Folia Biologica et Oecologica 14: 1–13 (2018)

Acta Universitatis Lodziensis

Understanding cannabinoid receptors: structure and function

ANGELIKA ANDRZEJEWSKA[1], KLAUDIA STASZAK[1], MARTA KACZMAREK-RYŚ[1], RYSZARD SŁOMSKI[1,2], SZYMON HRYHOROWICZ*[1]

[1]Institute of Human Genetics, Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznan, Poland
[2]Department of Biochemistry and Biotechnology, University of Life Sciences, Dojazd 11, 60-632 Poznan, Poland
E-mail: szymon.hryhorowicz@igcz.poznan.pl

ABSTRACT

The endocannabinoid system (ECS) consists of the endocannabinoids, cannabinoid receptors and the enzymes that synthesize and degrade endocannabinoids. The whole EC system plays an important role in the proper functioning of the central and autonomic nervous system. ECS is involved in the regulation of the body energy and in the functioning of the endocrine system. It can affect on the regulation of emotional states, motoric movement, operations of the endocrine, immune and digestive system. Many of the effects of cannabinoids are mediated by G coupled –protein receptors: CB1, CB2 and GPR55 but also of transient receptor potential channels (TRPs) which not only induce the sensation of pain but also support inflammation via secretion of pro-inflammatory neuropeptides. In this review work we briefly summarize the role and action of cannabinoid receptors CB1 and CB2, protein-coupled receptor 55 (GPR55) and transient receptor potential vanilloid 1 (TRPV1).

KEY WORDS: cannabinoid receptors, CB1, CB2, TRPV1, GPR55

Introduction

Cannabinoid receptors are one of the most abundant neuronal receptors that are linked through G proteins (the guanine-nucleotide-binding proteins) to the effectors system (called G-protein coupled receptors, GPCRs). This group includes two well characterized types of receptors – CB1 and CB2. They are part of the endocannabinoid system (ECS), consisting of lipophilic substances stimulating their function – endocannabinoids and exogenous agonists, as well as enzymes governing the synthesis and degradation (Pawlak et al., 2011). In recent years, researches have provided evidence that the system is more complicated and additional receptor types should exist to explain ligand activity in

DOI: 10.1515/fobio-2017-0004

many physiological processes. To date other types of cannabinoid receptors, non-CB1 and non- CB2, have been reported such as orphan GPR55 and TRPV1 receptors, which could explain not completely understood pathway system (Bisogno et al., 2001; Ryberg et al., 2007; Befort, 2015).

It is known that mammalian tissues may produce endogenous ligands of cannabinoid receptors. First discovered the substance of this type was the amide derivative of arachidonic acid - arachidonoyl ethanolamide - AEA, which was called anandamide. Later was discovered another endocannabinoid of lipid structure - 2-arachidonyl glycerol (2-AG). These substances can activate receptors and are synthesized on demand in response to elevations of intracellular calcium. Studies show that repeated administration of cannabinoid receptor agonists may cause the development of tolerance to some of their effects (Pertwee, 2006; Pertwee, 2009).

Exogenous cannabinoids has been termed substances that are extracted from the marijuana plant Cannabis sativa or are synthesized artificially. The most popular examples of these substances are cannabinol (CBN), cannabidiol (CBD) and delta-9-tetrahydrocannabinol (Δ9-THC). The last of them is the major immunomodulatory and psychoactive component of the marijuana (Cabral and Griffin-Thomas, 2009).

The discovery of CB1 and CB2 receptors was followed by the development of CB1- and CB2-selective cannabinoid receptor antagonists. These substances block the normal operation of the receptor and may weaken or suppress agonist (Pertwee, 2006).

The whole endocannabinoid system plays an important role in the proper functioning of the central and autonomic nervous system. The reason for such action of cannabinoids is their easy passage through the blood-brain barrier. ECS is involved in the regulation of the body energy and in the functioning of the endocrine system. It can affects on the regulation of emotional states, motoric movement, operations of the endocrine, immune and digestive system (Komorowski and Stępień, 2007). This system plays a major role in the control of pain, in reward processing and in the development of addiction (Befort, 2015).

Although the relationship between activity of cannabinoid receptors and cell proliferation is not fully understood. It is known that certain agonists of these receptors, including anandamide, inhibit proliferation of cultured human breast cancer cells. It is worth noting, that the tests on the effectiveness of cannabinoids in the treatment of neurodegenerative diseases (such as Parkinson disease) are carried out, because of their neuroprotective properties (Konarska and Ellert, 2004).

Methods

Publication search was performed in Medline and PubMed database. The key words used were cannabinoids receptors, cannabinoids, CB1, CB2, GPR55, TRPV1. We decided to use both, the older papers describing the discovery of cannabinoid receptors and more recent publications, describing possible new interactions between potential novel receptors and cannabinoids.

Cannabinoid receptor type 1 - CB1 receptor

CB1 and CB2 receptors belong to G-protein coupled receptor (GPCR) family. The cannabinoid receptor type 1 is primarily located in central and peripheral nervous system. The CB1 receptors expressed predominantly in the brain. The highest density of cannabinoids binding sites are in first and fourth layer of the cerebral cortex, hypothalamus, pyramidal cell layer of the hippocampus, pariaqueductal gray dorsal horn and in the

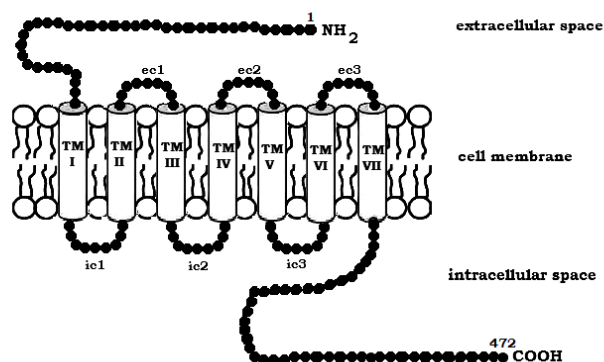
limbic system. These areas associated the endocannabinoid system with memory processes, modulation of emotional states and mobility, as well as anticonvulsant properties of cannabinoids. CB1 receptors are also observed in basal ganglia and cerebellum but in lower density than for example in rodents. Lower density of CB1 is also observed in the structure of the vental tegmental area and nucleus accumbens, which is responsible for the development of happiness and reward feelings, also known as reward system (Komorowski and Stępień, 2007).

Initially it was believed that these receptors are present only in the brain, but their presence in peripheral locations was also demonstrated. CB1 receptors were observed in inter alia in the pituitary gland, immune cells, tissues, digestive tract, reproductive system, heart, lung, intestine, tonsil, thymus, spleen and placenta (Rutkowska and Jamontt, 2005; Ryberg et al., 2007; Busquets-Garcia et al., 2016).

The cannabinoid receptor type 1 is the product of CNR1 gene expression. Medium sized protein is 52 858 Da and it is composed of 472 amino acids (Pawlak et al., 2011). For the first time the complementary DNA of this receptor was isolated from a cDNA library of rat cerebral cortex in 1990 (Konarska and Ellert, 2004; GeneCards). In 1995, Shire and his team have isolated a shorter isoform of the receptor - composed of 411 amino acids, as a result of deletion of 167 base pairs in the nucleotide sequence. This change resulting protein product, a variant CB1A, has a molecular weight of 46 kDa (Shire et al., 1995). In 2004, Ryberg et al. identified a second isoform of the receptor CB1 - a variant CB1B - with a molecular weight of 49 kDa, constructed of 439 amino acids. Both variants have altered the ability of the ligand binding and activation as compared to the original length transcript (Ryberg et al., 2005).

The construction of CB1 receptors is well characterized, comprising a single, highly fold to polypeptide chain. Chain completes its structure through the cell membrane seven times to form seven hydrophobic transmembrane domains (TM I - TM VII) (Kazula, 2009). Figure 1 shows a general scheme for the construction of CB1 receptor in human.

Figure 1. Construction of cannabinoid receptor type 1 (CB1). The numbers at the beginning (1) and end (472) of the chain indicate the direction of numbers of amino acids. Terminal residues, amino (N-) and carboxy (C-), transmembrane domains (TM I-VII), intracellular (ic) and extracellular (ec) loops are also marked.

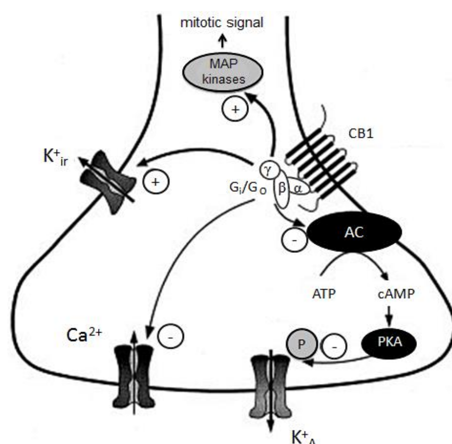


Polypeptide chain ends of the receptor, are located on different side of the cell membrane. The N-terminus is located outside the cell and the C-terminus have

an intracellular localization – in the cytoplasm. In addition, the receptor has in its structure a three extracellular and three intracellular loops. The second extracellular loop and the third transmembrane domain may bind CB1 agonists, while the third loop, which is located inside the cell, binds the inhibitory protein G. Construction of receptor shows a significant interspecies preservation. Comparing the nucleotide and amino acid structures of the CB1 receptor in human and rat, the similarity is equal respectively to 93% and 97% (Konarska and Ellert, 2004; Kazula, 2009).

The primary activity of cannabinoid receptors is the regulation of the permeability of adjacent ion channels. This is done through interaction with the trimeric protein Gi/Go, which act as adapter proteins. General scheme of functioning CB1 receptor is shown in Figure 2.

Figure 2. Diagram of the mechanism of signal transduction by the CB1 receptor in presynaptic bulb (based on Ameri, 1999 and Konarska, Ellert, 2004).



Start of the signalling reaction is conditioned by the appearance of the appropriate agonist (e.g. THC) or by an endogenous ligand (e.g. anandamide and 2-arachidonoylglycerol) which binds to the receptor directly (Nicoll et al., 2012).

When the ligand join the outer loop of the receptor, located in the presynaptic membrane, following its stimulation by changes the conformation of the internal receptor. The result is a Gi/Go protein attachment to the inner loop of the CB1 receptor, which then, due to the exchange of guanosine 5'-diphosphate (GDP) by guanosine 5'-triphosphate (GTP) dissociate an active subunits α, β and γ. The result of the G proteins activation is the start of multiple MAP kinase cascades (mitogen-activated protein kinase), which play a role i.e.: in gene expression, division, differentiation and apoptosis of cells. Cascade of reactions is responsible for inhibition of adenylyl cyclase (AC) activity, which reduces the amount of cyclic AMP (Cyclic adenosine 3 ', 5'-monophosphate, cAMP), or intracellular relay II row. This can lead to a reduction of protein kinases (PKA), which are responsible for phosphorylation of the potassium channel KA (depending on voltage) causing a greater flow of these ions. Activation of G-proteins, by CB1 receptors, directly produces inhibition of calcium channels, and activation of

potassium channels Kir and thus transporting accordance with the electrochemical gradient. As a result the above signal cascade, occurs to a reduced release of neurotransmitters from presynaptic terminals (Sullivan, 2000; Rutkowska and Jamontt, 2005; Tilley, 2011).

Over the years, a number of population studies that focus on the CNR1 gene and polymorphisms selected because of their possible impact on the functionality of the CB1 receptor, encoded by this gene (Albert, 2011). It is known that both the number encoded by the gene, CB1 receptors and their function can be changed in response to the altered gene expression in various conditions or disease development, and in response to contact with various substances, including drugs (Laprairie et al., 2012).

Cannabinoid receptor type 2 – CB2 receptor

CB2 was first cloned from the human leukemia cell line HL-60 in 1993. The receptor was identified among cDNAs based on its similarity in amino-acid sequence to the CB1 receptor, discovered in 1990. The discovery of CB2 helps to provide a molecular explanation for the established effects of cannabinoids on the immune system. While the location of the CB1 receptor is mainly focused in the central nervous system, whereas CB2 receptor is positioned peripherally (Befort, 2015). CB2 receptors are present primarily in the immune system: the spleen, tonsils and in the cells of the immune system, particularly in B cells and NK cells (natural killers), as well as in T cells, monocytes, macrophages, mast and Hortegi cells (microglia). This location of CB2 receptors indicates that they are responsible for immunomodulatory properties of cannabinoids. There have also been reports about the presence of these receptors in the retina of adult rats and other animals, and in mouse brain and peripheral nerves of mice and guinea pigs. (Rutkowska and Jamontt, 2005).

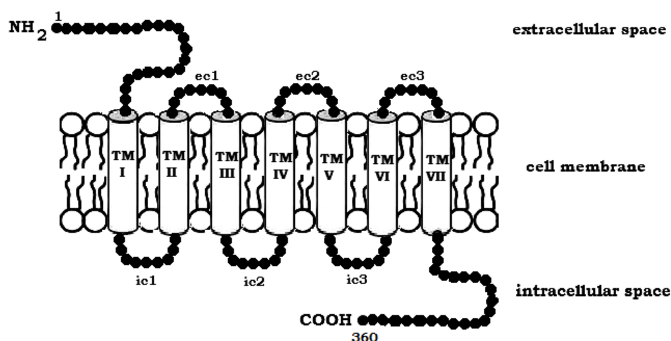
Studies suggest that these receptor is also present in small quantities in the several brain structures i.e.: hippocampus, striatum, thalamus, and also into ventral tegmental area neurons (Onaivi et al., 2006; Zhang et al., 2014). There is evidence that points to the role of CB2 receptor in addictive processes, for example of nicotine (Navarrete et al., 2013).

Recently, there a growing number of new connections between the activity of the CB2 receptor and the occurrence of certain diseases. Furthermore, CB2 receptors are related with malignancies of the immune system and may serve as potential targets for the induction of apoptosis. Also, because CB2 agonists lack psychotropic effects, they may serve as novel anticancer agents to selectively target and kill tumors of immune origin (Mc Kallip et al., 2002). Studies have shown, that the modulation of CB2 receptor signaling may represent a promising therapeutic target that can be used to reduce neuronal degeneration in neurodegenerative diseases, such as Parkinson's, Alzheimer's or Huntington's disease (Cassano et al., 2017). CB2 cannabinoid receptors expressed on malignancies of the immune system may serve as potential targets for the induction of apoptosis (Herrera et al., 2006). Findings also indicate an increased risk of schizophrenia for people with low CB2 receptor function (Ishiquro et al., 2010).

The human CB2 receptor is composed of 360 amino acid residues and a molecular weight of about 40 kDa (GeneCards). The CB2 receptor is a member of the G-protein-coupled receptor family. CB2 is also a single polypeptide chain of seven hydrophobic transmembrane domains TM I to TM VII,

which have α-helis structure and pass through the cytoplasmic membrane. Its structure contains three extracellular and three intracellular loops. It was found to have an extracellular, glycosylated N-terminus and an intracellular C-terminus too (Figure 3.) (Konarska and Ellert, 2004).

Figure 3. Construction of cannabinoid receptor type 2 (CB_2). The numbers at the beginning (1) and end (360) of the chain indicate the direction of numbers of amino acids. Terminal residues, amino (N-) and carboxy (C-), transmembrane domains (TM I-VII), intracellular (ic) and extracellular (ec) loops are also marked.



Most known cannabinoid compounds has a similar affinity for both receptor CB2 and CB1, despite differences in their amino acid structures. Compatibility between the amino acid sequences of these (CB1 and CB2) receptors in humans is 48%. It is worth noting that the coding sequences form CB2 are less conservative than the form CB1 (Konarska and Ellert, 2004; Kazula, 2009).

The CB2 receptor is mainly associated with the regulation of immune response processes. Modification of the receptor activity is associated with impaired cell proliferation and the generation of the defensive response modulators such cytokines. The CB2 receptor stimulation also results in the inhibition of adenylate cyclase (Konarska and Ellert, 2004). Studies showed that in CHO (Chinese hamster ovary) cells expressing CB2 receptor, cannabinoids activate MAP kinase. The CB2 receptor activation passed through the MAPK is carried out by G-protein subunits - γ and ß. One component of this pathway, in contrast to the CB1 receptor is a C protein kinase. In the molecular pathway by a signal from CB2 to MAPK may be also involved p21/Ras, Raf-1 and MEK kinase (MAPKK), Anandamide is example for endogenously produced CB2 ligands (Howlett and Mukhopadhyay, 2000; Konarska and Ellert, 2004).

There is growing evidence that selective antagonists of the CB2 cannabinoid receptor inhibit or abolish most of the immunosuppressive effects of cannabinoids. The cannabinoid ligands may be potential therapeutic agent in several pathophysiological situations, so the CB2 cannabinoid receptors have been widely studied in this direction. (Racz et al., 2008). The researchers demonstrated, that in contrast to the CB1 receptor, the CB2 receptor activation does not affect the ion channels (Pietrzak et al., 2011).

The CB2 partly exerts its effects through initiation of phospholipase C (PLC) and inositol 1, 4, 5-triphosphate (IP3) signaling pathways that lead to increased levels of intracellular calcium (Zoratti et al., 2003).

Presumably novel cannabinoid receptors

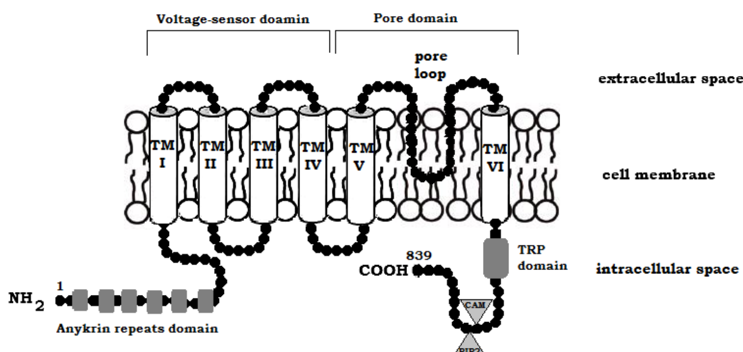
TRPV1 receptor

TRPV1 (transient receptor potential cation channel subfamily V member 1) belongs to the transient receptor potential (TRP) family, whose members act as ion channels. The TRP family is divided into subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like). To date, more than 100 TRP-encoding genes have been identified in animals (Clapham, 2005; Nilius, 2011).

TRPV1 is an integral membrane protein, a non-selective cation channel that is gated by a broad array of noxious ligands (GeneBank; Cui, 2016). TRPV1 affects on many functions including inflammation, painful stimuli sensation, and mechanotransduction (Cui, 2016).

The human TRPV1 gene is located on the short arm of chromosome 17 (17p13.2). It consists of 19 exons, includes 43 968 bases. The same protein is coding by four transcripts variant. The protein is composed of 839 amino acids and it can be subject to post-translational modifications: phosphorylation by PKA (mainly Ser117), phoshphorylation by CAMKII (regulate binding to vanilloids), glycosylation (Asn 604) (GeneBank; GeneCards). TRPV1 usually occurs as a homotetramer. Whereas it oligomerizes with other TRP receptors (e.g. TRPA1, TRPV3) it functions as promiscuous channel (Staruschenko et al., 2010; Cui, 2016). TRPV1 in the solved state is composed of four symmetrical subunits, and each of them consists of four modules: in the transmembrane region – the voltage sensor and pore domain and in the cytosol – N- and C-terminal modules (Fig. 4.) (Lee et al., 2015; Cui, 2016). The central ion-conducting pore opens up, when ligands are binding. In the N-terminus occur six ankyrin (ANK) repeats (33 amino acids), which serve as binding sites for inhibitors and allow interactions between proteins. There are also several phosphorylation sites (Lishko et al., 2007; Cui, 2016). The C-terminus contains a TRP domain, which encompasses an α-helix that runs parallel to the membrane. Moreover, there are a PIP2-binding site, CaM-binding site, and several sites for phosphatases and kinases (Numazaki et al., 2003; Garcia-Sanz et al., 2004; Cui, 2016). In the structure of TRPV1 also occur characteristic dual gates: G1 and G2, whose conformational changes lead to entry into active state (Liao et al., 2013; Cui, 2016).

Figure 4. Construction of TRPV1 receptor. The numbers at the beginning (1) and end (839) of the chain indicate the direction of numbers of amino acids. Terminal residues, amino (N-) and carboxy (C-), transmembrane (TM I-VII) and others domains, intracellular (ic) and extracellular (ec) loops are also marked.



Receptor activity is regulated in response to the binding of specific ligands including CAPS, plant and animal toxins, protons, heat. This is possible through the gating mechanism, which involves structural rearrangements within the channel. The result is a transition from the closed state (nonconducting) to open state (conducting) (Hille, 1978; Cui, 2016).

TRPV1 can be regulated in many ways via interactions with agonists or antagonists. There are a lot of agonists that activate TRVP1 channel. Anandamide was the first identified endogenous TRPV1 agonist (Zygmunt et al., 1999). The group of endogenous agonists includes also lipids derived from the metabolism of arachidonic acid, N-arachidonoylethanolamine (endocannabinoid), N-arachidonoyldopamine (endocannabinoid), Noleoylethanolamine and N-acylsalsolinols (Appendino, Minassi, Pagani, & Ech-Chahad, 2008). Several endogenous chemical substances also activate TRPV1: ammonia, adenosine, ATP, protons, ethanol, and polyamines (Szallasi et al., 2007; Cui, 2016). TRPV1 is also induced by a plethora of nonselective stimuli, such as intracellular redox states, acidic pH (<5.3), heat (>43°C) and electrostatic charge. CAPS (capsaicin, the substance from hot chilli peppers) and RTX (the substance from black pepper corn) belong to the natural TRPV1 agonists have been studied clinically as potential treatments for inflammatory and neuropathic pain. Furthermore, there are several other naturally occurring agonists for TRPV1 channel including cannabinoids (tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) (Bisogno et al., 2001; Cui,2016). These findings suggest that TRPV1 receptors might mediate some of the pharmacological effects of CBD and its analogues (Bisogno et al., 2001).

TRPV1 antagonists block the receptor channels so they can be treated as an promising therapeutic target. The first TRPV1 antagonist – capsazepine, was created via modifying the chemical backbone of natural agonist capsaicin. Antagonists are subdivided into two types: competitive and noncompetitive. Competitive TRPV1 antagonists bind to the active site and switch TRPV1 channel into closed state. Other competitive antagonists include cinnamide, quinazoline, benzimidazole analogues. In turn, antagonists like tetrabutylammonium and thapsigargin, are classified as noncompetitive when they block pores in receptor by interacting with allosteric sites of the channel (Brito et al., 2014; Cui, 2016).

The functioning of the TRPV1 receptor is involved into several physiological functions, among others: thermo-sensation (heat), autonomic thermoregulation, nociception, pain management, synaptic plasticity in the brain (long-term depression). TRPV1 is also involved in the endocannabinoid signaling in the brain (Nilius, 2011).

Furthermore, expression of the TRPV1 gene is increased in many disorders. Researchers observed the altered expression of the TRPV1 in human preeclamptic placenta (Martínez, 2016). TRPV1 is known to be expressed in peripheral sensory neurons and at lower levels in the spinal cord, brain. Besides neurons, TRPV1 is also expressed a wide-range in non-neuronal cells (e.g. keratinocytes, urothelium, T-cells, mast cells) (Martin, 2008). Therefore the role of TRPV1 in other pathological diseases, for example respiratory- and bladder-related diseases, diabetes, and cancer (Cui, 2016).

GPR55 (G protein-coupled receptor 55)

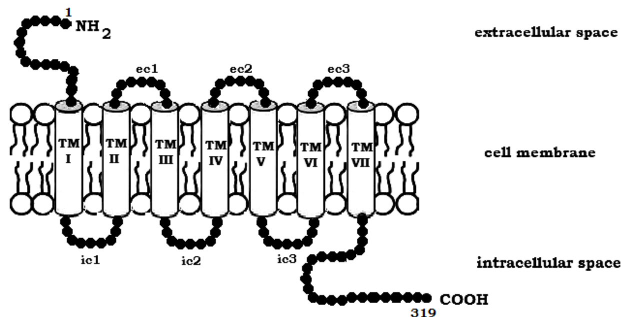
It has been suggested that GPR55 is a novel cannabinoid receptor (Mackie and Stella, 2006). It belongs to a G-protein-coupled receptor superfamily, precisely to the rhodopsin-like (ClassA) family of GPCRs. This protein plays significant role in signal transduction from the external environment (GeneBank).

The human gene that encodes GPR55 protein is located on the long arm of

chromosome 2 (2q37.1), encompasses 53,910 bases and contains 4 exons. There are 5 splice variants (GeneCards, GeneBank, Ensmbl). The GPR55 receptor is consists of 319 amino acids and 7 hydrophobic domains characteristic for GPRs. The molecular mass of the protein is 36637 Da. (GeneCards, OMIM). It is an integral membrane protein and its structure reminds structure of cannabinoid receptor (GeneBank). The GPR55 is de-orphanized as a cannabinoid receptor (Shore and Reggio, 2015).

The amino acid sequence is the most similar to the GPR35 (27%), P2Y (29%), GPR23 (30%), CXCR4 (26%) but also to CB2 (14.4%) and CB1 (13.5%) receptors. Amino acid structure of the human GPR55 is shown on Figure 5. (Sawzdargo et al., 1999; Shore and Reggio, 2015).

Figure 5. Construction of GPR55 receptor. The numbers at the beginning (1) and end (319) of the chain indicate the direction of numbers of amino acids. Terminal residues, amino (N-) and carboxy (C-), transmembrane domains (TM I-VII), intracellular (ic) and extracellular (ec) loops are also marked.



Expression of GPR55 is very high in large dorsal root ganglion neurons. Activation by defined cannabinoids increases amount of intracellular calcium in these neurons. GPR55 activation also inhibits potassium current through M-type potassium channels. It can suggests that activation of GPR55 enhances neuronal excitability (Lauckner et al., 2008). Expression of the GPR55 gene has been observed also in the: central nervous system, adrenal glands, gastrointestinal tract, lung, liver, uterus, bladder, kidney, and bone. The GPR55 receptor is also involved in regulation of energy intake and expenditure. Therefore, this receptor plays a role in the maintenance of energy homeostasis (Simcocks et al., 2014; Shore and Reggio, 2015) In addition, Ryberg et al. analyzed GPR55 receptor ability to bind and to mediate GTPγS binding by cannabinoid ligands. This analysis showed that GPR55 is activated by endocannabinoids as 2-AG, virodhamine, noladin ether, oleoylethanolamide and palmitoylethanolamide in small concentrations and the atypical cannabinoids Cannabidiol and abnormal-cannabidol (synthetic regioisomer of cannabidiol). (Ryberg et al., 2007; Shore and Reggio, 2015).

Above findings establish GPR55 as an additional cannabinoid receptor that can activate signaling pathways distinct from CB1 or CB2, and that may increase neuronal excitability and can be involved in nociception, particularly in neuropathic or inflammatory pain states. (Ryberg et al., 2007; Lauckner et al., 2008).

Summary

The discovery of the cannabinoid receptors in the 1990s led to the characterisation of the endogenous cannabinoid system in the context of its composition and many fundamental

physiological functions. This day understanding of the cannabinoid receptors and other receptors which binds cannabinoids ligands is still not yet fully understood. The existence of additional cannabinoid receptors has long been suspected, due to the effects of some of the compounds causing cannabinoid-like effects on blood pressure and inflammation, but not activating CB1 and CB2 receptors. (Járai et al., 1999; McHugh et al., 2008) Reports suggest that, due to the fact of structural similarity of GPR55 to CB1 and CB2 and sequence homology should be characterized as a third cannabinoid receptor (Ryberg et al. 2007; Johns et al., 2007)

Cannabinoids have a wide spectrum of activity, it exhibits the properties for antiinflammatory, neuroprotective, antiemetic and analgesic effects (McHugh et al., 2008). Studies suggested that they can also relieve intraocular pressure (benefit for glaucoma patients) and have an anti-proliferative effect on cancer cells. According to the National Institute of Health cannabinoids-based medications studies are concentrated on multiple sclerosis research, chronic obstructive pulmonary disease, sickle cell disease, spinal cord injury pain, inflammatory bowel disease (Crohn’s disease), cancer-related pain and brain tumors (U.S. National Institutes of Health, 2013. https://clinicaltrials.gov).

The use of cannabinoids is associated with adverse side effects profile, their action is non-specific, affects the entire central nervous system, causing dizziness, drowsiness, difficulty in concentrating, sleep disturbances and thinking and vision disturbances. In addition, there are also hallucinations, psychosis, depression and tachycardia. After prolonged use of these compounds there is a risk of developing a tolerance to cannabinoids psychotropic activity and any other side effects, which causes significant reduction of the therapeutic indications (Karjnik and Żylicz, 2008) Therefore, more hope for the use of cannabinoids compounds involves the treatment of pathophysiological mechanisms which closely correlate with the endocannabinoid system. There are also suspicions that a deeper understanding of receptors interacting with cannabinoids and its interconnections with the cardiovascular system may be important in the prevention and treatment of diseases of this system (Kazula2009). One way of overcoming the problems associated with the side effect of cannabinoids may be compounds which potentiate the action of endogenous cannabinoids or influence the cannabinoid system indirectly. In this context, gain greater knowledge of receptors interacting with cannabinoids seems to be extremely valuable.

Funding

This work was supported financially by The National Centre for Research and Development (Grant Number INNOMED/I/ 11/NCBR/2014) from the Innovative Economy Operational Programme founds, in the framework of the European Regional Development Fund.

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Streszczenie

Układ endokannabinoidowy (ang. endocannabinoid stystem-ECS) składa się z receptorów kannabinoidowych CB1 i CB2, agonistów egzogennych i endogennych: kannabinoidów i endokannabinoidów oraz enzymów regulujące syntezę i degradację endogennych ligandów tego układu. Jest systemem biorącym udział w wielu procesach fizjologicznych organizmu. Odgrywa on istotną rolę w prawidłowym funkcjonowaniu centralnego i autonomicznego układu nerwowego. Układ endokannabinoidowy reguluje również m.in. gospodarkę energetyczną, aktywność motoryczną, układ endokrynny oraz układ hormonalny poprzez regulacje powiązań neurohormonalnych i neuroimmunologicznych. Wiele jego wspomnianych funkcji związanych jest z receptorami sprzężonymi z białkami G, takimi jak receptory CB1, CB2 czy GPR55. Co raz więcej mówi się również o znaczącej roli receptorów przejściowego potencjału TRPs (ang. transient receptor potential), które nie tylko pośredniczą w odczuwaniu bólu, ale także są odpowiedzialne za wspomaganie stanu zapalnego poprzez wydzielanie pro-zapalnych neuropeptydów.

W niniejszej pracy przeglądowej postanowiliśmy krótko scharakteryzować oraz opisać rolę i działanie receptorów kannabinoidowych CB1 i CB2, receptora sprzężonego z białkiem G (GPR55) i receptora przejściowego potencjału waniloidowego (TRPV1).

Folia Biologica et Oecologica 14: 14–22 (2018)

Acta Universitatis Lodziensis

Biodegradation of 3,5-dinitrosalicylic acid by *Phanerochaete chrysosporium*

RAFAŁ MADAJ[1], HALINA KALINOWSKA[2], WITOLD SROCZYŃSKI[1], JAKUB SZELĄG[2], ELŻBIETA SOBIECKA[1]

[1] Institute of General Food Chemistry, Lodz University of Technology, Stefanowskiego 4/10 90-924 Lodz, Poland
[2] Institute of Technical Biochemistry, Lodz University of Technology, Stefanowskiego 4/10 90-924 Lodz, Poland
E-mail: rafal.madaj@edu.p.lodz.pl

ABSTRACT

Despite intensive efforts put on prevention of environment pollution by nitroaromatic compounds, these xenobiotics have not been eliminated from the biosphere. The physicochemical properties make nitroaromatics extremely recalcitrant to biodegradation. Therefore, microbial degraders of these pollutants are sought after. This paper reports preliminary results of the study on degradation of 3,5-dinitrosalicylic acid (DNS) by a basidiomycetous fungus *Phanerochaete chrysosporium* under stationary conditions in a culture medium containing 0.05–0.5% v/v of DNS. The results obtained suggest that the fungus degrades DNS through the reductive pathway.

KEY WORDS: nitroaromatic compounds, white-rot fungi, fungal biodegradation

Introduction

Aromatic nitrocompounds are chemicals containing one or more nitro (-NO_2) groups attached to the aromatic ring. These substances are known to be highly toxic and probably carcinogenic for humans as well as highly recalcitrant to natural biodegradation, thus they pose very serious threat to the environment (Williams *et al*. 2015, Lipczynska-Kochany 1995, Price 1997, Gong *et al*. 2003). Their content in natural environment increases every year due to

DOI: 10.1515/fobio-2017-0005

military operations, combustion of fossil fuels or civil application of explosives based on nitrated aromatic compounds (Sekhar & Wignes 2016, Rezaei 2010, Anasonye *et al*. 2015). There is a number of commercial applications of aromatic nitrocompounds. Vast majority of nitroaromatic products are employed in military, for instance chemicals like hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) or 2,4,6-trinitrotoluene (TNT) are examples of the most commonly applied explosives, whereas substances like 2,4-dinitrophenol (DNP) were widely used as defoliants, e.g. during Vietnam war (Haberman 2014, Grundlingh 2011). Nevertheless, aromatic nitrocompounds are also used in other areas, as e.g. pharmaceuticals (an antibiotic Chloramphenicol) or general purpose chemical reagents such as nitro-based aromatics like nitrobenzene or 3,5-dinitrosalicylic acid (DNS), which is used for reducing sugars assays (Kumar 2016).

The low affinity to water combined with high electron-withdrawing character are main factors responsible for the highly hazardous character of aromatic nitrocompounds (Shen *et al*. 2009, Mathieu and Alaime 2015). Most of the nitrocompounds are viscous liquids or solid compounds characterized by poor water dissolvability. Moreover, not only nitro groups impart the xenobiotic character, but also explosophoric properties (Infante-Castillo & Hernandez-Rivera 2012), what may explain their abundance in biosphere.

3,5-dinitrosalicylic acid is an organic, aromatic compound with physicochemical properties resembling other nitroaromatic compounds. The biggest difference lies in its solubility in water (Apelblat & Manzurola 1999), making DNS potentially more susceptible to biodegradation than chemicals like TNT or DNP.

There is a small number of microorganisms that degrade nitroaromatic compounds. One could distinguish two types of them: bacteria, that degrade nitrocompounds via reductive pathway through nitroso- and hydroxyamino- derivatives (Singh *et al*. 2015, Claus 2013, Lenke & Knackmuss 2016), often yielding products that are sometimes claimed to be more toxic than the substrate (for example amino- and hydroxyamino- derivatives of TNT) (Kulkarni & Chaudari 2007), and fungi, capable of even complete mineralizing of toxic nitroaromatics.

One of the fungal species, being under intense research is the basidiomycete fungus *Phanerochaete chrysosporium*, producing enzymes evincing very low substrate-specificity. Due to that, this white-rot family fungus is capable of degrading a wide range of aromatic substances, including lignin alcohols (Nousiainen *et al*. 2014, Asina *et al*. 2016), polychlorinated biphenyls (Cvancarova *et al*. 2012) and nitroaromatics (Spain 1995).

Therefore, this organism was investigated in terms of 3,5-dinitrosalicylic acid biodegradation. The results of preliminary investigations on the biodegradation of DNS by *P. chrysosporium* as well as the putative biodegradation pathway are presented in this paper.

Materials and methods

Experimental setup

The *P. chrysosporium* strain was maintained on agar slants with a modified Czapek medium, containing glucose instead of sucrose (glucose 30 g/l, sodium nitrate 2 g/l, dipotassium phosphate 1 g/l, magnesium sulphate 0.5 g/l, potassium chloride 0.5 g/l, ferrous sulphate 0.01 g/l) and enriched with oat flakes (5 mg per 5 ml) as a source of thiamine (Maza *et al*. 2015). Liquid cultures under stationary

conditions were carried out in 200 ml Erlenmeyer's flasks, in the modified Czapek's medium (20 ml) enriched with 50 mg of sugar beet pulp instead of oat flakes. The sterile medium (after autoclaving at 121 °C for 15 min) was inoculated with 1 ml of fungal spores washed from the agar slants using sterile demineralized water (5 ml per slant). The fungus was cultivated at 30 °C for 7 days and then 20 ml of a tested (0.05%, 0.25% and 0.5%) DNS solution in water (its pH was adjusted to 5.0 with 0.1 M NaOH) was added to each flask and the flasks were weighted because the metabolic activity of *P. chrysosporium* in the DNS-containing culture medium was monitored, among others, based on the decrease in the weight, caused by carbon dioxide emission from the culture.

The controls without DNS contained 20 ml sterile demineralized water instead of DNS solution. To eliminate the error caused by water evaporation during the culture, which also causes a decrease in weight, three 40 ml portions of the sterile culture medium were incubated along with the inoculated samples. The decrease in the weight of the culture medium controls was compared with that of the inoculated samples and controls. Another set of three controls, containing 20 ml of the sterile culture medium (not inoculated with the fungus) and supplemented after the first 7 days with 20 ml of suitable DNS solution was prepared and used to monitor the spontaneous DNS degradation under experimental conditions.

Extraction of 3,5-DNS

Every fourteen days the content of three weighted flasks was filtered through filter papers and 6 ml of each filtrate was analysed for the content of residual DNS and its degradation products. DNS was extracted from each filtrate three times with 12 ml of ethyl acetate (analytical grade) and then the extracts were pooled, and 6 ml aliquots of the pooled extracts were evaporated. The efficiency of extraction was above 98%. The solids (residues of DNS and its degradation products) were dissolved in 1 ml of methanol (analytical grade), and the solutions were filtered using syringe filters (4.5 µm), transferred into eppendorf tubes and subjected to HPLC-UV analysis.

HPLC-UV analysis

The extracted compounds were analysed using an HPLC Knauer system equipped with a C18 RP column (Supelco 4.8 µm x 4.8 mm x 10 cm), a Knauer HPLC pump and an UV-Vis detector. The mobile phase contained 70% methanol and 30% acetonitrile (both HPLC-grade). The temperature of column was set at 25 ˚C and the injection volume was 20 µl.

The measurements of absorbance were conducted at the wavelengths of 210, 254, 278 and 340 nm.

Presentation of results

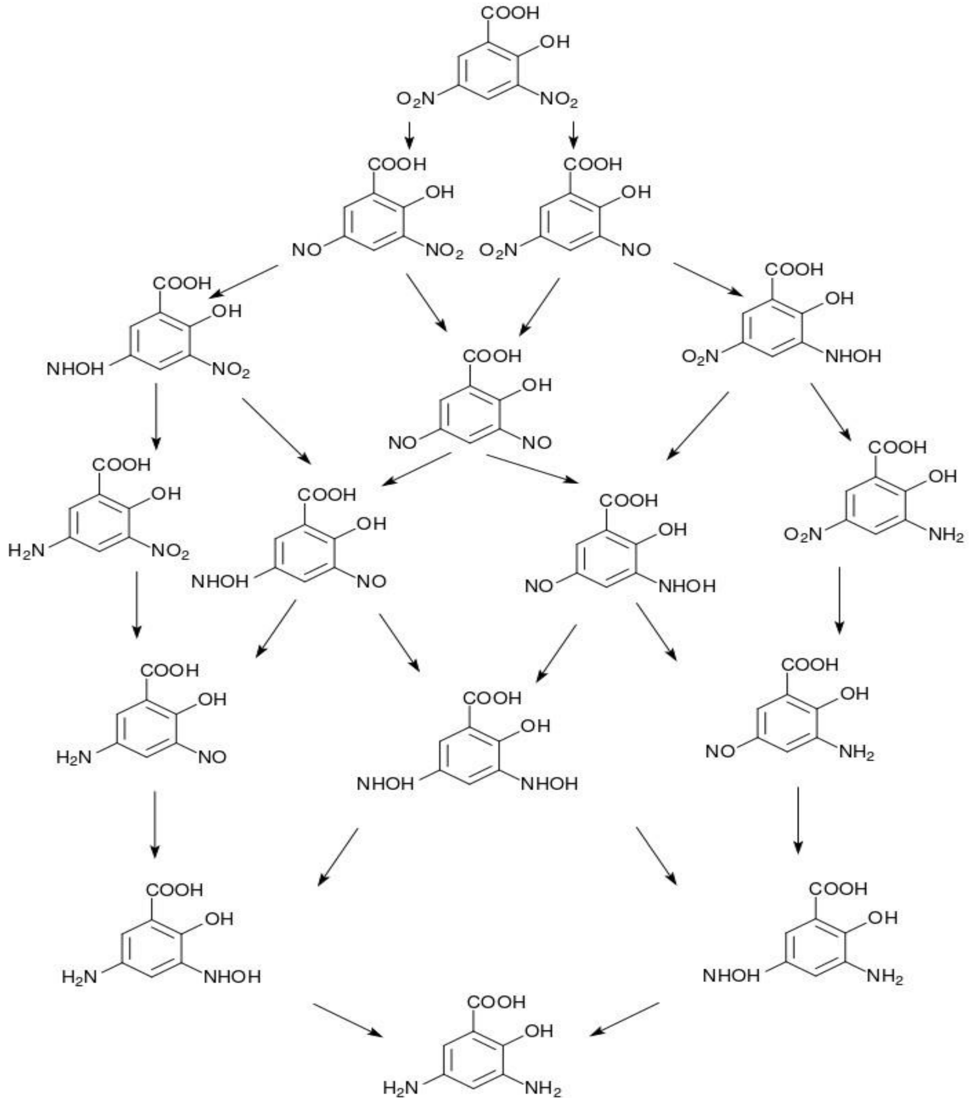
The results presented in figures 2, 3 and 5 are means + SD of triplicate samples. All structures presented in this work as well as a theoretical pathway of DNS biodegradation by *P. chrysosporium* were prepared using a BKchem GNU software (ver. 0.13.0). The figures were created using a RStudio Open Source Edition ver. 3.4.0.0 and MS Excel 2007.

Results

Putative biodegradation pathway

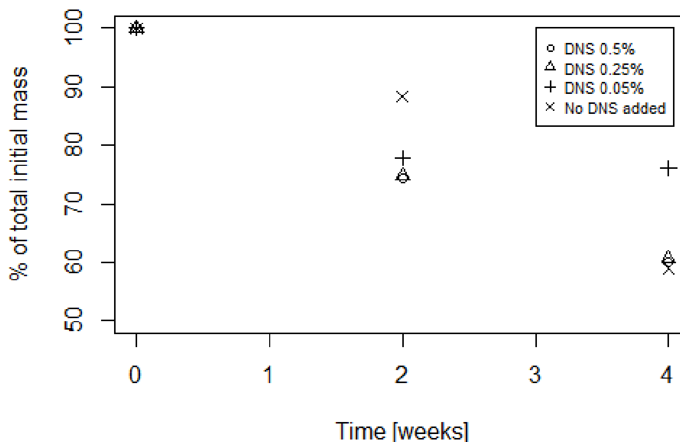
The investigation of TNT biodegradation (Spain 1995, Bayman *et al.* 1997) showed that not only oxidoreductaes (LiP, MnP, laccase) take part in detoxication and biodegradation, but also a range of nitroreductases (Maza *et al.* 2015). Based on that, the putative pathway concerning initial steps of 3,5-DNS biodegradation was proposed (Fig. 1).

Figure 1. Putative denitrification pathway of 3,5-DNS by *P. chrysosporium* involving nitroreductases and leading to 3,5-diaminosalicylic acid through nitroso- and hydroxyamino- derivatives.



It was assumed that the presence of 3,5-dinitrosalicylic acid might affect the general fungal metabolism and influence the rate of carbon dioxide emission by the growing *P. chrysosporium*. Production of carbon dioxide increases with the intensity of oxidative metabolism, and when the latter is growing, the decrease in weight of the culture medium is faster. Therefore, each flask with the fungus was weighted before filtration and its weight was compared with the initial one. The dynamics of weight decrease with time, either in the absence or presence of 0.05, 0.25 and 0.5% DNS, is shown in Fig. 2.

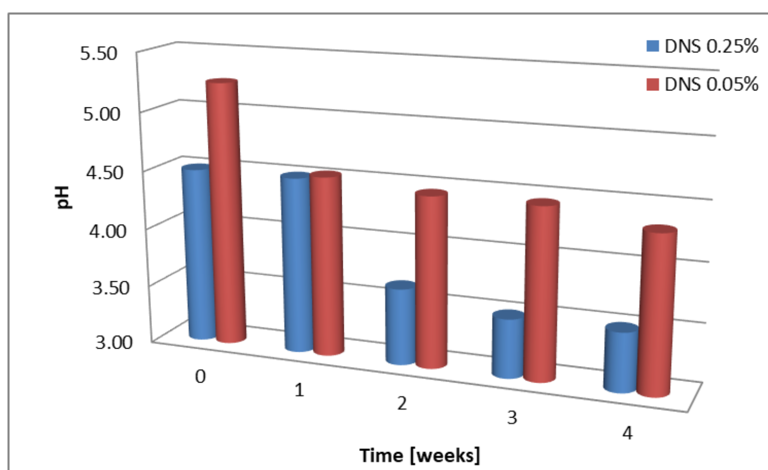
Figure 2. The average weight decrease with time.



The data shown in Fig. 2 demonstrate that the presence of 0.05–0.5% DNS in the culture medium only slightly affected the dynamics of the medium's weight decrease during the growth of *P. chrysosporium*. It was also visible that at these DNS concentrations, the fungus could grow like in the DNS-free medium. The decrease in weight of the sterile culture medium controls was negligible and therefore it was not presented in Fig. 2.

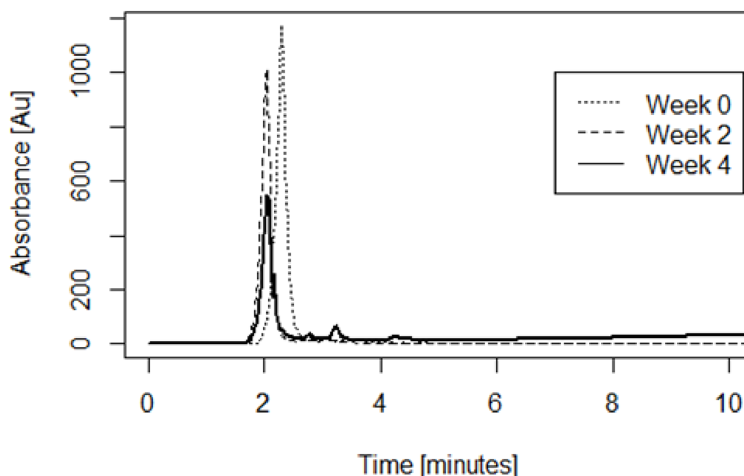
As *P. chrysosporium* is known to produce organic acids (oxalic) and acidify the environment (Bonnarme & Jeffries 1990), changes in pH of the culture medium with time were monitored. The results of these measurements are presented for the DNS concentrations of 0.05% and 0.25% in Fig. 3. It was observed that in the medium containing 0.05% DNS, the values of pH were slightly higher on the same days than in the other samples that suggested the occurrence of basic intermediates of DNS conversion. At the higher DNS concentrations, the pH values were nearly the same as in the DNS-free samples.

Figure 3. The change of pH with time.



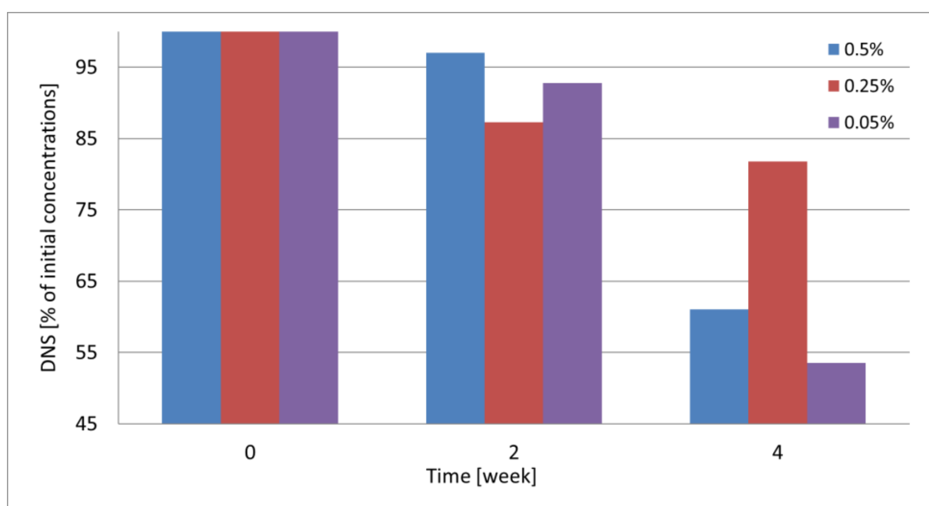
The analysis of extracts from culture medium samples using HPLC-UV showed that DNS concentration decreased with time of *P. chrysosporium* growth (Fig. 4). Because the analysis of the DNS-containing controls, which were not inoculated with the fungus, showed that this compound was not spontaneously degraded under the experimental conditions, so the reduction of DNS content in the inoculated samples may be ascribed to its degradation by the fungus.

Figure 4. An exemplary chromatogram of three samples. The first peak at 2 min corresponds to DNS whereas the visible small peaks may represent the unknown intermediates of DNS degradation.



The decrease in DNS concentration in the culture medium and occurrence of peaks that may represent DNS conversion products suggest that *P. chrysosporium* metabolizes 3,5-dinitrosalicylic acid. The collective results of HPLC-UV analyses are shown in Fig. 5.

Figure 5. A decrease in 3,5-DNS content with time.



The relatively large reduction of DNS content in the culture medium supplemented with 0.05% and 0.5% DNS after 4 week cultivation may be ascribed to its partial precipitation from the culture medium, which was not observed at 0.25% concentration. The results presented in Fig. 5 demonstrate that the rate of DNS removal for the highest and lowest concentrations is significant, up to 50% of initial substrate concentration. The lesser extent of DNS content reduction at its initial concentration of 0.25%, compared to that at the lowest initial concentration (of 0.05%), may suggest that in the latter case DNS was metabolized by the fungus like 'a co-substrate' and therefore its concentration was decreased below 55% of the initial one within 4 weeks. When the initial concentration of DNS was increased 10-fold (to 0.5%) the fungus was unable to efficiently metabolize the substrate and had to precipitate its excess to survive. Also at the initial concentration of 0.25%, the metabolism of DNS was disturbed, however, the fungus was able to synthesize enzymes that are responsible for this process and therefore precipitation of DNS was less intensive. The observed significant drop in pH in the latter case might be favorable for the solubility of degradation products, which contain amino groups (Fig. 1).

Discussion

The assumption of the reductive character of 3,5-dinitrosalicylic acid biodegradation by *P. chrysosporium* is based on the gradual decrease in DNS concentration in the culture medium filtrates. Furthermore, these filtrates contained putative intermediate products that absorbed light at wavelengths characteristic of nitroaromatic compounds. Furthermore, the gradual browning of culture medium during the 4 week cultivation may be also caused by reduction of the nitro groups attached to the aromatic ring of DNS. The gradual decrease in pH of the culture medium was ascribed to the synthesis of organic acids by *P. chrysosporium*. It may be also considered one of the mechanisms enabling the removal of DNS from the environment by precipitation.

The results obtained in this study are consistent with findings concerning the removal of other pollutants like 2,4,6-trinitrotoluene or 2,4-dinitrophenol by *P. chrysosporium* and other wood-decaying fungi. These organisms are capable of either partial or complete degradation of TNT (Spain *et al*. 2000, Tashes *et al.* 1990). However, the reported initial concentrations of the substrates were in most cases much lower than in our work that was beneficial for the biodegradation process. Although further studies are necessary to describe in detail the DNS degradation pathway, the observed browning of culture medium and decrease in the DNS concentration provide evidence that *P. chrysosporium* may be used for the removal of this nitroaromatic compound from aqueous systems.

Summary

The reported results suggest that *P. chrysosporium* metabolizes 3,5-dinitrosalicylic acid. Further experiments will focus on determination of the structure of intermediates that appear in the culture medium, and identification of the genes encoding nitroreductases in the genome of the fungus. Also the conditions of DNS degradation will be optimized to increase the rate of this process.

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Streszczenie

Związki nitrowe to szeroka grupa ksenobiotyków, które ze względu na swoją silną toksyczność, wyjątkową odporność na rozkład biologiczny oraz skłonność do bioakumulacji, stanowią bardzo poważny problem dla biosfery. Prowadzi się obecnie wiele badań nad mikroorganizmami, które zdołały wykształcić szlaki metaboliczne pozwalające na rozkład takich związków jak 2,4,6-trinitrotoluen, kwas pikrynowy czy kwas 3,5-dinitrosalicylowy. Jednym z takich mikroorganizmów jest podstawczak *Phanerochaete chrysosporium*, należący do grupy grzybów białej zgnilizny drewna.

Artykuł ten poświęcony jest badaniom nad rozkładem kwasu 3,5-dinitrosalicylowego przez *P. chrysosporium* w warunkach hodowli stacjonarnej w pożywce zawierającej 0,05–0,5% masowego kwasu 3,5-dinitrosalicylowego. Uzyskane wyniki wskazują na zdolność wybranego mikroorganizmu do rozkładu substratu na drodze redukcji grup nitrowych.

Folia Biologica et Oecologica 14: 23–32 (2018)

Acta Universitatis Lodziensis

The use of *in vitro* assays for the assessment of cytotoxicity on the example of MTT test

MAGDALENA TWARUŻEK*, EWA ZASTEMPOWSKA, EWELINA SOSZCZYŃSKA, IWONA AŁTYN

Department of Physiology and Toxicology, Institute of Experimental Biology, Faculty of Natural Sciences, Kazimierz Wielki University, Chodkiewicza 30, 85-064 Bydgoszcz, Poland
E-mail: twarmag@ukw.edu.pl

ABSTRACT

In recent years, biological tests have been developed based on cell cultures and successfully used to the hygienic assess of a variety of samples. *In vitro* assays become the complement of conventional chemical methods. They do not narrow the results only to the quantitative and qualitative information on toxic substances, but also increase knowledge on the direct impact on the organism. They are also an alternative for animal testing, which are currently given up for ethical reasons. At present, the market is steadily increasing in the number of tests and bio-assay techniques. Based on our own studies we conclude that the MTT test is perfect as a diagnostic method for evaluating the cytotoxicity of materials of different composition such as mycotoxins, pesticides, bacterial cultures, moulds isolates, food, feed, as well as a vast spectrum of other environmental samples.

KEY WORDS: MTT test, SK cells, cytotoxicity

Introduction

Due to the significant development of industry, a lot of harmful substances are released into the water, air, soil, and to our bodies, which contributes significantly to the development of environmental toxicology. Interests in issues of xenobiotics and compounds interfering the proper functioning of cells and organisms as a result of a number of their toxic effects has increased. An increase in exposure of humans and animals through food additives, to the industrial toxins or secondary fungal metabolites, has been observed. The exposure became a major problem in laboratory diagnostics. Therefore, it is important to develop rapid and cost-effective *in vitro* assays for identification of potentially toxic substances. The possible impact of new and existing chemical substances has led to the development of a wide range of methods for testing their biological effects. Methods for toxicity evaluating using cellular models (*in vitro*) have

DOI: 10.1515/fobio-2017-0006

become an alternative for tests performed on animals. They are relatively simple and easy to make, while increasing their accuracy and sensitivity. They now constitute the basis for determining safe levels of chemical compounds, thus constantly increasing the information database concerning degree of toxin harmfulness. It should be remembered that the use of animals for biological tests is costly, time consuming, and often relates to ethical issues. Therefore, the Committee of Toxicology and Environmental Factors Assessment of US National Research (NRC) undertakes the initiative to develop the non-invasive *in vitro* tests and computer methods based on human biology in order to prevent the reaction evaluations based on groups of animals. It is also postulated the use of cells and change of paradigm of carrying out the experiments on animals towards the *in vitro* direction (Sikorski & Adomas 2010).

Cytotoxicity is one of the basic mechanisms of the evaluation of toxic substances. To determine the *in vitro* cytotoxic activity multiple techniques for measuring changes related to a disturbance of physiological processes within human or animal cells caused by the test substance, are applied. Tests used to evaluate the cytotoxicity allow for a direct or indirect measurement of different changes reflecting the cytotoxic activity, e.g. number of live and dead cells, sometimes, in combination with determination of the cell membrane integrity, assay the enzyme activity related to cell metabolism, determining the ability to cell division, or determination of the total protein or DNA in the cell culture (Krzysztoń-Russjan *et al.* 2009).

Some of the tests and techniques for bio-assays

Depending on the method used, cytotoxicity testing can be divided into two groups. One type of tests measures the total protein content, which include, among others, Kenacid Blue test and sulforhodamine B staining. The second group of tests is based on methods based on measurements of redox activity. That group includes Alamar Blue assay, XTT test, and MTT test.

Kenacid Blue test allows to determine the overall amount of cellular protein measured by its fast coloration. The amount of pigment incorporated into the cell culture determines the degree of cytotoxicity caused by the test agent (Putnam 2002).

Sulforhodamine B staining also allows the measurement of the total protein content within the cell, on the basis of which the cytotoxic activity is determined. Sulforhodamine B is a pigment that is bound to the basic amino acids of cellular proteins. The amount of protein in the sample is directly proportional to the number of living cells (Anuszewska 2010).

One of the fundamental and rapid tests by means of living cells measuring is neutral red uptake by lysosomes. This pigment passes through the cell membrane of living cells only and accumulates in the lysosomes. This test distinguishes living and undamaged from dead cells by determining the number of viable cells stained red (Stokłosowa 2004).

LDH test is a method based on enzymatic reactions resulting in a colored by-product determined by spectrophotometry. Lactic dehydrogenase (LDH) is a cytosolic enzyme, which under physiological conditions is not released into the environment. Mechanical damage of the plasma membrane and the cell death caused by the action of harmful factors, invokes the release of LDH from cells (Szliszka *et al.* 2007). The enzymatic reactions in the assay take place in two stages: first, lactate is converted to pyruvate (LDH catalyzes the reaction),

whereas during the second stage, diaforase catalyzes the transfer of H^+ onto tetrazoline that is reduced to formazan. Currently, the LDH level is used as a general indicator of the presence and severity of acute or chronic tissue damage (Hak 2006).

Example of another test indicating a lack of cell membranes integrity is the NAG test. It allows for the demonstration of the lysosomal enzyme N-acetyl-beta-D-glucosaminidase presence in a culture medium, which is possible only when the cells are damaged (Krzysztoń-Russjan *et al.* 2009).

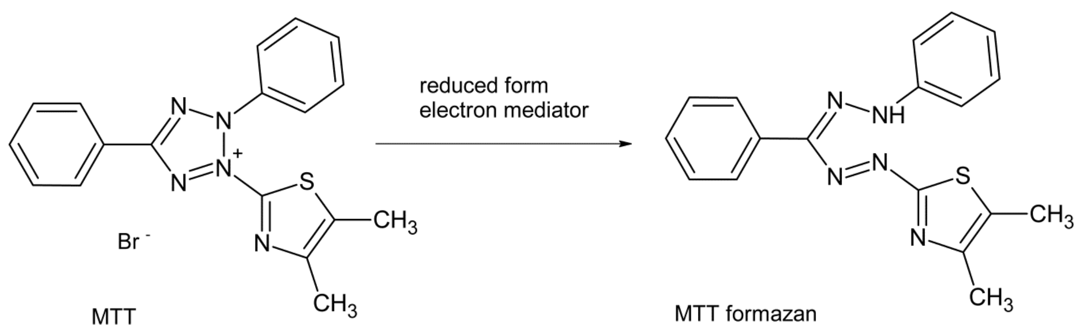
Acridine orange stain is versatile and most widely used to distinguish live cells from apoptotic and necrotic ones. The assay utilizes the ability of pigment passing through the cell membrane, due to which it colors the whole cell. Fixed as compared to the live cells vary in cytoplasm and nucleoli color. This is because cell membrane is damaged during the fixing process, and thereby rapid inflow and accumulation of the pigment inside the cells is enabled (Noatyńska 2011).

Alamar Blue assay uses the natural metabolic processes in cells. Alamar Blue is stable and non-toxic compound and contains the oxidation-reduction indicator (redox). When it is oxidized, turns blue (and it is weakly fluorescent then), while after placing this compound in living cells, gets reduced and turns red. The size of this change is a reflection of cell viability (Nakayama *et al.* 1997).

XTT assay is one of colorimetric methods, which are based on the reduction of pigmented indicator added to the growth medium. The method uses the process of XTT tetrazoline ring cleavage involving mitochondrial dehydrogenases of living cells, leading to the formation of orange formazan molecules. The rate of reduced formazan formation is proportional to the number of living cells (Dzierżewicz *et al.* 2005, Sajduda 2007).

The MTT test is a quantitative colorimetric test for toxicity, which is based on conversion of yellow-colored tetrazolium salt (MTT) into violet-colored, water-insoluble formazan (Fig. 1). This reduction occurs proportional, as only living cells have such ability.

Figure 1. Reduction of MTT to formazan (Stokłosowa 2004, p. 251).



This test was developed in the 80's by Mosmann and is one of the most commonly used methods for rapid, quantitative assessment of sample cytotoxicity against cells (screening method). It gives no information on the type of occurring toxins, but their biological effects in the form of cytotoxicity (Hanelt *et al.* 1994).

Cellular MTT bio-reaction is associated with enzymes of the endoplasmic reticulum and affects the reduction of pyridine. Only metabolically active cells carry out this process. Thus, if cells have previously been damaged or destroyed by a toxin, the reaction is less intense or does not occur at all, which can be determined due to the change of color and photometric determination. Full analysis involves the sample testing (contaminated or potentially contaminated material) and control (pure material) as a reference for the cytotoxicity assessment, because this test can detect, e.g. in addition to the contamination with mycotoxin, also other causes of cytotoxicity, as well as those causing a range of allergic reactions (Grajewski & Twarużek 2004).

Due to the fact that the cell culture test (MTT) is characterized by a high sensitivity and is a quick tool for evaluation of cytotoxicity, it can be used to test a large number of samples (Woźniak *et al.* 2004). Gareis (2006) reports that the colorimetric MTT test can be used as a diagnostic means for evaluating the cytotoxicity of materials of different composition, i.e. mycotoxins, pesticides, mould isolates, bacterial cultures, foods, feeds, as well as environmental samples. In 1999, Gareis with Kuss demonstrated the usefulness of the MTT test for the analysis of organic and inorganic compounds, that often charge the foodstuffs, posing a risk to the consumer. In general, this test was useful to demonstrate the traces of pesticide residues - not only to indicate the insecticides, but also less toxic herbicides, fungicides, or growth regulators. Among tested plant protection means, for up to 24 of 28, they found the decrease in vitality of cells by 10%, while for the half of substances they observed the drop in cell vitality by 50%. They also studied selected systems of these xenobiotics combinations. Of the 6 analyzed ones, 2 mixtures showed an independent activity, while the remaining 4 were characterized in part by synergistic effect. There was no antagonism. Our own findings suggest that the MTT test is useful for determining the cytotoxicity of bacterial strains pathogenic to humans and animals (Zastempowska & Twarużek, 2016).

The principles of MTT test

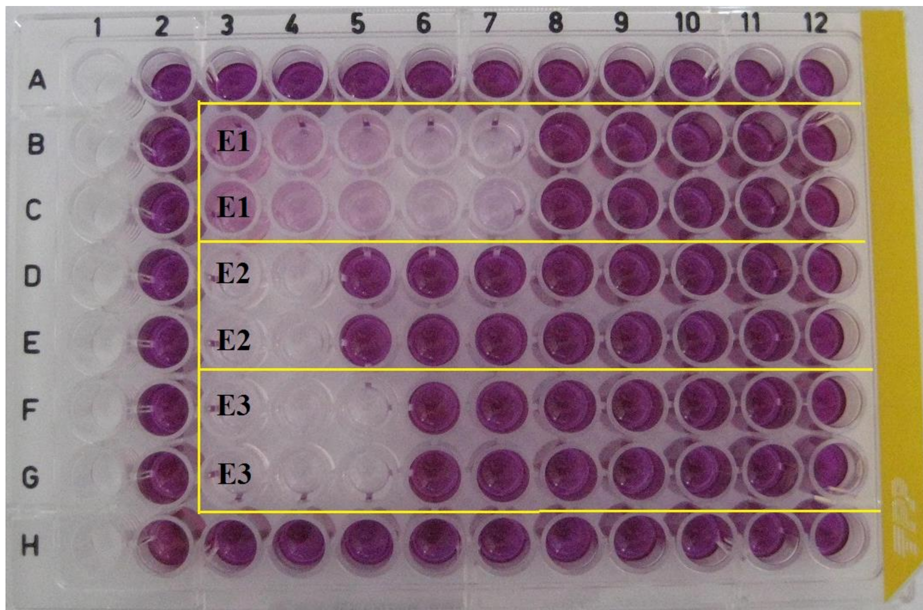
Properly prepared extracts are placed in the wells of the pre-dilutions plate (96-well plate) according to the protocol for test samples distribution. Most often, three extracts in horizontal distribution in a suitable number of dilution – 10, are tested at one micro-plate (Fig. 2). The highest and the lowest rows constitute a cellular barrier and the first column is left free as a zero value for the reader. Released cells brought into the suspension state with a precise indication of their quantity are added to the second plate (test plate). They are transferred to all wells of the plate leaving only the first column free. Such prepared plates are placed in an incubator (37°C, 5% CO_2, 98% humidity) for 48 hours. After this time, the inspection of cells growth is carried out under inverted microscope and MTT salt solution is prepared. A sterile salt is added to the wells and plate is re-incubated for 4 hrs. Then the entire contents of the wells is pulled off using vacuum pump and DMSO is added to each well.

Wells to control the cells are dark purple and correspond to 100% of the division activity. Wells containing contaminated (cytotoxic) sample are discolored, or their color is much less intense (Fig. 3). The absorption values of samples less than 50% of the division activity are considered to be toxic. Based on the degree of dilution, the limit toxic concentration can be determined, i.e. the lowest tested sample that caused the toxic effects to cells.

Figure 2. Scheme of extracts distribution on testing plate.
E – dissolved extract, e – diluted extract, S+K – SMEM (special medium) with cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
C			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
D			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
E			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
F			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
G			E	E S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K	e S+K
H												

Figure 3. Image of MTT micro-plate with 3 cytotoxic extracts



The cytotoxicity evaluation of bacterial cultures can be carried out without dilution. The cell suspension with determined density is plated on 96-well plate and incubated for 24 hours (37°C, 5% CO_2, 98% humidity). After achieving the confluent culture, cells are rinsed with phosphate buffered saline (PBS) solution and then 50µl medium is added to the cell culture (Minimum Essential Medium, MEM) and 50µl of bacterial suspension prepared in the substrate adapted to the type of bacteria, e.g. Tripticase Soy Broth (TSB). Control contains, instead of bacterial suspension, 50µl of sterile TSB medium. Each bacterial strain is tested in several replicates. After 20-hour incubation, the medium is removed together with bacteria, and MTT salt is added to the wells. After a 2-hour incubation and removal of MTT salt, 99.8% ethanol is added. Then, the absorbance is read using micro-reader and cytotoxicity is calculated from the following formula:

*% cytotoxicity = [1-(absorbance of infected culture/absorbance of control)]*100*

Own study

Mycological and cytotoxic analysis of the following samples was performed at the Mycotoxin Analytical Laboratory, Kazimierz Wielki University in Bydgoszcz: building materials, foodstuffs, cereals, and isolates of moulds. The SK cells (swine kidney) were used to MTT test, because they are characterized by high sensitivity to the most common mycotoxins - as demonstrated by Hanelt *et al.* (1994). These authors compared the three cell lines were grown in monolayers: SK, HeLa (cells isolated from human cervical carcinoma), and MDCK cells (canine kidney) against mycotoxins. Swine kidney cells proved to be the most sensitive towards 43 analyzed mycotoxins.

The MTT test rated the cytotoxicity of the mycobiota on mouldy building walls. Table 1 presents the analysis results of samples collected from the mouldy compartments.

Table 1. The results of analyzes of building materials samples using the MTT test.
- no cytotoxicity; + low cytotoxicity; ++ moderate cytotoxicity; +++ high cytotoxicity.

Sample type	Step	IC_{50} [mg/ml]	Rate
Sample from walls from bathroom of mould flat	3	250	++
Sample from walls from living room of mould flat	5	62.5	+++
Sample from damp flat	-	-	-

The first two samples were taken from the walls of residential pavilions situated within the post-flood area. In both cases, there was a very high level of moulds. They showed respectively moderate and high cytotoxicity against control (Twarużek 2005). The third sample collected from mouldy tenant apartment in the city of Bydgoszcz showed no cytotoxic effects. In further detailed mycological and toxicological studies of samples it was found only the presence of mould species not producing the toxic secondary metabolites. The indoor moulds have been recognized as an important factor of air pollution. They cause adverse health effects both in humans and animals. They are also associated with an intense occupational

exposure; high levels of exposure were recorded, among others, at farmers, hospital stuff, and at people working indoors with the faulty ventilation system (Johanning *et al.* 1998).

Table 2 shows the part of own studies on the contamination with moulds and assessment of cytotoxicity of food products purchased in retail trade in Bydgoszcz city.

Table 2. Analysis of food products samples using the MTT test.
- no cytotoxicity; + low cytotoxicity; ++ moderate cytotoxicity; +++ high cytotoxicity

Sample type	Dominating mould species[1]	Step	IC_{50} [mg/ml]	Rate
Raisins	100% *Aspergillus niger*	2	200	-
Dried figs	57% *Aspergillus niger* 43% *Penicillium* spp.	2	200	-
White powdered pepper	44% *Aspergillus sydowi* 30% *Aspergillus fumigatus*	7	6.25	++
Ground nutmeg	93% *Endomyces fibuliger*	9	1.56	+++
Hot pepper	67% *Alternaria alternata*	6	12.5	++
Chili pepper Cayenne	73% *Eurotium herbariorum*	10	0.78	+++
Coffee powder	100% *Aspergillus flavus*	6	12.5	++
Rye flour	34% *Eurotium repens* 25% *Penicillium*	4	50	+
Wheat bran	45% *Aspergillus candidus* 23% *Aureobasidium pullulans*	7	6.25	++
Buckwheat	58% *Aspergillus candidus*	5	25	++
Barley flakes	43% *Eurotium* spp. 42% *Cladosporium sphaerosporum*	4	50	+
Angel hair pasta made from wheat flour	78% *Aspergillus candidus*	1	400	-
Nuts	85% *Penicillium* spp. 5% *Aspergillus* spp.	4	150	+
Pistachios	72% *Cladosporium* spp. 8% *Penicillium* spp.	3	300	-

Samples that were characterized by high mould infection were tested applying MTT (samples having lower degree of infestation were used as a control). The detection of mycotoxins in seeds or fruits brings a lot of problems and can be subject to errors due to their irregular occurrence within such raw material. The instrumental methods of analysis are not for determining the derivative forms of studied toxins and "masked" forms are completely missed, hence the MTT test was used to bio-assays (Grajewski 2003).

Several years of research performed by many authors confirm that with increasing interest in maize cultivation, the importance of this plant fungal diseases that cause a significant decline and deterioration in quality of crops, increases as well (Rataj-Gutarowska & Frąckowiak 2006). On the basis of research, it is estimated that every year the

diseases of maize cause losses in the yield size reaching in some years up to 30%, as well as the deterioration in the quality of grain and silage mass. Mycotoxins produced by fungi colonizing mouldy maize and other raw materials can cause many human diseases (including various allergies, hormonal disorders, and cancer). In particular, their presence in the feed is a major threat to the health and life of animals (Tekiela 2008). It should be remembered that some of the mycotoxins can be ingested by humans due to the carry-over.

Table 3 shows the use of MTT test to assess the mould-contaminated wheat and maize grain as well as the by-product – dried distillers grains with solubles (DDGS).

Table 3. The results of analyzes of cereal samples using MTT test.
- no cytotoxicity; + low cytotoxicity; ++ moderate cytotoxicity; +++ high cytotoxicity

Type of sample	Step	IC_{50} [mg/ml]	Rate
Maize	7	6.25	++
Wheat	3	100	+
DDGS	4	50	+

The study of plant material again demonstrated the usefulness of the MTT test application. During many years of the research, the problem that occurred when testing the oats samples, was noted. Regardless of the degree of fungal infestation of samples (in pure material), there was the cytotoxic effect towards SK. More detailed analysis revealed that substances found in oats – saponins and trypsin inhibitors – are characterized by toxic action (Grajewski *et al.* 2008).

Subsequent studies also used MTT test to assess *Aspergillus* genus isolates (Table 4).

Table 4. The results of the cytotoxicity analysis of Aspergillus genera isolated from air-conditioned hospital environments.
- no cytotoxicity; + low cytotoxicity; ++ moderate cytotoxicity; +++ high cytotoxicity

Type of sample	Step	IC_{50} [cm^2/ml]	Rate
Aspergillus ochraceus	9	0.122	+++
Aspergillus niger	5	1.953	++
Aspergillus fumigatus	10	0.061	+++
Aspergillus flavus	8	0.244	+++

Strains of *Aspergillus* genus fungi occurring in air-conditioned hospital environments were subject to cytotoxicity assessment (Table 4). In all cases, high cytotoxic potential was found (Gniadek *et al.* 2009). These fungi are characterized by the production of highly toxic metabolites such as aflatoxins, ochratoxin A, sterigmatocystin, and cyclopiazonic acid. In assessing the toxicity of these mycotoxins at different animal species, the nephropathic, hepatotoxic, embriotoxic, teratogenic, mutagenic, and carcinogenic influences were observed (Grajewski 2006).

Summary

Several years of own research confirmed that the cell cultures MTT test has a high sensitivity relative to moulds and their

secondary metabolites and is a fast tool to assess their cytotoxicity. It was confirmed that due to the MTT test, it is possible to identify threats to our health, especially by mouldy or contaminated building materials or raw materials and finished products infected by secondary metabolites. It should be noted, however, that no single *in vitro* test is sufficient to consider a substance safe for human health (Chomiczewska *et al.* 2009).

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Streszczenie

Testy biologiczne oparte na hodowlach komórkowych z powodzeniem stosowane są do oceny cytotoksyczności leków, artykułów spożywczych, pasz. Testy *in vitro* stają się uzupełnieniem konwencjonalnych metod chemicznych, a także alternatywą dla badań przeprowadzanych na zwierzętach. Na podstawie badań własnych można stwierdzić, że test MTT jest czułą metodą diagnostyczną przydatną w ocenie cytotoksyczności nie tylko mikotoksyn, pestycydów, bakterii, pleśni, żywności, pasz, ale także próbek uzyskanych z materiałów budowlanych.

Folia Biologica et Oecologica 14: 33–46 (2018)

Acta Universitatis Lodziensis

The spread of alien species along the touristic routes of the Słowiński National Park

Anastazja Krzyżanowska[1], Przemysław Piotr Tomczyk[1]*, Paulina Pruszkowska-Przybylska[2], Katarzyna Maria Zielińska[1]

[1] Department of Geobotany and Plant Ecology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90–237 Łódź
[2] Department of Anthropology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90–237 Łódź

E-mail: przemyslaw.tomczyk@biol.uni.lodz.pl

Abstract

Touristic routes have a great meaning for the spread of alien species through the protected areas like national parks. The aim of the study was to assess the spread of the chosen species along the different kinds of touristic routes of the Słowiński National Park and asphalt roads lying in the direct vicinity of the Park, in its protective zone. The presence of 108 localities of eight alien species were recorded (*Padus serotina*, *Quercus rubra*, *Acer negundo*, *Robinia pseudoacacia* from woody species and *Conyza canadensis*, *Impatiens parviflora*, *Juncus tenuis* and *Oxalis fontana* from herbaceous plants). The localities of the woody plants were found in areas where they were formerly deliberately planted or near routes leading to such areas. All of the analysed woody plants can be the source of ecological threat and should be actively removed. Interestingly, regarding herbaceous plants, the species which according to literature are not a threat to untransformed habitats – *Juncus tenuis* and *Oxalis fontana* – were noted quite commonly. Their populations should be monitored. The populations of *Impatiens parviflora*, classified to the IV class of invasiveness and *Conyza canadensis*, which can invade sand dunes, should be destroyed as soon as possible till they occupy small areas.

Key words: invasive species, anthropopression, protected areas, synanthropic plants

Introduction

Touristic routes are the paths and roads used for hiking, biking or even driving through the areas of special touristic value. The very important feature of touristic routs is their linear character what means very big ratio of their length to width. This is the reason why they can act as a kind of ecological corridors (Forman *et al*. 2003, Lugo & Gucinski 2000). Despite usually small area of the touristic routes in the context of the whole protected area their impact can be meaningful.

DOI: 10.1515/fobio-2017-0007

Roads are a type of habitat well known for the significantly bigger number of invasive plant species in comparison to the surrounding habitats (Damschen *et al.* 2014, Flory & Clay 2006, Flory & Clay 2009, Parendes & Jones 2000, Pauchard & Alaback 2006, Rooney 2005, Watkins *et al.* 2003). Transport is to a large extent associated with an unintentional transfer of propagules and roadsides function for many invasive plant species as a prime habitats. It means that roadside populations are the source ones from which species spread into seminatural or natural plant communities (Joly *et al.* 2011, Rooney 2005). The alien species usually concentrate along roads because the roads create disturbed habitats in which the competition from native species is lower. In forests narrow paths of open habitats also facilitate the dispersion of seeds, sometimes over very long distances (McKinney 2002).

It is widely believed that the ecological role of roads depends on the intensity of human traffic. Tourism is one of the factors generating the traffic around the world and increasing the phenomenon of alien species spreading (Weber 2000). The aim of this article was to present the role of different kinds of routes in the Słowiński National Park, from the small paths used for hiking to the asphalt roads (Figure 1). The Park is a huge attraction for tourists because of the beautiful beaches at the seaside. Thus the most popular are the trails leading to the seashore, however those going through the belt of moving dunes or to the old lighthouse in Czołpino are very popular also. The number of tourists visiting the Park each year is difficult to determine, but it is circa 275 000 people, what means 12 people per one ha (data from 2008) (Partyka 2010). Touristic traffic within the Park is not distributed evenly. The total length of touristic routes is 144.3 km (Partyka 2010), but they are very diversified. Many of them are up to three meters wide and used by a large number of tourists getting to the seashore. Among them, the hardened road leading from Łeba to the interior of the Park, where the movement of electric vehicles is allowed, draws attention. It gives possibility not only to get closer to the seashore and the belt of moving dunes, but also enables setting out on a boat trip on Łebsko Lake and to see the military exhibition of rocket launcher. However very long fragments of touristic routes in the Park are used by few tourists ready for walking trips covering many kilometers or by bikers. In the analysis we included different kinds of routes crossing the National Park and also the asphalt roads typical for Łeba and other urbanized areas lying in the direct vicinity of the Park. Four woody and four herbaceous species of alien plants were selected to the analysis. Among these taxa there were the ones considered to be dangerous for native plant biodiversity as well as the ones that accordingly to the literature have not been regarded as invasive yet.

The aims of the study were:

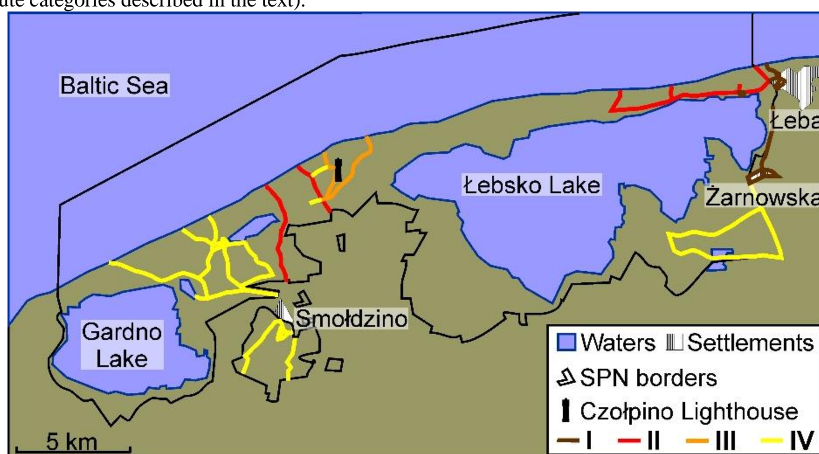
- to evaluate the spread of the chosen alien species along the different kinds of touristic routes going through the Słowiński National Park and its protective zone;
- to evaluate the necessity of the active removal of specimens of these species.

Materials and methods

The Słowiński National Park (pol. Słowiński Park Narodowy, SPN) is located in the north part of the Poland, on the coast of the Baltic Sea. In the area of the Park there is well preserved system of zonal vegetation associated with a seashore. Close to the sea a narrow zone of ephemeral, pioneering dune flora

involving halophytic plants is located. Beyond, there is a zone of white dune plant communities, further in the hinterland, there is a zone of gray dunes and finally a wooded area. The forest phytocoenoses of the National Park consist of birch and alder stands characteristic for swamp and alluvial sites, deciduous stands of hornbeam, beech, birch and oak trees, and coastal and inland pine stands. In the SPN there are also two big eutrophic lakes: Gardno and Łebsko, and several smaller lakes and fens with typical for such sites natural vegetation (Piotrowska 1997, Wojterski 1979).

Figure 1. The area of the Słowiński National Park with different kinds of routes taken to the analysis (I – IV – the route categories described in the text).



The research was conducted in July in the years 2013 and 2014, within the framework of the project "Inventory of the alien plant species along the touristic routes of the Słowiński National Park" led by the Botany Section of The Biological Student Association of University of Lodz. The study included four species of woody plants brought to Poland for commercial purposes and for gardening: *Padus serotina* Ehrh., *Quercus rubra* L., *Acer negundo* L. and *Robinia pseudoacacia* L. We investigated both the mature trees and seedlings. Another four species of herbaceous plants unintentionally dragged to the country or escaped from botanical gardens were chosen: *Conyza canadensis* (L.) Cronquist, *Impatiens parviflora* DC., *Juncus tenuis* Willd., *Oxalis fontana* L. The characteristic features of the chosen species are presented in Table 1. The occurrence of the mentioned species was noted alongside the touristic routes of four different categories:

I – the asphalt roads lying in the direct vicinity of the Park (in its protective zone) or constituting the border of the Park – these roads are connected with the routes coming directly through the Park;

II – the shortest routes to the beach, with the parking areas lying at the Park borders – these paths are used by a large number of people interested in the rest on the seashore;

III – the routes leading to the tourist attractions of the Park (route through the moving dunes or to the old lighthouse) – these are used by the tourists who are ready to take the effort of longer hike to see some interesting places from different point of view;

IV – the long routes leading through the forests and near lakes or lying in the

south, less frequently visited by tourists, parts of the Park – these are used by few people enjoying long hiking, biking or by the local society.

We assumed that our categories represent different intensity of the human traffic. The length of the surveyed routes in the particular categories varied (I category: 9.5 km; II: 13 km; III: 5 km; IV: 37 km), but was representative for the Park (with the exception of the asphalt roads – these were analysed as a comparative sample at the east side of the Park protective zone). The routes chosen for the analysis are presented on Figure 1.

The occurrence of the chosen alien species was noted with the standard GPS device (application GPS Essentials uploaded to Samsung Galaxy Tab 8.9) so their accuracy (usually about 25 meters) decided about placing the next GPS point. In that way we recorded the separate localities of alien species specimens and we were setting the series of points if the population of the alien species was spread alongside the longer than 25 meters part of the route. In each point we were assessing the number of specimens or the surface area occupied by the species in square meters. The chosen method depended on the species and the degree of its spread (counting of specimens was possible in the case of the tree seedlings but in the case of such species like *Juncus tenuis* it would be ineffective). The following three-step scale of the population size evaluation was developed on the basis of the preliminary studies:

1 – one to three individuals of alien species,

2 – four to twenty individuals or covering smaller than one square meter,

3 – over twenty individuals or population covering at least one square meter.

The alien species which were noted during the studies grew directly on the routes or in the zone not wider than 1.5 m from the road surface.

The nomenclature of species was based on Mirek *et al.* (2002).

Table 1. The characteristic features of the chosen species

Species	**The place of origin**	**Causes / way of entering into Poland**	**Main properties of species conducive to its invasion**	**Native habitats threatened by invasive species**	**Ways to combat (safe for the environment)**	**Category of invasiveness (Tokarska-Guzik *et al.* 2012)**
Acer negundo	North America	Planted as a roadside tree and in parks, also promoted as a melliferous tree and used for the restoration of degraded land (Packiewicz *et al.* 2006, Frączek *et al.* 2009).	Species with high resistance to high and low temperatures, drought and chemical contamination of the soil. It has light fruits that are spread by wind and water (Packiewicz *et al.* 2006, Frączek *et al.* 2009, Bijak *et al.* 2014, Danielewicz & Wiatrowska 2014).	willow-poplar forests (*Salici-Populetum*) (Mędrzycki 2014)	The most common method is felling trees at the base of the root collar (Credit Valley Conservation 2011, Mędrzycki 2014).	IV
Padus serotina	North and South America	Due to the rapid and luxuriant growth it has been used in the plantings in the forests (Namura-Ochalska 2012).	Dispersed by birds, especially starlings (Krzysztofiak & Krzysztofiak 2015). It has ability of very intense vegetative propagation and strong competitiveness due to allelopathy (Robakowski & Bielinis 2011).	Forest communities, mostly old acidophilous oak woods (Tokarska-Guzik *et al.* 2012)	The easiest way is to eliminate the juveniles that can be pull out by hand. For mature specimens the most effective treatment is grubbing individuals (Namura-Ochalska 2012).	IV

Table 1. The characteristic features of the chosen species (cont.)

Species	The place of origin	Causes / way of entering into Poland	Main properties of species conducive to its invasion	Native habitats threatened by invasive species	Ways to combat (safe for the environment)	Category of invasiveness (Tokarska-Guzik *et al.* 2012)
Quercus rubra	Eastern and central USA and south–central Canada	Species grown for wood (has less knots in wood than native species) and as an ornamental tree (Kuc *et al.* 2012).	Resistant to pollution, drought and frost. The species heavily shadows the ground, releases allelopathic compounds and has slowly decaying litter. It is spread mainly by zoochory (Danielewicz & Wiatrowska 2014).	*Quercus robur* forests and mixed *Quercus robur* and *Pinus sylvestris* forests (Woziwoda & Obidziński 2016)	Cutting down mature trees and removing juveniles for many years (Tałałaj 2013).	IV
Robinia pseudoacacia	Appalachia and Plateau Ozark and Ouachita Mountains	It is used for decorative and economic reasons (among others because of its valuable wood, melliferous flowers and root system preventing erosion) (Tokarska-Guzik *et al*. 2015).	It tolerates drought, air pollution and soil salinity (Danielewicz & Wiatrowska 2014, Kołaczkowska 2008). It releases allelopathic compounds (Tokarska-Guzik *et al.* 2015).	Xeric and calcareous grasslands, dry heaths, Euro-Siberian steppe woods with *Quercus sp.* (Tokarska-Guzik *et al.* 2012)	It is recommended to cut down the trees and then eliminate all growing shoots (Obidziński & Woziwoda 2014).	IV
Conyza canadensis	North America	Unknown / random	One specimen can produce from thousand to 100 thousands seeds (Regehr & Bazzaz 1979). The seeds are very easily dispersed by wind and water (Weaver 2001).	Meadows and sand dunes (Thebaud & Abbott 1995)	Unexplored	I
Impatiens parviflora	Central Asia	Unknown / random	It produces a large number of seeds (on average 1-2 thousand) which are ejected under the pressure of 12 atmospheres at a distance of 3–4 meters (Sołtys-Lelek & Barabasz-Krasny 2010).	The vegetation of river banks, calcareous rocky slopes, forests. (Tokarska-Guzik *et al.* 2012)	Mechanic removal of plants by pulling them out before the release of seeds or mowing the young specimens (Adamowski & Bomanowska 2016).	IV
Juncus tenuis	North America	Unknown / random	The species is resistant to trampling or running over. In each capsule it produces an average of 270 seeds which can be carried by animals, people or wheels (Richards 1943).	Lowland hay meadows (Tokarska-Guzik *et al.* 2012)	Unexplored	I
Oxalis fontana	North America	Unknown / random	It can spread vegetatively by rhizomes (Hantz 1979, Rutkowski 2006, Small 1896).	Lack	Unexplored	I

Results

The total number of 108 GPS points reflected the spread of the chosen alien species along the routes of the National Park. The number of the points for particular species and the most frequently recorded category of abundance (in parentheses) are as follow:
Conyza canadensis: 8 (3),
Impatiens parviflora: 10 (3),
Juncus tenuis: 23 (1),
Oxalis fonatana: 15 (2),
Padus serotina: 13 (1),
Quercus rubra: 27 (2),
Robinia pseudoacacia: 8 (1),
Acer negundo: 4 (1).

The details relating to particular GPS points are included in the Appendix. The presence of the species was noted along almost each of the penetrated touristic routes in the whole area of the Park (Figure 2,3). The high concentration of the localities was connected with the asphalt roads in the protective zone of the SPN (category I), besides we did not confirm the meaning of the road category. Along each of them we found the species in different categories of population size. Also each of analysed alien species was represented by populations of different size category, including those of the biggest abundance (Figure 2, 3).

Figure 2. Localisation of the GPS points corresponding to the chosen woody alien species localities in the area of the Słowiński National Park (marked by a dots, 1-3 – the categories of population size described in the text) and diagrams with the number of these GPS points noted along different kinds of routes (I-IV – the route categories described in the text).

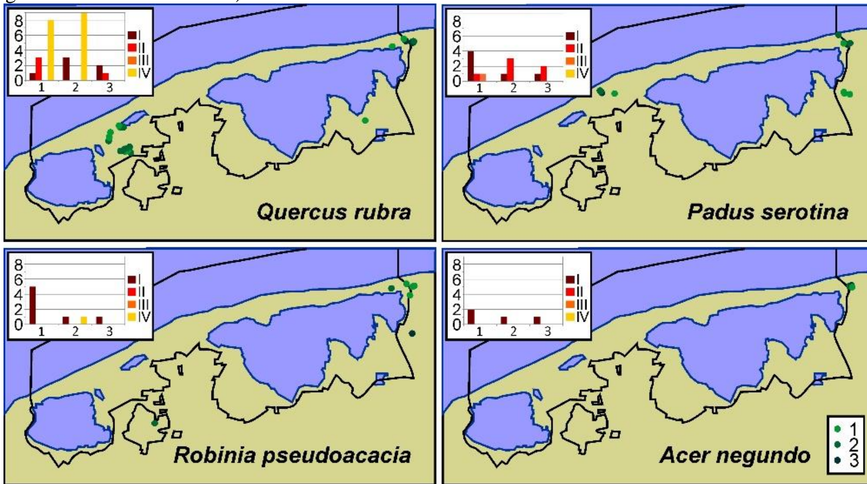
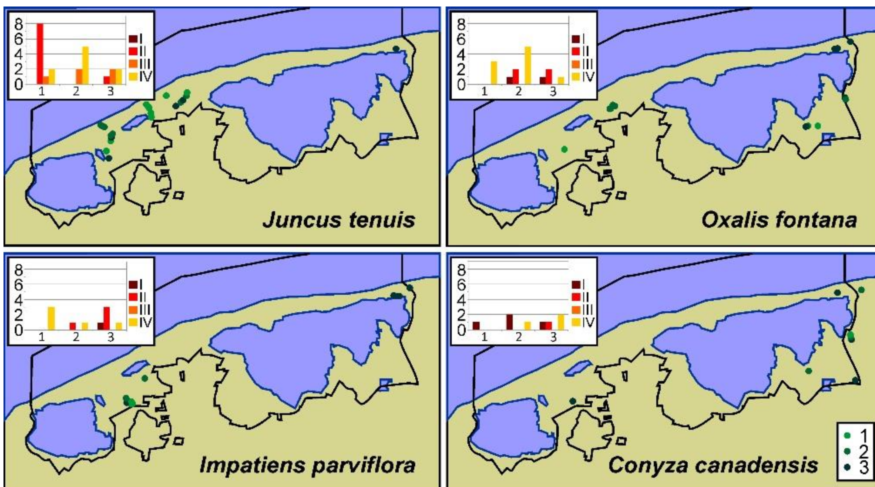


Figure 3. Localisation of the GPS points corresponding to the chosen herbaceous alien species localities in the area of the Słowiński National Park (marked by a dots, 1-3 – the categories of population size described in the text) and diagrams with the number of these GPS points noted along different kinds of routes (I-IV – the route categories described in the text).



Discussion

The alien woody species were found more often along asphalt roads connecting towns or villages (I category). However, two of those species occurred also in the vicinity of touristic paths lying deep in the Park, we found a lot of young specimens of *Quercus rubra* and *Padus serotina* there. Those species are a residue of earlier forestry. They were deliberately introduced to the area, like to the other forest complexes in Poland (Gazda & Augustynowicz 2012). *Quercus rubra* was found in the southern part of the Park, characterised by more fertile soils in comparison with the coastal area. Young seedlings were located in the vicinity of the mature trees. On the contrary, seedlings and young specimens of *Padus serotina* where found in places where the older individuals were not in sight. However those specimens grew near routes leading to the vicinity of the old military training area (to the north of Łebsko Lake), where the species was planted. Nowadays it spreads due to zoochory (Jagodziński *et al*. 2015). Thus the earlier plantations became a source populations for contemporary observed seedlings. On the borders of the National Park *Quercus rubra* and *Padus serotina* were observed near the human settlements. Similarly two other analysed tree species *Acer negundo* and *Robinia pseudoacacia* were connected with human settlements where they were planted (especially near Łeba). Our study did not confirm the importance of the road category for the spread of selected tree species, while the presence of the source populations was of crucial importance. The other authors confirm that the presence of specimens of the alien tree species in the vicinity of protected areas can be the real threat (e.g. Dajdok *et al*. 2007, Mędrzycki 2007, Namura-Ochalska 2012, Tałataj 2013).

Among all of the examined herbaceous species only *Juncus tenuis* was not present near the asphalt roads. This species was the most common from the analysed herbaceous plants and grow directly on the dirt roads and along them, very often in the small hollows in which the pools of stagnant water can occur after rainfalls. It was found most often on sandy soils on the roads leading to the sea. The others of the analysed alien herbaceous species occurred near roads of different categories, but in different parts of the SPN. For example *Impatiens parviflora* could not grow on very poor soils in the north part of the Park. So it was the presence of proper sites what was crucial for the spread of herbaceous plants. In this context special attention should be paid to *Conyza canadensis*. Although Tokarska-Guzik *et al*. (2012) classified this taxon to the lowest category of invasiveness and Veawer (2001) described it as a ruderal species (colonizing abandoned agrocoenoses and other disturbed habitats), there are reports in literature that it can also grow in meadows and on sand dunes (Thebaud & Abbott 1995). Because the moving dunes are one of the most valuable and unique features of the SPN, species which are able to occupy such habitats can be a real threat.

Each of the analyzed species was present in different classes of abundance, including the biggest one (over twenty individuals or population covering at least one square meter). That means that despite the chosen species were classified to different classes of invasiveness (Tokarska-Guzik *et al*. 2012), in the area of the SPN all of them have potential for successful spreading. We did not found the dependence between the type of touristic routes and the size of populations. It seems that the presence of the road is more important than its size. Along all of the investigated routes, even those rarely used, the alien species can be

found and successfully spread. Other authors confirm that even small interference to the compactness of natural phytocoenoses may facilitate the alien species encroachment (Fornal-Pieniak 2011, Banaszek & Szymczyk 2014).

Conclusions

Considering above, we postulate the active removal of the all of analysed woody species from the Park and its vicinity. Removal of mature trees and shrubs will reduce the number of emerging seedlings. In the case of herbaceous plants we postulate the destruction of patches of *Impatiens parviflora* and *Conyza canadensis*. The populations of these taxa are still relatively small, but they can function as sources of the future spreading. Their fast removal can reduce the threat and costs of possible future activities in the fight with alien species. Whereas in the case of very common, but classified to the lowest category of invasiveness species *Juncus tenuis* and *Oxalis fontana*, we propose monitoring them to determine whether they are expanding the area of occurrence. Because the sources of propagules of alien species (especially herbaceous) can be parking areas and places which are frequently chosen for the organised tourist trips, such areas should be regularly monitored.

Acknowledgements

We gratefully acknowledge the Authorities of the Słowiński National Park for any help and enabling of conducting field research. We also thank to all persons who provided us funds for the organization of scientific camps, during which the study was conducted: Rector of University of Lodz, prof. dr hab. Włodzimierz Nykiel; Dean of Faculty of Biology and Environmental Protection, prof. dr hab. Elżbieta Żądzińska; Director of Institute of Ecology and Environmental Protection, prof. dr hab. Piotr Frankiewicz and Chair of Department of Geobotany and Plant Ecology, prof. dr hab. Józef K. Kurowski.

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Streszczenie

Szlaki turystyczne mają wielkie znaczenie dla rozprzestrzeniania się obcych gatunków na obszarach chronionych, takich jak parki narodowe. Celem badań była ocena rozprzestrzeniania się wybranych gatunków wzdłuż różnego rodzaju szlaków turystycznych przebiegających przez Słowiński Park Narodowy oraz dróg asfaltowych leżących w bezpośrednim sąsiedztwie Parku, w jego otulinie. Stwierdzono obecność 108 stanowisk ośmiu obcych gatunków: *Padus serotina*, *Quercus rubra*, *Acer negundo*, *Robinia pseudoacacia* spośród gatunków drzewiastych oraz *Conyza canadensis*, *Impatiens parviflora*, *Juncus tenuis* i *Oxalis fontana* spośród roślin zielnych). Stanowiska roślin drzewiastych odnotowano na obszarach, gdzie wcześniej były celowo sadzone oraz wzdłuż szlaków prowadzących do takich obszarów. Wszystkie analizowane rośliny drzewiaste mogą być źródłem zagrożenia ekologicznego i powinny być aktywnie usuwane. W przypadku roślin zielnych, gatunki, które zgodnie z literaturą nie stanowią zagrożenia dla nieprzekształconych zbiorowisk roślinnych – *Juncus tenuis* i *Oxalis fontana* – notowano dość powszechnie. Ich populacje powinny być monitorowane. Populacje *Impatiens parviflora*, gatunku zaklasyfikowanego do IV klasy inwazyjności oraz *Conyza canadensis*, który zgodnie z literaturą może rozprzestrzeniać się na wydmach, powinny zostać zniszczone jak najszybciej, dopóki zajmują małe obszary.

Appendix

The GPS points noted during the studies conducted in the years 2013 – 2014. Abundance and road categories in accordance with the description in Material and methods.

Species	Locality (GPS)	Abundance category	Road category
Conyza canadensis (L.) Cronquist	N 54° 40.282' E 17° 11.235'	3	IV
	N 54° 41.832' E 17° 28.899'	2	IV
	N 54° 41.472' E 17° 32.670'	3	IV
	N 54° 45.455' E 17° 32.658'	2	I
	N 54° 43.343' E 17° 32.054'	3	I
	N 54° 43.450' E 17° 31.778'	2	I
	N 54° 43.484' E 17° 31.749'	1	I
	N 54° 45.297' E 17° 30.858'	3	II
Impatiens parviflora DC.	N 54° 40.293' E 17° 11.410'	1	IV
	N 54° 40.298' E 17° 11.336'	1	IV
	N 54° 40.309' E 17° 11.048'	3	IV
	N 54° 40.328' E 17° 11.135'	1	IV
	N 54° 40.380' E 17° 10.929'	2	IV
	N 54° 41.448' E 17° 12.521'	2	II
	N 54° 45.680' E 17° 32.450'	3	I
	N 54° 45.289' E 17° 31.359'	3	II
	N 54° 45.274' E 17° 31.254'	3	II
	N 54° 45.237' E 17° 31.111'	3	II
Juncus tenuis Willd.	N 54° 40.185' E 17° 09.609'	3	IV
	N 54° 40.483' E 17° 09.442'	1	IV
	N 54° 40.982' E 17° 09.537'	1	IV
	N 54° 41.110' E 17° 09.594'	3	IV
	N 54° 41.216' E 17° 09.694'	2	IV
	N 54° 41.359' E 17° 09.821'	2	IV
	N 54° 41.518' E 17° 09.131'	2	IV
	N 54° 41.590' E 17° 08.832'	2	IV
	N 54° 41.662' E 17° 08.736'	2	IV
	N 54° 41.978' E 17° 12.671'	1	II
	N 54° 42.233' E 17° 12.572'	1	II
	N 54° 42.321' E 17° 12.488'	1	II
	N 54° 42.536' E 17° 12.345'	1	II
	N 54° 42.572' E 17° 12.278'	1	II

Species	Locality (GPS)	Abundance category	Road category
Juncus tenuis Willd	N 54° 42.617' E 17° 12.189'	1	II
	N 54° 42.641' E 17° 12.144'	1	II
	N 54° 42.648' E 17° 14.580'	3	III
	N 54° 42.769' E 17° 14.752'	2	III
	N 54° 42.887' E 17° 14.937'	3	III
	N 54° 42.949' E 17° 13.722'	1	II
	N 54° 43.089' E 17° 15.239'	2	III
	N 54° 43.164' E 17° 15.275'	1	III
	N 54° 45.270' E 17° 31.079'	3	II
Oxalis fontana L.	N 54° 40.486' E 17° 10.589'	1	IV
	N 54° 42.472' E 17° 13.897'	2	IV
	N 54° 42.487' E 17° 13.942'	2	IV
	N 54° 42.500' E 17° 14.015'	2	IV
	N 54° 42.509' E 17° 14.071'	2	IV
	N 54° 42.526' E 17° 14.098'	2	II
	N 54° 43.096' E 17° 31.692'	3	I
	N 54° 41.952' E 17° 29.575'	1	IV
	N 54° 41.865' E 17° 28.875'	1	IV
	N 54° 41.832' E 17° 28.899'	2	IV
	N 54° 41.852' E 17° 28.834'	3	IV
	N 54° 43.218' E 17° 31.570'	2	I
	N 54° 45.720' E 17° 32.220'	3	II
	N 54° 45.312' E 17° 30.801'	2	II
	N 54° 45.384' E 17° 30.783'	3	II
Padus serotina Ehrh.	N 54° 42.884' E 17° 13.765'	3	II
	N 54° 42.902' E 17° 14.395'	1	III
	N 54° 42.922' E 17° 13.750'	3	II
	N 54° 42.993' E 17° 13.712'	2	II
	N 54° 43.003' E 17° 13.720'	2	II
	N 54° 45.437' E 17° 32.316'	1	I
	N 54° 45.437' E 17° 32.337'	3	I
	N 54° 45.448' E 17° 32.464'	2	I
	N 54° 45.880' E 17° 31.980'	2	II
	N 54° 43.351' E 17° 32.031'	1	I
	N 54° 43.321' E 17° 32.110'	1	I

Species	Locality (GPS)	Abundance category	Road category
Padus serotina Ehrh.	N 54° 43.422' E 17° 32.574'	1	I
	N 54° 45.616' E 17° 32.395'	1	II
Quercus rubra L.	N 54° 40.293' E 17° 11.410'	2	IV
	N 54° 40.352' E 17° 11.045'	1	IV
	N 54° 40.380' E 17° 10.929'	1	IV
	N 54° 40.426' E 17° 10.804'	2	IV
	N 54° 40.457' E 17° 10.743'	2	IV
	N 54° 40.469' E 17° 10.826'	2	IV
	N 54° 40.472' E 17° 10.672'	2	IV
	N 54° 40.607' E 17° 11.297'	2	IV
	N 54° 40.813' E 17° 09.515'	2	IV
	N 54° 40.918' E 17° 09.637'	1	IV
	N 54° 40.982' E 17° 09.537'	1	IV
	N 54° 41.110' E 17° 09.594'	1	IV
	N 54° 41.468' E 17° 10.487'	1	IV
	N 54° 41.488' E 17° 10.914'	2	IV
	N 54° 41.491' E 17° 10.834'	2	IV
	N 54° 41.538' E 17° 10.245'	1	IV
	N 54° 45.616' E 17° 32.395'	1	II
	N 54° 45.216' E 17° 31.100'	1	II
	N 54° 45.628' E 17° 32.475'	3	II
	N 54° 45.679' E 17° 32.340'	1	II
	N 54° 45.499' E 17° 32.487'	2	I
	N 54° 45.478' E 17° 32.445'	1	I
	N 54° 45.441' E 17° 32.466'	2	I
	N 54° 45.453' E 17° 32.525'	3	I
	N 54° 45.463' E 17° 32.540'	3	I
	N 54° 45.473' E 17° 32.685'	2	I
	N 54° 41.944' E 17° 29.338'	1	IV
Robinia pseudoacacia L.	N 54° 39.327' E 17° 13.442'	2	IV
	N 54° 45.592' E 17° 32.514'	1	I
	N 54° 45.255' E 17° 30.913'	2	I
	N 54° 43.422' E 17° 32.574'	3	I
	N 54° 45.433' E 17° 32.419'	1	I
	N 54° 45.036' E 17° 32.291'	1	I

Species	Locality (GPS)	Abundance category	Road category
Robinia pseudoacacia L.	N 54° 45.441' E 17° 32.622'	1	I
	N 54° 45.455' E 17° 32.658'	1	I
Acer negundo L.	N 54° 45.441' E 17° 32.466'	1	I
	N 54° 45.448' E 17° 32.464'	2	I
	N 54° 45.463' E 17° 32.540'	3	I
	N 54° 45.455' E 17° 32.658'	1	I

Folia Biologica et Oecologica 14: 47–54 (2018)

Acta Universitatis Lodziensis

Virological aspects of non-human primates or swine-to human xenotransplantation

NATALIA MAZURKIEWICZ[1]*, AGNIESZKA NOWAK[1], MAGDALENA HRYHOROWICZ[1], JOANNA ZEYLAND[1], DANIEL LIPIŃSKI[1], RYSZARD SŁOMSKI[1,2]

[1] Department of Biochemistry and Biotechnology, Poznan University of Life Sciences, ul. Dojazd 11, 60-632 Poznań

[2] Institute of Human Genetics of Polish Academy of Sciences, ul. Strzeszyńska 32, 60-479 Poznań
E-mail: nmmazurkiewicz@gmail.com

ABSTRACT

There are a number of human diseases, which can lead to organ failure. The consequence is often the need for a transplant. The number of performed operations is very low due to the shortage of organs for transplantation. As a consequence, the number of people waiting for transplant is still growing. The solution to this situation may be xenotransplantation. Xenotransplantation word comes from the Greek xenos meaning stranger, the other. It is defined as any procedure that involves the transplantation, implantation or infusion of tissues or zoonotic organs into a human recipient, but also human body fluids, cells, tissues, organs (or fragments) that have ex vivo contact with zoonotic cells, tissues or organs. One of the obstacles of the xenograft transplantation is the risk of animal pathogens transmission to the humans. Viruses that pose risk in the non-human primates-to-human xenotransplantation includes: the human immunodeficiency virus - HIV and the Marburg virus described in this paper. In addition viruses, which is a problem in pig-to-human xenotransplantation have also been described, including: porcine endogenous retrovirus - PERV, porcine cytomegalovirus - PCMV, porcine lymphotropic herpesvirus - PLHV and hepatitis E virus - E - HEV. This review of literature is the latest knowledge of the microbiological safety of xenotransplantation.

KEY WORDS: PERV, HIV, zoonoses, transgenic animals

Introduction

Transplantation involves the removal of an organ, tissue, or cells from the donor's body and implantation into the recipient's body. The field of science that deals with the subject of an organ transplantation is transplantology. The organs are transplanted to recipients, whose do not have fully functioning organs due to the illness or mechanical damage. Transplantation of organs or its fragments is a life saving method that improves quality and prolongs lives of

DOI: 10.1515/fobio-2017-0008

people for which there is no other treatment available (Smorąg *et al.* 2011, Saxena *et al.* 2016). We can distinguish four types of transplantations:

• autotransplantation – a transplantation within the body of the same person, an example may be transplanting skin from one place to another,

• allotransplantation – a transplantation between representatives of the same species, for example kidney transplantation between two people,

• isotransplantation – a kind of allotransplantation that is carried out between individuals with same genotype, for example monovular twins,

• xenotransplantation – a transplantation between individuals belonging to different species; an example is the transplantation between a pig and a human (Gołąb & Basak 2012, Saxena *et al.* 2016).

Many human diseases can lead to organ failure. The consequence is often the need for a transplant. The number of the performed operations is very low due to the shortage of organs for transplantation. Therefore, the number of people waiting for transplant is still growing (Saxena *et al.* 2016). The way out of this situation may be xenotransplantation. The concept of organ transplantation in a pig-to-human or non-human-primate-to-human system is designed to keep the patient alive. Xenotransplantation research focuses on obtaining animals whose organs can be transplanted until a suitable human donor is found and ultimately to replace the human organs as a functionally and physiologically equivalent (Zeyland *et al.* 2015, Jura *et al.* 2006).

Xenotransplantation is defined as any procedure that involves the transplantation, implantation or infusion of tissues or zoonotic organs into a human recipient, but also human body fluids, cells, tissues, organs (or fragments) that have ex vivo contact with zoonotic cells, tissues or organs. There is a fundamental problem here, because of the presence of species specific antigens on the animals cells surface that are not present on the human cells. This incompatibility causes the side effects of transplantation of cells, tissues or organs from unmodified animals. It is a severe immune response that results in immediate or chronic rejection of the transplant (Cooper *et al.* 2015, Zeyland *et al.* Słomski 2015).

Since xenotransplantations have been considered as a viable option for dealing with the deficiency of organs required for transplantation to all people on the waiting lists, primate monkeys were tested as the most suitable animals. This choice was made because the non-human primates are closely related phylogenetically with humans. What is the biggest advantage of such a solution is its biggest disadvantage at the same time. Viruses that infect primates can infect humans as well. This is one of the factors that has determined the change of the subject matter of the research from primates to cloven-hoofed animals (especially pigs) (Smorąg *et al.* 2011, Jura *et al.* 2006, Cooperet *et al.* 2015).

Virological risks in the nonhuman primates – to – human xenotransplantation

The first animals that were taken into account in the context of xenotransplantation are primates. Apart from the obvious advantages of these animals associated with physiological similarity, they also have drawbacks, including one that limits the development of research in this direction. This obstacle is the virological risks. An example of a virus that has been transmitted from this animals to human and could be a problem in xenotransplantation is the human immunodeficiency virus – HIV. HIV is a lentivirus (a subgroup of retrovirus) that

causes HIV infection. It causes an acquired immunodeficiency syndrome (AIDS) (Specke *et al.* 2002, Boneva *et al.* 2001).

Human immunodeficiency virus is transmitted by exchange or contact with body fluids of the host. HIV causing AIDS is one of the major pathogens - the sixth leading cause of mortality in the world. The life expectancy of people infected with HIV has increased over the years and is approaching the average in the population. This is the result of the efficacy of antiretroviral therapy (ART). The use of ART drugs has improved the quality of life of infected people and reduced morbidity and mortality (Rumbwere *et al.* 2016).

There are several strains of the virus that cause AIDS as a result of the human infection. The HIV-1 strain is derived from chimpanzees (*Pan troglodytes*) and its predominance is restricted to the inhabitants of Europe and America. The HIV-2 strain is derived from the sooty mangabey (*Cercocebus atys*) and is found predominantly in the populations of central Africa. HIV attacks the cells which have CD4 (clustered differentiation 4) receptors and also the cells with co-receptors CXCR4 or CXCR5 (C-X-C Chemokine receptor type 4 or 5) on their surfaces. HIV infection causes AIDS that develop by reducing the number of lymphocytes in the blood and thus weakening of the immune system. The death of a viral carrier is the result of infection with pathogens that a properly functioning immune system would be able to fight. HIV is one of the most variable viruses that precludes the immune system from fighting infection by producing the right antibodies, and makes it difficult to develop antiviral drugs and vaccines (Boneva *et al.* 2001, Rumbwere *et al.* 2016).

Another virus, which is a problem in non-human primates-to-human xenotransplantation is the Marburg virus, which belongs to the Filoviridae family. This virus was transferred to a man from the green monkey (*Chlorocebus sabaeus*). The Marburg virus genome is single-stranded nonsense RNA (-ssRNA). It causes a disease called haemorrhagic fever which is very difficult to treat. The virus can be transmitted through contact with an infected person, or it can be transmitted via droplet path (Günzburg & Salmons 2000).

Viruses, which pose a direct threat in non-human primates-to-human xenotransplantation are very numerous, and combating them has not produced measurable results so far, so the researchers left the concept of using primates for xenotransplantation. (Cooper *et al.* 2015).

Virological risks in the pig – to – human xenotransplantation

An animal that seems to be more suitable for use in xenotransplantation is the domestic pig (*Sus scrofa domestica*). Despite the phylogenetic remoteness of the pig from human, the functionality of its organs and size is similar to human organs. However, despite of the fact that the virological problem in this xenotransplantation system is smaller, it also worries the researchers (Fischer *et al.* 2016). The pig is a carrier of the porcine endogenous retrovirus – PERV. This is the biggest problem because of the integration of the viral genome into many loci of the pig genomes. PERVs are the acquired elements that are present in the majority of the pig genomes. What is more, PERV like other retroviruses often recombine and it is able to "transposing" in the pig genome as transposon. It also has the ability to recombine with human retroviruses. This recombination is a big problem, as it can lead to the uprising of the virulent strains of viruses that will be

unpredictable. PERV provirus sequences have been classified as A, B, and C types, where A and B are predominantly present in the genome of all pigs, while the presence of type C is strongly reduced or absent (Plotzki *et al.* 2015). A transmission of porcine endogenous retrovirus type A and C recombinants to human cells *in vitro* has been observed. But the presence of these recombinants in the genome of the pig *in vivo* has not been proven. In addition, the virus is only capable of infecting human cell lines *in vitro*. However, the transfer of the virus from pig to human was not observed *in vivo* (Wilson 2008, Scobie & Takeuchi 2009). This may be related to the fact that human cells have many mechanisms to fight viral infections. In particular, a group of proteins capable to combating viral infections called: apolipoprotein B mRNA-editing catalytic polypeptide cytidine deaminases - APOBEC. The expression of these proteins in transgenic animal cells could reduce the PERVs potential infection and thereby protect patients after xenotransplantation (Dörrschuck *et al.* 2008). The complement lysis system can also protect cells from infection with the porcine endogenous retrovirus. This suggesting that there is no evidence of infection with this virus in vivo, which may be caused by the human-host immune system (Fujita *et al.* 2003). It should be noted that the mechanism will be no longer effective in animals with a knock-out of the gene encoding α-1,3-galactosyltransferase (Gal-KO animals), so some of the concerns may involve the use of animals with this modification (Kuwaki *et al.* 2005). Therefore, PERV provirus genes should be eliminated from the pig genome. Due to the large number of components of the PERVs in the swine genome this seemed to be impossible. However, endogenous pig retroviruses were inactivated in 2015 using the CRISPR/Cas9 system. In experiments, the complete inactivation of all the identified genome provirus copies in one individual has been achieved. There were as many as 62 copies of the virus. For this purpose, specific gRNA was designed that was complementary to the very conservative catalytic center of the *pol* gene present in the sequences of each type of PERVs. This gene encodes the protein that acts as a reverse transcriptase and is essential for proper replication and virus infection (Yang *et al.* 2015). This is a huge success that brings researchers closer to completely eliminating the PERVs problem in xenotransplantations. However, these are not the first successes in the study of the PERV virus in this aspect. Earlier attempts were made to inactivate porcine endogenous viruses with siRNA silencing mechanism with very good effects, but not as spectacular as in the case of application of the CRISPR/Cas9 system (Dieckhoff *et al.* 2008, Yang *et al.* 2015).

The second virus, which can be a problem in pig-to-human xenotransplantation is porcine cytomegalovirus – PCMV. It belongs to the herpesvirus family. Studies in the pig-to-non-human primates xenotransplantation have shown an increased replication of PCMV in transplanted tissues due to immunosuppression (Morozov *et al.* 2017). In addition, it has been shown that enhanced replication leads to damage of the endothelial cell layers and the massive coagulopathy, which in turn can accelerate the process of the xenograft rejection. However, no transfer of the virus from swine transplanted tissues to non-human primates tissues has been reported. Additional studies indicate the susceptibility of human fibroblast *in vitro* cultures to PCMV infection and decreased susceptibility to antiviral drugs used in CMV cytomegalovirus therapy. An

effective way of eliminating this virus is the early withdrawal of piglets from the sow. This appears to be sufficient in the context of the use of these animals in xenotransplantations (Gollackner *et al.* 2003, Morozov *et al.* 2016).

The third swine virus posing a problem in xenotransplantation is the porcine lymphotropic herpesvirus – PLHV. There are three types of this virus: 1, 2 and 3. It belongs to the γ-herpesvirus family. This virus is homologous to the human herpesvirus 8 – HHV-8 (otherwise known as Kaposi's sarcoma-associated herpesvirus – KSHV or Epstein-Barr virus – EBV) (Plotzki *et al.* 2016). Although transmission of the PLHV from pig to non-human primates tissues has not been shown after xenotransplantation. The high PLHV homology with human HHV-8 and EBV viruses may induce PLHV-1 activation after recombination, which was observed experimentally (Gazda *et al.* 2016). No effective method of controlling this virus was found, only the monitoring of swine breeding is suggested (Santoni *et al.* 2006, Morozov *et al.* 2016).

The next virus posing a problem in the pig-to-human xenotransplantation is hepatitis E virus (HEV). This type of virus (subtype 1 precisely) occurs in tropical and subtropical climates. A human can get infected with this virus by drinking water contaminated with animal manure. In recent years, there has been an increase in the number of infected with subtype 3 of this virus, which is common in pigs. The infection occurs also during allotransplantation or by ingestion of infected pork. This direct mechanism of infection makes the virus one of the major problems of xenotransplantation at the moment (Banks *et al.* 2004, Morozov *et al.* 2016).

Despite the wide variety of pig viruses potentially endangering the human health after xenotransplantation, it has not been proven that such infections would be possible in an *in vivo* system. The exception is the hepatitis E virus, but it can be prevented by keeping the appropriate conditions for animals and regular veterinary testing (Onions *et al.* 2000).

Summary

The modern transplantology problem of insufficient quantity of tissues and organs for transplantation is increasingly alarming. Statistics show that more and more patients are waiting for organs. The solution to this urgent problem could be xenotransplantation (Saxena *et al.* 2016).

After many years of comprehensive research on non-human primates-to-human xenotransplantation, the attention has been placed on another animal – the domestic pig. The change in concept was dictated primarily by the very high risk of transmitting viruses from primates. Viruses from non-human primates that threaten humans includes human immunodeficiency virus (HIV) or Marburg virus. These are not the only viruses that could be a problem in xenotransplantation. There is a high likelihood of infection with viruses that have not yet been reported in humans and appears in non-human primates. This threat is due to the high phylogenetic proximity of humans and primates. Moreover, there are no effective methods to preventing these infections or their effective treatment.

Hence the change in the direction of xenotransplantation and the interest in the domestic pig. The great advantage of this animal is the functionality and size of its organs, which is similar to human organs. However, the topic of threat to zoonoses also exists in this case. Although viruses that may be a problem in pig-to-human xenotransplantation have been also described, but in most cases this is a hypothetical threat that has

not occurred *in vivo*. The viruses described in the context of pig-to-human xenotransplantation includes porcine endogenous retrovirus – PERV, porcine cytomegalovirus – PCMV, porcine lymphotropic herpesvirus – PLHV and hepatitis E virus (E-HEV). However, there are effective methods for eliminating all of the above viruses and others can be excluded by genetic engineering or maintaining appropriate conditions for animals and regular veterinary examinations.

Acknowledgements

Presented work was financially supported by The National Centre for Research and Development (grant number INNOMED/I/17/NCBR/2014) from the Innovative Economy Operational Programme founds, in the framework of the European Regional Development Fund.

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Streszczenie

Istnieje wiele chorób człowieka, które mogą doprowadzić do niewydolności narządów. Konsekwencją często jest konieczność przeprowadzenia przeszczepu. Liczba wykonywanych operacji jest bardzo niska ze względu na niedobór narządów do transplantacji. W konsekwencji liczba osób oczekujących na przeszczep ciągle rośnie. Wyjściem z tej sytuacji może być ksenotransplantacja. Pojęcie ksenotransplantacja pochodzi od greckiego xenos oznaczającego obcy, inny. Jest to każdy zabieg polegający na transplantacji, implantacji lub infuzji biorcy – człowiekowi – komórek, tkanek lub organów odzwierzęcych, również płynów ustrojowych, komórek, tkanek, narządów człowieka (lub ich fragmentów), które miały kontakt ex vivo z komórkami, tkankami lub organami zwierzęcymi. Jedną z przeszkód w przeszczepach ksenogenicznych jest zagrożenie przeniesienia patogenów zwierzęcych i zainfekowanie organizmu człowieka. Wirusami, które stanowią zagrożenie w przeszczepach, w układzie małpy naczelne-człowiek to między innymi: ludzki wirus upośledzenia odporności HIV (z ang. human immunodeficiency virus) i wirus Marburg, które zostały opisane w niniejszej pracy. Ponadto przedstawiono wirusy stanowiące problem w transplantacjach w układzie świnia-człowiek, czyli: endogennego retrowirusa PERV (z ang. porcine endogenous retrovirus), wirusa cytomegalii świni PCMV (z ang. porcine cytomegalovirus), wirusa limfotroficznego świni PLHV (z ang. porcine lymphotropic herpesvirus) oraz wirusa

zapalenia wątroby typu E - HEV (z ang. hepatitis E virus). Niniejszy przegląd literatury stanowi najnowszy stan wiedzy na temat mikrobiologicznego bezpieczeństwa ksenotransplantacji.