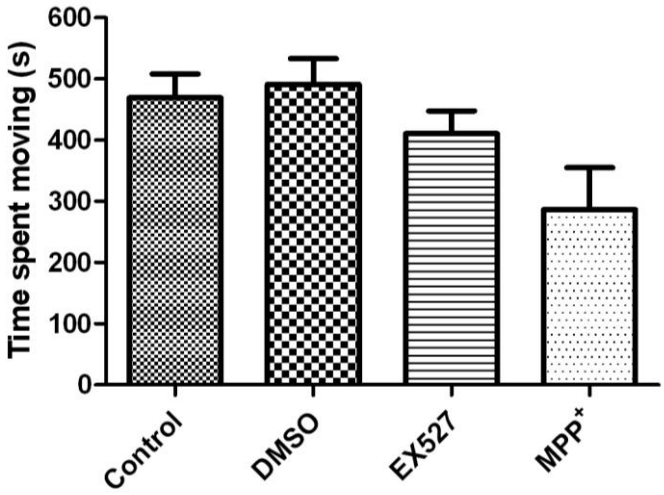
Fig. 23 Touch response of larvae 6dpf

6.5. Locomotion assay

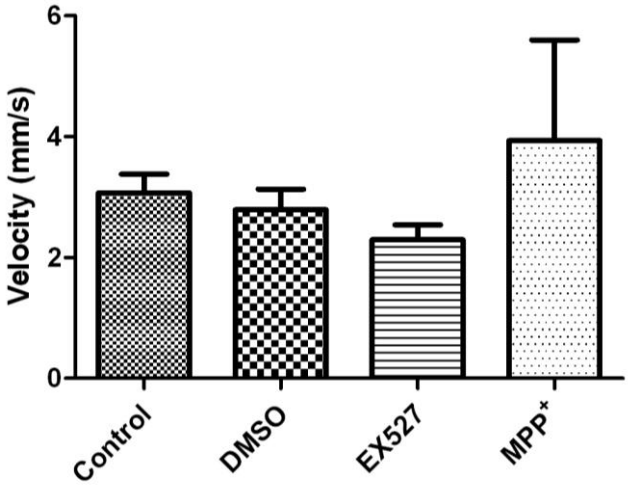
In Fig. 24 are shown the results of locomotion video analysis with larvae 6dpf. The assay was not absolved by larvae treated with MPP^+ and EX527 in combination, since this combination turned to be highly toxic and only one larva managed to survive the treatment.

The total time that larvae spent by swimming was 10% reduced by EX527 treatment (68%), but 30% by MPP^+ (48%) in comparison to the embryo medium control (78%). The elevation of MPP^+ bar in graph B is caused by one highly mobile larva – 10 times in comparison with other MPP^+ treated larvae. Otherwise the speed of MPP^+ treated larvae is very close to speed of EX527 treated larvae. The total length of larvae trajectories corresponds with the time they spent in motion. MPP^+ treatment does not seem to influence the number of times larvae started to swim, however EX527 seems to raise this number.

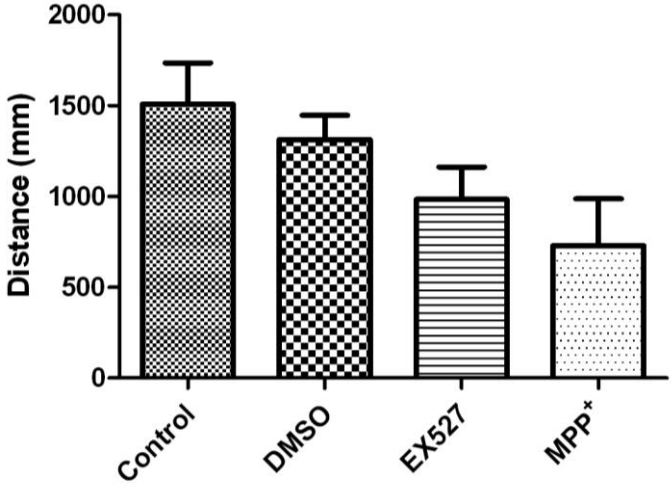
Graph A: Time (s) larvae spent moving



Graph B: velocity (mm/s) of larvae swimming



Graph C: total length of larvae trajectory (mm) when swimming



Graph D: total number of swimming initiations of one larva during the video

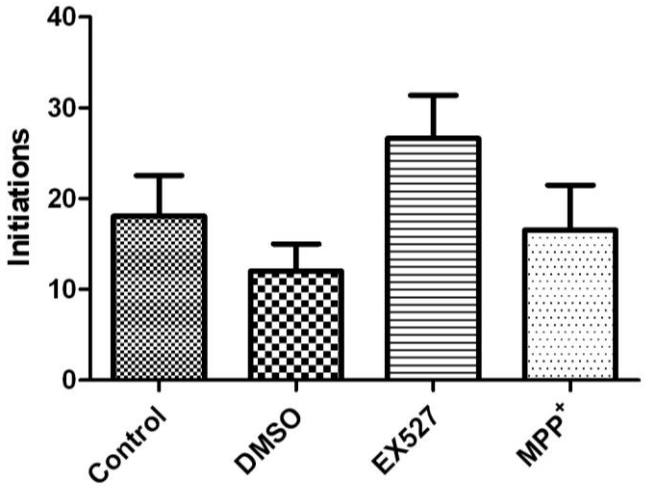


Fig. 24 Locomotion video analysis. Graphs showing behavior of larvae treated with 10μM EX527, 1mM MPP^+ and 1% DMSO and embryo medium controls. Graph A shows the amount of total 600s of the video, that larvae spent in motion; Graph B shows the average larvae velocity during the locomotion; Graph C shows length of the trajectory that larvae transposed during the whole video; Graph D shows the number of swimming initiations during the video.

7. DISCUSSION

This study confirms that SIRT1 indeed plays significant role during embryo development. SIRT1 inhibition was attained by EX527, selective SIRT1 inhibitor without any other known pharmacological mechanism. This led to abnormal phenotype (Fig. 8 and 13) and death in dose dependent manner. In embryos treated with 100μM concentration of EX527 in period between 6hpf and 78hpf after the first 24 hours of treatment 42% lethality was observed (Fig. 10). Those embryos that survived displayed abnormal phenotype in comparison to the controls (Fig. 9 and 13). A part of the abnormalities was caused rather by delay in development, which was observed by delayed somite formation, slower translocation of the otic vesicle towards the eye, lack of pigmentation. Also cardio-vascular system was significantly affected. The heart formation was delayed and cardiac oedema was often present. In those embryos where heart beat developed, it was very slow and no blood circulation to the body was noted. Next day, 54hpf, 48% showed signs of life but apart from abnormalities described from previous day also malformations of tail and unidentified cellular structures (Fig. 11 and 13). Some of them demonstrated first signs of pigmentation. None of these embryos was ever found hatched and the lethality was 100% by the next 78hpf reading (Fig. 12). 50μM EX527 concentration in 30hpf caused 20% death, which is almost three times higher in comparison with 7% lethality of both DMSO and embryo medium control (Fig. 9 and 10). 30hpf surviving embryos treated with 50μM EX527 did not show any disturbing level of abnormalities. However, 54hpf 36% of embryos treated with 50μM EX527 displayed abnormal phenotype such as cardiac oedema, bradycardia and accumulation of erythrocytes before heart (Fig. 11 and 13). By 78hpf 50μM EX527 treatment resulted in 20% death and 76% embryos/larvae showing abnormal phenotype (Fig. 12 and 13). Lethality and abnormal development noted in10μM EX527 treated embryos is very similar to both controls, leading us to the conclusion that this EX527 concentration is non-toxic for zebrafish at this stage of development (Fig. 9, Fig. 10, Fig. 11, Fig. 12). This deduction was supported by results of touch response test where larvae at age 78hpf treated

with 10μM EX527 behaved in the same manner as control larvae (Fig. 22).

In larvae treated in period 3 to 6dpf SIRT1 inhibition caused more severe damage compared to treatment in earlier period. After 24hours of treatment with 100μM EX527 62% larvae were found dead and 30% displayed severe abnormalities like cardiac oedema, gnarled tails and brain bleeding (Fig. 17). By the next day, at the age 5dpf all of these larvae were found dead (Fig. 18). 50μM EX527 concentration did not show any toxicity after first 24 hours of treatment, after 48 hours all of these larvae were affected showing brain bleeding and heart defects (Fig. 17 and 18). Only one larva managed to survive till third day of treatment suffering many defects linked to SIRT1 inhibition. Concentration 10μM of EX527 seems to be quite safe, though on the last day of treatment 18% of larvae suffered from defects mainly cardiac oedema. There is a possibility that larvae ability to response to the touch stimuli was also influenced, because 20% of larvae that survived 10μM EX527 treatment failed to give the swim away response to the touch stimuli (Fig. 23). These results are in conformity with locomotion assay results (Fig. 24), where 10μM of EX527 caused that larvae started to swim more times in comparison to the control, but swam for shorter time and also their speed was little reduced. These findings lead us to that older larvae are more sensitive to the SIRT1 inhibition than embryos, though lack of SIRT1 also leads to developmental complications and death in embryos as well.

These findings are in accordance with results Wang et al. (2008), Cheng et al. (2002) and McBurney et al. (2003) got from generating SIRT1 deficient mice embryos, which when survived displayed abnormal shape of head, lack of hindlimb bud and in general they were smaller thanks to nuclear fragmentation and cell death. Our embryos/larvae after 50μM EX527 treatment showed on second and third day very similar phenotype – cardiac oedema and bleeding - as Potente et al.(2007) attained with anti SIRT1 morpholinos at tg(fli1:eGFP) line of zebrafish embryos, which are special for their endothelial cells being fluorescent. There is a possibility that we also observed extra branching of intersomitic vessels which was described by Potente team, but we cannot be certain about this since we were using wild-type zebrafish embryos which are

transparent.

In zebrafish embryos/larvae treated with 1mM MPP^+, concentration used to induce PD like symptoms in zebrafish, in their 6-78hpf during the monitoring with microscope no visually evident toxicity was noticed (Fig. 14). However, when we exposed these larvae to the gentle tail-touch stimuli, we recorded negatively altered escape reaction (Fig. 22). The exact percentage which is shown in touch response graph might be distorted thanks to a small larvae sample that underwent the test; however it is evident that MPP^+ treatment negatively influences larvae ability to tactile perception. The hypothesis that simultaneous SIRT1 inhibition with 10μM EX527 which itself seems to be nontoxic at this stage of development might be able to rescue zebrafish larvae from MPP^+ toxicity, proved to be false. The larvae that were co-treated with 1mM MPP^+ and 10μM EX527 appeared to be absolutely normal during microscope monitoring, but their ability to escape after touch stimuli was even more reduced than their MPP^+ treated littermates (Fig. 15 and Fig. 22).

In larvae between 3 and 6dpf MPP^+ treatment seems to have more severe effect in comparison with earlier application, but 75% managed to survive not displaying any abnormalities (Fig. 20). However, 50% of these larvae failed to answer to the touch stimuli to the tail (Fig. 23). Co-treatment with 10μM EX527 did not help to reduce the toxicity, but right to the contrary resulted in almost 100% lethality (Fig. 21). The larvae that were treated with both drugs died during the whole course of therapy and none of them displayed any visually evident defect that might be reason of death.

The experiments done with EX527 bring evidence that EX527 is capable of selective SIRT1 inhibition which can be in dose dependent manner compared to SIRT1 knock down by morpholinos. The results we attained by combining non-toxic concentration of EX527 with MPP^+ in zebrafish embryos/larvae, in other words inhibiting function of SIRT1 in vertebrate *in vivo* PD model, strongly suggest that SIRT1 inhibition is not a way to treat PD. A dose of EX527 which was supposed to be safe not only failed to rescue the embryos/larvae from MPP^+ treatment, but in zebrafish treated between 6-78hpf worsened their tactile perception, though visually left them undistinguishable from their untreated

littermates. In larvae treated in period of 3 to 6dpf the SIRT1 inhibition with simultaneous exposure to MPP^+ caused even much worse damage, nearly total lethality. Why larvae that were treated in very early developmental stages managed to survive, while their older siblings died after the same treatment is a curious fact, we do not know the explanation to, neither is known the mechanism by which SIRT1 inhibition potentiates the MPP^+ toxicity. It seems safe to say that MPP^+ toxicity was increased by non-toxic SIRT1 inhibition, rather than that SIRT1 inhibition was increased to toxic level by MPP^+, because combination of these drugs did not cause abnormal phenotype which was observed on larvae treated with EX527 in doses capable of inhibition of SIRT1 on toxic levels. This would be in accordance with existence of specific SIRT1 promoter sequences linked to PD (Zhang et al. 2012).

Regarding the fact that SIRT1 is predominantly located in nucleus, it is probable that the mechanism by which it influences PD development happens in the nucleus as well. Whether it is the lack of Hsp70 activation caused by SIRT1 reduced function or more likely other yet unknown mechanism remains a question (Donmez et al. 2012). Another question is whether activation of SIRT1 would help to suppress PD development, though regarding research conducted by Kakefuda et al. (2009) this option also seems unlikely.

So is interference of SIRT1 function a way to slow down or even cure PD or is it more likely a dead end? This is a question that requires time and further investigation to be answered.

8. CONCLUSION

We brought further evidence and confirmed current knowledge of SIRT1 function. We show that selective SIRT1 inhibitor EX527 is capable in dose dependent manner of SIRT1 inhibition comparable to that achieved by morpholinos. On *in vivo* vertebrate model we confirmed that SIRT1 is needed for normal embryonic development and thriving, confirming that the malfunction results in wide range of developmental defects. Most frequently noted abnormalities were linked to delay in development and to cardiovascular system – heart swelling, bradycardia, and brain bleeding. In later stages zebrafish larvae seem to be even more sensitive to SIRT1 inhibition. The phenotype we reached on zebrafish is comparable to phenotype reached on SIRT1 down-regulated mice models confirming zebrafish as a valuable animal model for studying mechanisms linked to human diseases.

When we exposed zebrafish embryos at the age of 6hpf to combined EX527 in nontoxic concentrations and MPP^+ treatment in hope that SIRT1 inhibition might protect them from PD symptoms development, we got rather contradictory results. SIRT1 inhibition not only fails to save embryos from MPP^+ toxicity, but seems to potentiate it. With larvae exposed between 3 to 6dpf the SIRT1 inhibition in PD model zebrafish has very severe impact resulting in death. According to these results we conclude that SIRT1 inhibition does not have protective effects in PD development.

There is growing body of evidence that sirtuins and other means of epigenetic modulation might be the causative in pathogenesis of e.g. neurodegenerative diseases. Better knowledge of epigenetic modulation, thus, opens for us new ways for effective therapy. However, all the findings in SIRT1 and other sirtuins area indicate that their activity is very complex and includes regulatory function of broad range of cellular mechanisms. In previous ten years sirtuins became extremely interesting target that still requires a lot of investigation, which is being carried in the laboratories all over the world.

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10. APPENDIX

Glossary

Blastoderm – cellular part of the embryo, excluding the yolk cell, derived from the blastodisc by early morphogenesis; refers particularly to the time when the cell array is sheet-like, between 30%-epiboly and the end of gastrulation (Kimmel et al. 1995)

Blastodisc – dome of cytoplasm that segregates from the yolk toward the animal pole during and after one cell-stage, and which undergoes cleavage (Kimmel et al. 1995)

Cleithrum - transversely oriented bone connecting the occipital region of the skull dorsally and pectoral girdle ventrally; appears near the end of embryogenesis (Kimmel et al. 1995)

Epiblast – the outer of the two layers of the blastoderm that form during gastrulation, corresponding to primitive ectoderm during gastrulation and to the definitive ectoderm after gastrulation (Kimmel et al. 1995)

Epiboly – the thinning and spreading of both the yolk syncytial layer and the blastoderm over and across the yolk cell, eventually encompassing the yolk cell completely; epiboly begins at dome stage, converts the blastodisc to the bastoderm, and is considered to be over when the yolk plug closes over (at 100% epiboly) (Kimmel et al. 1995)

Epigenetic code - is a defining code in eukaryotic cells consisting of the specific epigenetic modification in each type of cell (Turner 2007)

Epigenome – consists of a record of the chemical changes to the DNA and histone proteins of an organism. These changes can be passed down to an organism´s offspring. Changes in epigenome can result in changes to the structure of chromatin and changes to the function of the genome (Bernstein et al 2007)

Hypoblast (mesendoderm) - the inner of the two layers of the blastoderm that form during gastrulation and gives rise to the definitive mesoderm and endoderm (Kimmel et al. 1995)

Iridophore – reflective pigment cell (Kimmel et al. 1995)

Neuromasts - volcano-shaped lateral line sensory organ located in characteristic positions within the skin epithelium and containing hair cells and their support elements (Kimmel et al. 1995)

Neuromere - a brain subdivision recognized morphologically as a swelling bounded by constrictions (Kimmel et al. 1995)

Otic vesicle length - a staging device or index defined as the number of additional otic vesicles that could be fit in between the otic vesicle and the eye (Kimmel et al. 1995)

Polster (pillow) - the hatching gland rudiment at the time it underlies the forebrain during the early segmentation period (Kimmel et al. 1995)

Somite - undifferentiated mesodermal component of an early trunk or tail segment or metamere, derived from paraxial mesoderm; forms the myotome, sclerotome, and perhaps dermatome (Kimmel et al. 1995)

Xanthophore - a neural crest-derived cell pigmented yellow (Kimmel et al. 1995)

Yolk syncytial layer – peripheral layer of the yolk cell including nuclei and monyolky cytoplasm (Kimmel et al. 1995)

Drug solution preparation tables

Table 2. Drug concentration for the first exposure

Drug solution	Volume prepared (µl)	Embryo medium (µl)	2% DMSO (µl)	Drug solution (µl)
EX527 200µM	1100	1078	0	22 EX257 10mM
EX527 100µM	600	0	300	300 EX527 200µM
EX527 20µM	600	0	540	60 EX257 200µM
EX527 2µM	600	0	540	60 EX257 20µM
MPP^+ 2mM	600	480	0	120 MPP^+ 10mM
EX527 20µM+MPP^+ 2mM	600	420	0	60 EX257 200µM + 120 MPP^+ 10mM

Tab. 2 Drug concentration. First column: tested concentration of drug solution that needed to be prepared for drug exposure; second column: prepared volume of drug solution (µl); third column: volume (µl) of embryo medium used for preparation; fourth column: volume (µl) of DMSO solution used for preparation; fifth column: volume (µl) and concentration of drug solution used for preparation

Table 3. Drug concentration for the second and third exposure

Drug solution	Volume prepared (µl)	Embryo medium (µl)	1% DMSO (µl)	Drug solution (µl)
EX527 100µM	1100	1089	0	11 EX257 10mM
EX527 50µM	600	0	300	300 EX527 100µM
EX527 10µM	600	0	540	60 EX257 100µM
EX527 1µM	600	0	540	60 EX257 10µM
MPP^+ 1mM	600	540	0	60 MPP^+ 10mM
EX527 10µM+MPP^+ 1mM	600	480	0	60 MPP^+ 10mM + 60 EX257 100µM

Tab. 3 Drug concentration. First column: tested concentration of drug solution that needed to be prepared for drug exposure; second column: prepared volume of drug solution (µl); third column: volume (µl) of embryo medium used for preparation; fourth column: volume (µl) of DMSO solution used for preparation; fifth column: volume (µl) and concentration of drug solution used for preparation

For the first drug exposure the drug solutions and DMSO concentrations

needed to be doubled, because wells with the zebrafish embryos already contained 250µl of embryo medium. The drug solutions for the first exposure were prepared according to Table 2., for second and third according to Table 3.