CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy Hradec Králové

Department of Pharmaceutical Chemistry and Drug Control

DIPLOMA THESIS

2014 Kateřina Babková

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Katedra farmaceutické chemie a kontroly léčiv

UNIVERSITY OF EASTERN FINLAND

Faculty of Health Sciences

School of Pharmacy

Department of Pharmaceutical Chemistry

Microorganism based polyamines

Detection of prostate cancer

DIPLOMOVÁ PRÁCE

Školitel: Doc. RNDr. Veronika Opletalová, Ph.D.

Školitel specialista: Janne Weisell, Ph.D.

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DECLARATION

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ABSTRAKT

Univerzita Karlova v Praze
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Název diplomové práce: Polyaminy organismů. Detekce rakoviny prostaty

Rakovina prostaty je nejčastější nádorové onemocnění u mužů, hned po rakovině plic je druhým nejfrekventovanějším typem rakoviny, který je příčinou úmrtí u mužů. Každým rokem stoupá počet nově diagnostikovaných případů. Prevencí tohoto onemocnění je zdravý životní styl a nesmíme ani opomenout a podcenit pravidelné lékařské prohlídky, které by pomohly odhalit nemoc ještě v prvopočátcích, protože prevence a včasná diagnostika mohou zlepšit šance na uzdravení.

Rakovinu prostaty je možné detekovat mimo jiného i ze vzorku moči. Znakem toho, že je něco v nepořádku, je zvýšená hladina polyaminů. Polyaminy jsou produktem proteinového metabolismu a jsou přítomné ve všech savčích buňkách. Jejich enzymatické pochody nejsou zatím prozkoumány do posledního detailu, ovšem ví se, že pokud se v jejich metabolismu vyskytne problém (např. nadměrná produkce enzymů, zvýšené hladiny metabolitů), nevěstí to nic dobrého.

Cílem mé práce byla syntéza umělých a přírodních analogů polyaminů, které jsou produkovány mikroorganismy způsobujícími infekce močových cest u pacientů s rakovinou prostaty. Produkty jejich metabolismu mohou tak dávat falešně pozitivní výsledky při stanovení obsahu polyaminů z moči. Do budoucna by se mnou připravené látky mohly používat jako standardy při čichovém (eNose) testu moči.

Kromě produktů mikrobiálního metabolismu byla připravena i jejich deuterovaná analoga, protože při měření pomocí v sérii zapojené LC s tandemovým MS/MS slouží jako vnitřní standard a snižují tak chyby a nepřesnosti měření.

ABSTRACT

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Title of diploma thesis: Microorganism based polyamines. Detection of prostate cancer

Prostate cancer is the most prevalent type of solid malignant tumour among men and the second highest cause of cancer related mortality of men after lung cancer. Every year the number of new cases is growing. To prevent this disease it is important to think about a healthy lifestyle and not to underestimate regular health checks because prevention and early diagnosis can bring better therapy results.
Among others, prostate cancer can be diagnosed from urine samples. Increased levels of polyamines are the sign that something is wrong. Polyamines are ubiquitous in living mammalian cell, their metabolic pathways are still not clarified in depth but if a problem appears in their metabolism (for example enzyme overproducing, higher levels of any polyamine metabolite) it won´t mean anything good.
The aim of my work was to synthesize natural polyamines and their analogues which are produced by microorganism causing urinal infections of patients. Their metabolic end product can affect the urine results (false positive). Another use of my prepared compounds can be as future standards for olfactory measuring of urine samples.
Beyond preparing microorganism´s metabolism end products, I also synthesized their deuterated analogues because they are used as internal standards for liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) to minimalize mistakes and inaccuracies.

CONTENT

1. ABBREVIATIONS

AcPAO	Acetyl polyamine oxidase
Az	Antizyme
AzI	Antizyme inhibitor
Bn	Benzyl
Boc	*Tert*-butyl carbamate
BPH	Benign prostatic hyperplasia
Cbz	Benzyloxycarbonyl
CT	Computed tomography
DCM	Dichloromethane
DFMO	Difluoromethylornithine
DRE	Digital rectal examination
EtOAc	Ethyl acetate
EtOH	Ethanol
IGF-I	Insulin growth factor I
LAD	Lithium Aluminium Deuteride
LAH	Lithium Aluminium Hydride
LC-ESI-MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
$MgSO_4$	Magnesium sulfate
MeOH	Methanol
MRI	Magnetic resonance imaging
ODC	Ornithine decarboxylase
PAs	Polyamines
PCa	Prostate cancer
PHI	Prostate Health Index
PSA	Prostate-specific antigen
Pu	Putrescine
RT	Room temperature
SAMDC	S-Adenosylmethionine decarboxylase
SCFAs	Short chain fatty acids

SMO	Spermine oxidase
SPD	Spermidine
SPDS	Spermidine synthase
SPM	Spermine
SPMS	Spermine synthase
SSAT	Spermidine/spermine acetyltransferase
TFA	Trifluoroacetic acid
THF	Tetrahydrofurane
TMS	Tetramethylsilane
TPS	3-(Trimethysilyl)-1-propanesulfonate
TRUS	Transrectal ultrasonography
VOCs	Volatile organic compounds

2. AIM OF THE WORK

From various synthetic strategies I am going to use the Michael addition to build up C-N bonds. (Hyvönen, et al., 2011) I am going to make amines react with acrylonitrile to get PAs with end nitrile groups. After the protection of amine groups and the subsequent reduction of the intermediate diaminonitriles with LAH or LAD, deprotection step follows, which results in target compounds.

I am also going to try to synthesize other interesting substances which can occur in microorganism metabolism. These will be exploited as standards for electronic nose (eNose) measurements.

One of my aims will be to find out the best conditions of reactions – such as the best amount and ratio of chosen reagents, temperature, reacting time, optimal protecting and deprotective groups and also the way to purify the end products.

3. INTRODUCTION

3.1 PROSTATE CANCER

Prostate is men´s sexual gland producing liquid which protects and enriches sperm. It is situated below the bladder and it is surrounding the beginning of urethra. There is age-related benign prostatic hyperplasia (BPH), another prostatic disease is inflammation, called prostatitis, but the most serious of all of them is for sure prostate cancer. (PCa) (Kaplan & Pontari, 2014)

PCa is the most common solid neoplasm; elderly people suffer from PCa more often than young men. (Heidenreich, et al., 2012) Prostate cancer is the second most common non-skin cancer among men in most western populations. Although the PCa morbidity is high, the ethiology remains unclear. (Hsing & Chokkalingam, 2006)

3.1.1 EPIDEMIOLOGY AND INCIDENCE

Prostate cancer is the second most prevalent diagnosed cancer and also the second most common cause of death from cancer in men in the Czech Republic. (Damber & Aus, 2008) (WHO, 2014) (Zdravotnická statistika, 2013) PCa is recognized as one of the major medical problems facing the male population, with an incidence rate of 214 cases per 1000 men. (Heidenreich, et al., 2012) Each year, according to statistics (Uroweb, 2014), an estimated 2.6 million new cases of PCa are diagnosed in Europe. (Heidenreich, et al., 2008)

The number of new diagnosed cases is growing, this is also due to better diagnostic devices. In history, the biggest rise was in the late 1970s and early 1980s, then between 1986 and 1992, after increased use of prostate-specific antigen (PSA), and the last slow rise came after the 1990s. (Weir, et al., 2003) Anyway, the number of new diagnosed cases is really alarming.

The incidence differs widely between countries and ethnic population. The highest incidence of PCa is found in North America and Scandinavia and the lowest in Asia, especially in China. And concerning ethnic groups, the highest number of diagnosed cases was observed for African-Americans. (Grönberg, 2003)

3.1.2 STAGING AND RISK STRATIFICATION

The most often used system for staging and risk stratification is Gleason score. In the system, the values achieve a score between 2 and 10. Number 2 means the least aggressive and 10 the most aggressive form. “This score is a sum of two most common patterns (grades 1-5) of tumour growth found. To be counted, a pattern (grade) needs to occupy more than 5% of the biopsy specimen.” (Heidenreich, et al., 2008)

Table 2 Staging and risk stratification of prostate cancer

Adapted from (Kirby & Madhavan, 2010)

	PSA	**Gleason score**
Low risk	<10 ng/ml	≤6
Intermediate risk	10-20 ng/ml	7
High risk	>20 ng/ml	8-10

3.1.3 RISK FACTORS AND PREVENTION

First of all, PCa is an age-related disease, over 80% of diagnosed persons are older than 65 years. The risk increases with age and it´s a strong predetermining factor because the latency of PCa is long - the incidence increases over three fold. (Hsing & Chokkalingam, 2006)

If a first-degree relative is affected with PCa, the risk is two-fold to three-fold higher for developing this disease. (Kirby & Madhavan, 2010) If the number of affected family members increases, the risk of developing prostate cancer by individuals is also much higher. (Grönberg, 2003) The genes most plausibly linked to familial prostate cancer are *ELAC2/HPC2* (gene encoding tRNA 30 processing endoribonuclease*), RNASEL* (gene encoding $2^{'}, 5^{'}$-oligoadenylate(2–5 A)-dependent RNase L), *MSR1* (gene for macrophage scavenger receptors), *CHEK2* (an important regulator of p53 in the DNA-damage-signaling pathway), vitamin D receptor, and *PON1* (expression of the main human paraoxonase). (Dong, 2006)

After observations the highest rate of PCa is reported for African Americans while the lowest rate is reported for Chinese men. (Hsing & Chokkalingam, 2006) This can be caused by genetic predisposition – a genetic variant has been identified on chromosome 8q24 which often occurs in African Americans (Amundadottir, et al., 2006); different quality of health care and also by external risk factors which are connected with life style. (Grönberg, 2003)

Because testosterone and its main metabolite – 5-dihydrotestosterone regulate growth and function of prostate gland, correlations between development of PCa and high testosterone levels were studied, but the results are still inconclusive. (Hsing & Chokkalingam, 2006)

Two big independent cohort studies have found out that men in the highest quartile with increased insulin growth factor I (IGF-I) concentrations had an increased risk of PCa compared with people in the lowest quartile. (Hartman, et al., 2000) (Stattin, et al., 2000)

Hereditary factors play the most important role in determining the risk; exogenous factors may also affect and increase the risk of developing clinical PCa. (Heidenreich, et al., 2008) There is not enough evidence to recommend life style changes. But it is suspected that risk factors as alcohol consumption, sexual behaviour (risk of an infection) and chronic inflammation and lack of prevention in dietary habits, occupation and physical activity, may play a significant role in origin of PCa. (Hsing & Chokkalingam, 2006)

According to Kolonel (Kolonel, 2001) there is a link between the intake of red meat and fats; and higher incidence of PCa. Unfortunately, it is not clear if other factors might have an impact on this risk. Calcium is a regulator of vitamin D (down-regulation of vitamin D´s antiproliferative effects), which means if the intake of calcium is enormously high, this can have a negative influence on PCa development. (Chan & Giovannucci, 2001)

Lycopene contained in tomatoes is seen as a future chemoprotective compound and higher consumption showed a positive effect on prostate-specific antigen (PSA) levels which decreased. (Chen, et al., 2001) Higher intake of lycopene is as promising as the intake of selenium and vitamin E for their antitumorigenic and antioxidant effects, induction of apoptosis, enhancement of the immune system, and an effect on the production of testosterone. (Grönberg, 2003) (Clark, et al., 1998) Other risk factors are uncertain but their possible influence is studied.

3.2 SCREENING AND DIAGNOSIS

PCa belongs to adenocarcinomas and there are often no symptoms presented which means that the diagnosis is reliant on investigations – the most used are digital rectal examination (DRE), transrectal ultrasonography (TRUS), biopsy and serum concentration of PSA. Other

helpful tools are magnetic resonance imaging (MRI), computed tomography (CT) or whole-body bone scintigraphy. (Kirby & Madhavan, 2010)

3.2.1 CURRENT DIAGNOSTIC TOOLS

Digital rectal examination (DRE) is one of the options how to find out if there is something wrong with prostate from the functional point of view. It is an essential part of urological examination; because most PCa are in the peripheral zone of prostate, it´s possible to detect them using DRE. (Heidenreich, et al., 2012) With this examination the size, induration and the presence of nodules can be detected. (Kirby & Madhavan, 2010)

TRUS is also exploited but the reliability is not so high, it cannot detect all PCa areas properly. To be really sure biopsy is used but we try to avoid this invasive method. PSA needs to be assessed and a DRE has to be carried out first, then it comes to ultrasound guided biopsy. (Heidenreich, et al., 2012) But to be honest none of these methods is 100% reliable. For example, we might not have a representative sample for biopsy measurement (even when 6-12 cores are removed by needle). Other problems that are connected with biopsy: heterogeneity and multifocal nature of cancer foci within the prostate. (McDunn, et al., 2013) The overall cancer detection rate in the clinical trial cohort was 54.4%, it means more than 50% of prostate tumours might be missed exploiting the traditional biopsy. (Pinto, et al., 2011)

Nowadays prostate specific antigen (PSA) from serum is tested. PSA is a kallikrein-like serine protease produced almost exclusively by the epithelial cells of the prostate. After beginning to use this screening method the number of new diagnosed patients grew, it caused a kind of revolution in PCa diagnostics. This disease could be detected at early stages and the mortality rate was decreased. (Kirby & Madhavan, 2010) Unfortunately, the specificity of PSA test is relatively low because this method is organ-specific, not cancer-specific. So if the patient has prostatitis, benign prostatic hypertrophy or other non-malignant conditions, PSA levels will be high as well. (Heidenreich, et al., 2012) Measurement of serum prostate-specific antigen (PSA) for the detection of prostate cancer has poor specificity in men with PSA levels between 2 and 10 ng/ml. It was found out that there is more than one isoform of this antigen. We need to figure out which of them is the most specific one for PCa. In serum there is “free” PSA that is unbound to protease inhibitors (~30%) and “complexed” PSA that is bound to α_1-antichymotrypsin (~70%). In studies (Filella & Giménez, 2013), scientists have suggested that

in men with a total PSA (tPSA) level between 2 and 10 ng/ml; measurement of the free to total PSA (f/tPSA) ratio or complexed PSA (cPSA) can better distinguish between malignant and benign prostate disease than tPSA alone. And Roddam´s review (Roddam, et al., 2005) confirmed the thought. This could be one way how to improve the results of PSA test; another possibility would be exploiting the so-called Prostate Health Index (phi). Even free PSA has various isoforms which are precursor forms of fPSA and the most specific for PCa of all of them is [-2]pro PSA. (http://www.prostatehealthindex.org/laboratory-what.asp, 2014)

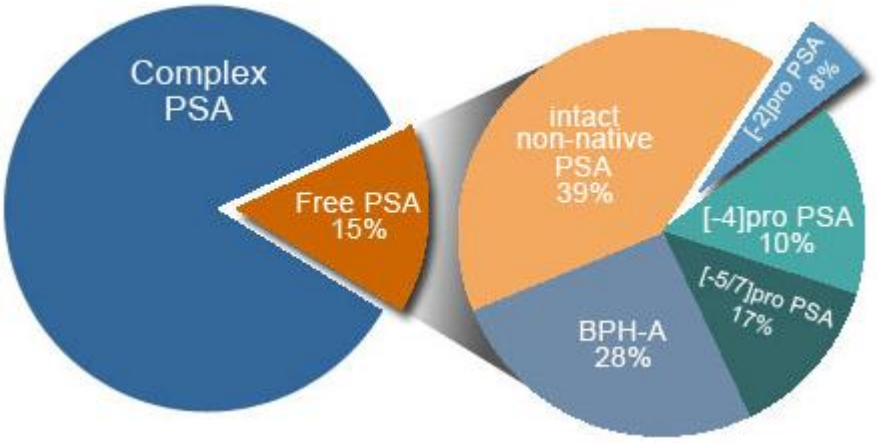

Figure 1 Molecular forms of PSA in serum

Using phi the results of studies demonstrated a significant improvement in clinical specificity for prostate cancer detection when p2PSA measurements are combined with free PSA measurements.

Adapted from (http://www.prostatehealthindex.org/laboratory-what.asp, 2014)

3.2.2 OLFACTORY DETECTION OF PROSTATE CANCER

Dogs are able to detect tissues containing cancer, first evidence of "sniffer dogs" was published in (Williams & Pembroke, 1989). After that another case happened: when a pet Labrador began to sniff a lesion of his 66-year-old owner. (Church & Williams, 2001) Appropriately trained dogs showed the ability to detect prostate, breast, ovary and lung cancer as well as melanoma due to recognizing a characteristic "odour signature" in body fluids and also in breath. Different types of cancer produce diverse compounds that are released, in case of PCa especially, in urine. We talk about volatile organic compounds (VOCs), such as alkanes, methylated alkanes, aromatic compounds or benzene derivatives. These substances

are selectively expressed or overexpressed by cancerous cells and nose epithelium (olfactory sense) detects them. (Lippi & Cervellin, 2011)

VOCs in urine have been proposed as cancer biomarkers and double-blind study with a trained Belgian Malinois shepherd showed that the dog correctly recognized the human cancer urine samples with 91% sensitivity. (Cornu, et al., 2011)

Unfortunately, this method has a lot of problems – the result depends on chosen training methodology, ability of the individual animal, other patient´s comorbidities, odour signature below certain limits and cost-effectiveness. On the other hand, these preliminary investigations on "sniffer dogs" may have consequences for the clinics and the laboratory. The animals sniffing might help identify suitable biomarkers and then be replaced with an electronic nose (eNose) based on matrix-assisted laser desorption/ionization (MALDI) and enhanced laser desorption and ionization (SELDI), time-of-flight mass spectrometry (TOF-MS) analysis or highly sensitive and fast-response array of sensors based on gold nanoparticles. (Lippi & Cervellin, 2011)

3.2.3 ELECTRONIC NOSE

An electronic nose (eNose) is an artificial gas sensing system which uses an array of sensors specific to different VOCs. The sensor array includes metal oxide semiconductors, optical and amperometric gas sensor as well as surface acoustic and piezoelectric sensors. (Zohora , et al., 2013)

An assumption is that there is a profile of volatile components that are released among others into the urine. These VOCs have different odours and they are associated with alterations in HLA expression (major histocompatibility complex, MHC, molecules, referred to in humans as HLA). And changes in HLA molecules are typical in cancer cells. (Balseiro & Correia, 2006) This all together gives us the conclusion that VOCs in urine are possible as cancer biomarkers.

A Finnish study (Roine, et al., 2012) wanted to figure out if it´s possible to differentiate the non-malignant from the malignant prostate cell lines. Studies have shown that benign and malignant prostate cell lines have diverse smell print (partly this is caused by oxidative stress). Utilizing a ChemPro® eNose, the samples were analysed with misclassification rates of 2.9-3.6%.

Advantages of eNose are sensitivity to a wider spectrum of VOCs, speed, cost and the fact that manipulation with this device is really easy. Unfortunately, eNose is not able to quantify accurately measured molecules.

Since PCa is the second most frequent cancer in men and because the performance of the most frequently used biomarker PSA is not optimal, new biomarkers and tools for PCa diagnostics are urgently needed. More research is needed to discover whether these findings can be used in clinical diagnostics.

3.3 MICROORGANISMS AND METABOLOMIC SIGNATURES OF PROSTATE CANCER

3.3.1 INTERACTIONS BETWEEN HOST AND ITS COMMENSAL MICROBES

Gut microbiota lives in symbiosis with the host and it is involved in the regulation of mammalian metabolic pathways. Gut microbiota is affected by the host´s genotype, diet, age, sex, and health status. (See **Figure 2**) This system is an easily adaptable, metabolically renewable, flexible ecosystem. (Li, et al., 2008) Changes in gut microbiota composition might be important in the progression of diseases; the influence seems to be bigger than suspected. (Nicholson, et al., 2005)

We talk about combinatorial metabolism, under this name we understand that compounds are subsequently metabolized by the host, and by the microbes presented in the host. Due to this combinatorial metabolism, there is an increased number of metabolites and metabolic moieties. (Nicholson, et al., 2005)

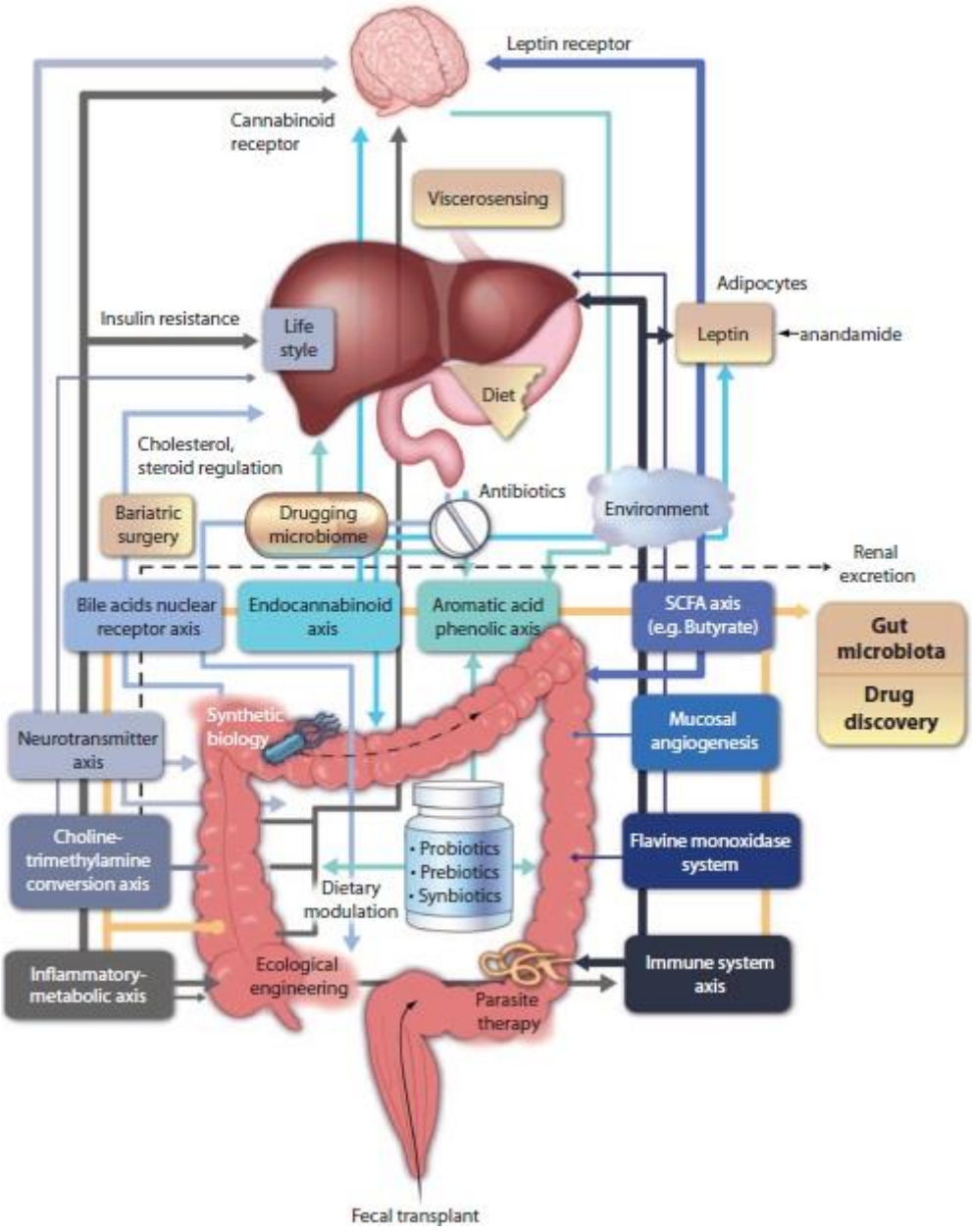

Figure 2

Microorganism activity influences and modulates human host metabolic reactions. Interactions between host and its commensal microbes and between microorganisms themselves lead to production of metabolites, which are essential for host health, such as short chain fatty acids (SCFAs), bile acids, and choline. (Adapted from (Holmes, et al., 2012))

3.3.2 MICROBES METABOLITES IN URINE AND THEIR VALIDATION

The end products of microbial metabolism together with human-microbial metabolites are excreted into the feces or/and into urine. These together with small molecules from human

host (body liquids and tissues) can be measured and the results exemplify the metabolic status of human body. Urine and other biofluids give us useful information about metabolites of microbial origin. (Xie, et al., 2013) In a Spanish study (Monleon, et al., 2009), fecal extracts from patients with colon cancer were compared to healthy specimens that showed that the gut microbes and the detected metabolites were significantly altered in subjects with colorectal cancer.

From analytical techniques we expect that they would be robust for various structures, comprehensive and we will get the results in a reasonable time frame. The analytical methodologies, including high-resolution NMR spectroscopy, gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography-mass spectrometry (HPLC-MS) and LC-NMR-MS, are used to recognize the spectrum of contained substances. (Nicholson, et al., 2005)

Analytical methodologies target to validate the specific metabolite or class of compounds using just one measurement that is often not feasible for systems as biofluids, or biological compartment. (Moco, et al., 2012) This is just a wish because no single analytical method can determine the entire metabolic composition. The metabolites are often determined using LC-MS and GC-MS in combination. (Xie, et al., 2013) For urine samples, NMR spectroscopy is often used; it does not require any sample pretreatment but it is less sensitive. (Martin, et al., 2007), (Li, et al., 2008), (Nicholson, et al., 2005)

From results of a Finnish study (Roine, et al., 2012) mentioned above we know that it´s possible to recognize which metabolites released in urine are from prostate cancer cell lines and which are from healthy ones. We can amplify this very useful information for future research concentrating on the distinction whether the urine sample really contained metabolites linked to PCa or if it is a metabolic product of bacterial infection which could mystify us. These findings could refine eNose measurements by the distinction between positive and false positive tests.

4. BACKGROUND

4.1 CHARACTERISATION OF POLYAMINES

Polyamines (PAs) are organic polycations, compounds that are present in every living cell and are essential for their growth. The most common PAs are putrescine (Pu), spermidine (SPD) and spermine (SPM) (See **Figure 3**). These three examples are typical for mammalian tissues but a wider spectrum of distinct PAs exists in other organisms. (Pegg & Casero Jr., 2011) Their primary and secondary amino groups are protonated at physiological pH that imparts electrostatic interactions with negatively charged compounds (like DNA, RNA, phospholipids, proteins). (Park & Igarashi, 2013) PAs interact differently from inorganic positively charged divalent cations because of their defined distances, distribution of space. PAs dispose of hydrophobic interactions (flexible methylene chains). Those interactions are stronger and more specific.

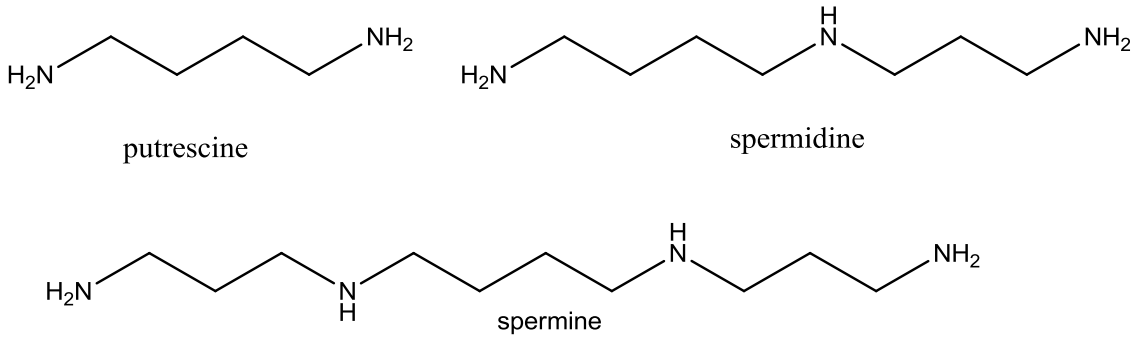

Figure 3 Structures of naturally occurring polyamines in mammalian cells

In normal cells the levels of PAs are really intricately controlled by biosynthetic and catabolic enzymes (Thomas & Thomas, 2003), by the cell membrane transport system and also by antizyme (a small protein compound that forms a complex with enzyme). Dysregulation in the control of the polyamine metabolism and uptake results to increased levels of PAs and of enzymes which is often associated with tumour growth. Accumulation of PAs is connected with transformation or apoptosis. That is why it is so important to observe the PAs levels which we can investigate among others from plasma or urine content. Those are very useful tools in the early diagnosis of cancer and stroke. (Igarashi & Kashiwagi, 2011)

4.1.1 BACTERIA CLASSES

(Hamana, et al., 2006) analysed the cellular content of PAs in order to create a synoptic classification of bacteria classes and subclasses. The bacteria were grown in the media and the concentration of PAs was determined by high-performance liquid chromatography (HPLC). Results from this study showed that for some bacteria classes the occurrence of PAs was specific which was used to reorganization of bacterial taxonomic system.

For example SPD was the major PA in the classes Deltaproteobacteria and Epsilonproteobacteria. On contrary, in some thermophiles (within Beta-/, Delta-/ and Epsilonproteobacteria) SPM was found in high concentration.

The intracellular content of SPD is higher than the presence of Pu, in average about 10 fold, in almost all bacteria. The cellular levels of SPM are closely dependent on exogenous presence of SPM in the medium. (Shah & Swiatlo, 2008)

4.2 THE ROLE OF POLYAMINE IN CELLS

Polyamines are essential for almost all prokaryotic and eukaryotic cells. From this postulate it is clear that they have to play a very important role – especially in proliferation. First they affect the DNA structure; micromolar concentration of PAs can provoke the transition of right-handed DNA (B-DNA) to left-handed DNA (Z-DNA). (Thomas & Messner, 1988) This facet influences the regulation of gene expression by unravelling DNA-protein interactions and chromatin structure. (Igarashi & Kashiwagi, 2010)

Recent studies have shown that PAs are responsible for ligand receptor-interactions. PAs control the activity of ion channels. They are able to inhibit or potentiate for example the function of the inwardly rectifying potassium channels or the activity of NMDA-receptors by glutamate response. (Pegg & Casero Jr., 2011) (Yamakura & Shimoji, 1999)

Another role of PAs is stimulation of the assembly of 30S ribosome, tRNA formation, frame shifting. More than 57% of polyamines are bound to RNA. A very important role that PAs play is the translation, they have multiple effects on posttranscriptional regulation, they may figure in regulation of gene expression and they can modulate protein kinase activities

(Flamigni, et al., 1999) , the cell cycle and membrane structure as well. (Igarashi & Kashiwagi, 2010), (Pegg & Casero Jr., 2011)

4.3 REGULATION OF POLYAMINE LEVELS IN MAMMALIAN CELLS

4.3.1 BIOSYNTHETIC PATHWAYS

Polyamines are natural products of amino acids reactions. Biosynthesis begins from arginine which is converted by urease to ornithine. (See **Figure 4**) The next reaction is decisive for the speed of the polyamine biosynthesis (Kuksa, et al., 2000) - ornithine is decarboxylated by ornithine decarboxylase (ODC) and putrescine is formed. ODC is known as a marker of tumour progression (O'Brien, et al., 1975).

S-adenosylmethionine decarboxylase (SAMDC) helps to form decarboxylated S-adenosylmethionine that gives the aminopropyl group the possibility to catalyse reactions by distinct aminopropyltransferases termed spermidine synthase (SPDS) and spermine synthase (SPMS), respectively. Due to these reactions (with appropriate enzymes), first spermidine (SPD) and later spermine (SPM) are built.

4.3.2 POLYAMINE CATABOLISM

It is necessary to regulate the increased levels of PAs with catabolic enzymes (see **Figure 4**) which allow declining of compounds that exist in high quantities. SPM can be converted back to SPD by spermine oxidase (SMO), another option which is the same as for SPD degradation is spermine/spermidine acetyltransferase (SSAT). Acetylated intermediates are decomposed by acetyl polyamine oxidase (AcPAO). These acetylated compounds have really big importance because they can be excreted from cells and we can detect them in animal body fluids, urine and plasma (Park & Igarashi, 2013).

When SPM and SPD are degraded, two highly toxic compounds can be formed acrolein and hydrogen peroxide. Acrolein is actually more toxic (Park & Igarashi, 2013) and the levels of protein-conjugated acrolein are used to recognize the seriousness of brain stroke (Tomitori, et al., 2005) and renal failure (Igarashi, et al., 2006)

4.3.3 ANTIZYME AND ANTIZYME-INHIBITOR

If ODC is more active, there is another way how to stop the enzyme – with antizyme (Az). The binding of ODC with Az facilitates the very fast degradation of ODC in 26S proteasome. (Persson, 2013). Another advantage of Az is the suppression of the PA transport system. The failure to up-regulate Az leads to the growth of prostate carcinoma cells. It means that if Az exists in high concentrations prostate cancer cells can´t grow so rapidly. (Koike, et al., 1999) But of course, even antizyme has to be regulated by its own inhibitor (AzI) that is more tightly bound to Az than Az to ODC. ODC could then be easily displaced from formed complex Az-ODC.

The aim of the metabolic circle is just to keep the balance and to have PAs levels in reasonable limits.

4.3.4 POLYAMINE TRANSPORT

The last possibility how to regulate levels of PAs in a cell is the cell membrane system. Today it´s known that the system is saturable and energy-dependent. Studies focusing on difluoromethylornithine (DFMO), an irreversible inactivator of ODC, demonstrated that the decrease of PAs caused by DFMO induces increased uptake of PAs despite to Az. (Thomas & Thomas, 2003)

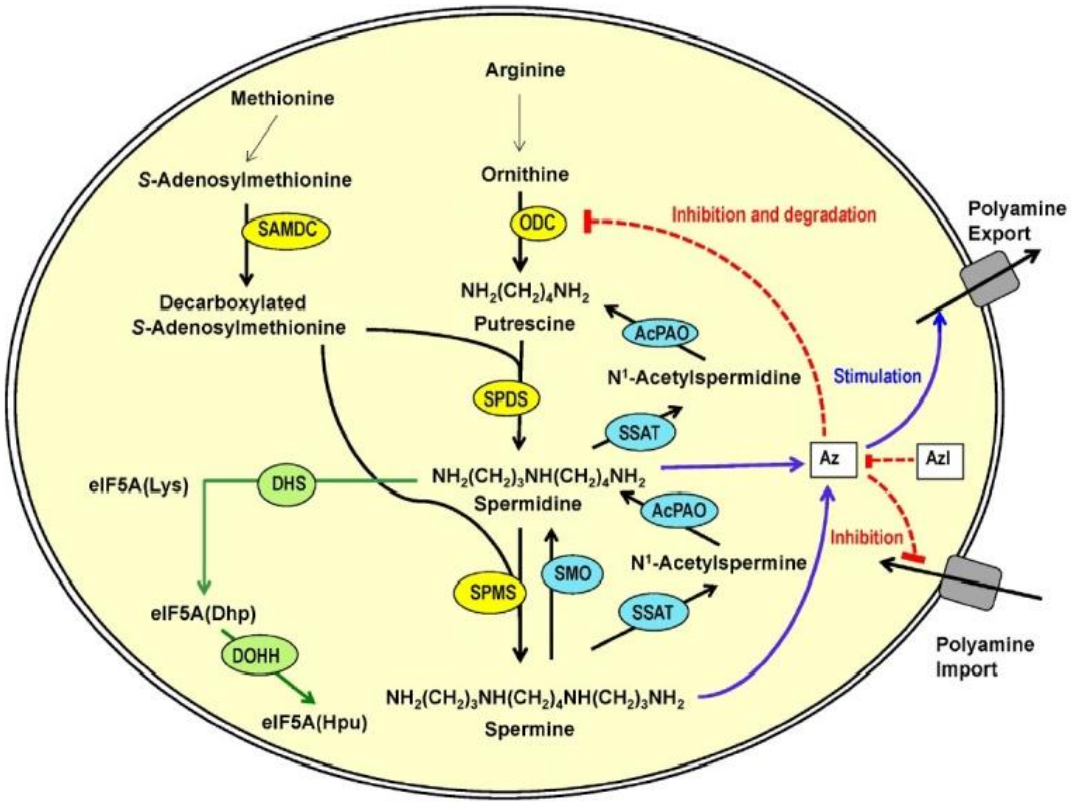

Figure 4

The biosynthetic enzymes are ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), spermidine synthase (SPDS) and spermine synthase (SPMS). Spermine monooxidase (SMO), spermine/spermidine N1-acetyltransferase (SSAT) and acetyl polyamine oxidase (AcPAO) belong to catabolic enzymes. The levels of ODC are influenced by antizyme (Az) and its inhibitor (AzI), respectively. Adapted from (Park & Igarashi, 2013)

4.4 POLYAMINES AND THEIR ANALOGUES

Polyamine analogues differ from their natural templates by altering substituents, by the lengths of the methylene-chain or by alkylating terminal amino groups. (Thomas & Thomas, 2003) These changes in structure evoke differences in their chemical and biological behaviour.

PAs analogues are investigated because of their promising potential as chemopreventive and antiparasitic agents, they may also figure as carriers for drug delivery, as metal chelators and as NMDA receptors modulators. (Häkkinen, et al., 2009)

If PAs analogues use the same transport system as PAs, it is possible to observe where they are distributed in cells and also to see what their metabolism looks like. After that, we can make conclusions to natural PAs which can help us to understand and subsequently to regulate their uptake to get information about the mechanism of action. We can also study the binding of PAs to receptors and ion channels. Some of the synthetic analogues can act as precursors, substrates, delivery vehicles for drugs or they might have antiproliferative effects. (Pegg & Casero Jr., 2011)

A really promising situation could arise if the analogues delivered the cytotoxic drug directly into tumour tissue. (Hyvönen, et al., 2011) We might decrease side effects of the drug by hitting just the target tissue. One big premise is that the polyamine-drug conjugates have to be metabolically stable.

4.4.1 DEUTERATED POLYAMINES

Polyamines with deuterium have the advantage that we can compare their results with results of non-deuterated compounds to get really reliable outcomes to confirm hypothesis

concerning these substances. Isotopically labelled analogues are used as internal standards to correct variations in sample preparation and to offset variability in MS detection. Deuterated compounds are the most appropriate internal standards in quantitative LC-MS/MS assays. Deuterium was chosen for its price and also because it is the most widely accessible stable isotope. (Häkkinen, et al., 2009)

5. MATERIALS AND METHODS

Materials and methods have been included into Attachment due to confidential reasons.

6. RESULTS AND DISCUSSION

Results and discussion have been included into Attachment due to confidential reasons.

7. CONCLUSION

The aim of the work was to prepare series of compounds with end amino groups with their deuterated analogues which are going to be used as internal standards in liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) measurements. Other compounds (products of microorganism metabolism) can be used as standards for e-Nose measurements. The best reaction conditions were identified. Future research will focus on the determination and improving of reaction conditions for 6-hydroxyspermidine synthesis. The information from this work brings the knowledge where to continue in future research concerning on diagnosis of PCa using e-Nose instead of invasive and non-specific diagnostic methods.

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