CHARLES UNIVERSITY IN PRAGUE
FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY

Metabolism of carcinogenic *o*-nitroanisole, its metabolite *o*-nitrophenol and environmental pollutants 2-nitrobenzanthrone and 3-nitrobenzanthrone

Summary of PhD Thesis

RNDr. Martina Svobodová

Supervisor: Prof. RNDr. Marie Stiborová, DrSc.

Prague 2010

INTRODUCTION

2-Nitroanisole

2-Nitroanisole (2-methoxynitrobenzene, 2-NA, *figure 1*) is an important industrial pollutant and a strong carcinogen for rodents causing neoplastic transformation in the urinary bladder and, to a lesser extent, in the spleen, liver and kidney [19, 30, 31]. 2-NA is also a toxic compound, causing anemia. 2-NA is used primarily as a precursor in the synthesis of *o*-anisidine (2-methoxyaniline), which is an intermediate in the production of many azo dyes. This compound is used in pharmaceutical industry as an intermediate in the synthesis of some medicaments [30, 31].

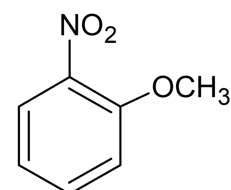

Figure 1
The structure of 2-nitroanisole

In spite of potent rodent carcinogenicity of 2-NA, this chemical is weakly mutagenic in the Ames test with the *Salmonella typhimurium*. This carcinogen also exhibits a low activity in cytogenetic tests. It induces a slight increase in chromosomal aberration and in sister chromatid exchanges, but only at high concentrations [31].

2-nitroanisole may be metabolized in two different pathways. The major route of 2-NA metabolism *in vivo* is oxidative demethylation to 2-nitrophenol, which appears in urine predominantly as a sulfate conjugate generated by the reaction with phosphoadenosinephosphosulfate catalyzed by sulfotransferase or a conjugate with glucuronic acid generated by the reaction with UDP-glucuronic acid [22]. Another metabolic pathway involves reduction to 2-methoxyaniline (*o*-anisidine). At blood concentrations at which the metabolism and elimination of 2-NA are linear, *o*-anisidine is a minor metabolite formed in liver.

This 2-NA nitroreduction, considered generally as an activation pathway for aromatic nitro compounds, was also clearly documented in the *in vivo* study [22]. Recently, it was shown that *N*-(2-methoxyphenyl)hydroxylamine is formed as a reduction metabolite of 2-NA after incubation with animal and human hepatic cytosol and buttermilk xanthine oxidase, and binds covalently to DNA *in vitro* [22]. Furthermore, it was demonstrated that 2-NA generates DNA adducts *in vivo* in rats treated with this chemical [37]. These results,

confirming 2-NA covalent binding to DNA after activation by human cytosolic reductases *in vitro* and in rats *in vivo*, indicate a genotoxic mechanism of 2-nitroanisole carcinogenicity.

3-Nitrobenzanthrone

3-Nitrobenzanthrone (3-NBA, 3-nitro-7H-benz[de]anthracen-7-on, *figure 2*) a polycyclic aromatic nitro compound, is a strong mutagen, carcinogen for rodent [16] and suspected carcinogen for human [20]. Its genotoxicity was demonstrated in many tests for mutagenicity [9, 16] and by the fact, it has a potential to form specific DNA adducts [2]. These adducts were detected *in vitro* in cell cultures and *in vivo* in rat and mouse [2, 3, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 27, 36].

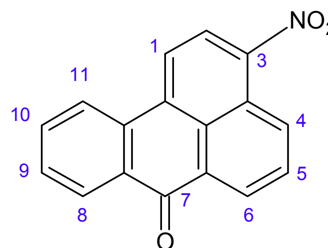

Figure 2

The structure of 3-nitrobenzanthrone

3-NBA occurs in natural elements, it was detected in soil and also in rainwater [24, 25, 40]. Its presence in exhaust fumes was demonstrated in 1997 [16]. 3-NBA was detected in exhaust fumes in range from 0.1 to 24 pmol/mg [16, 23], in the atmosphere from 1.4 to 249 fmol/m^3 [16, 17] and in soil in range from 4.3 to 4211 fmol/g [25, 40], which suggests that 3-NBA is mainly generated by imperfect combustion of diesel fuel. 3-NBA may also arise from reaction of parental polycyclic hydrocarbon with nitrogen oxides in the atmosphere (especially when ozone is present), however, to a lesser extent [16, 17]. Its genotoxicity may pose a high risk, especially to professional drivers, garage and gas stations personnel, auto mechanics and miners [18, 28, 29, 33].

Biotransformation of 3-NBA occurs namely in a reductive way [5, 7, 8, 35]. In the first biotransformation phase, the nitro group (-NO_2) of 3-NBA is firstly reduced to N-hydroxylamine (-NHOH). This reaction is catalyzed by cytosolic enzyme NAD(P)H:quinone oxidoreductase [7] and, to a lesser extent, by xanthinoxidase [1, 13] and microsomal NADPH:cytochrome P450 oxidoreductase (POR) [8]. The generated N-hydroxylamine is very unstable and splits into nitrenium ion, which either alone or after conversion to carbenium ion reacts with nucleophilic centers of DNA [2, 3]. In the second phase of biotransformation, N-hydroxylamine is conjugated with active sulfate or acetate,

which facilitates creation of nitrenium ion. N, O-acetyltransferases (NAT1 and NAT2) and sulfotransferases (SULT1A1 and SULT1A2) catalyze these reactions in human body [5, 7].

2-Nitrobenzanthrone

2-Nitrobenzanthrone (2-NBA, 2-nitro-7H-benz[de]anthracen-7-on, *figure 3*) is another polycyclic aromatic nitro compound, that occurs as a pollutant in air pollution. This isomer of 3-NBA was detected in polluted air in concentrations up to 70x higher than in the case of 3-NBA. 2-NBA is generated primarily by processes occurring in the atmosphere, while 3-NBA occurring especially in the exhaust fumes is formed from combustion processes [32, 39].

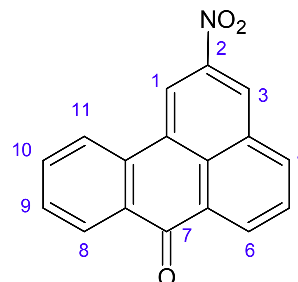

Figure 3

The structure of 2-nitrobenzanthrone

Genotoxicity of both isomers was tested in several *in vitro* and *in vivo* studies in rats. These experiments suggest that the 2-NBA is capable of producing DNA adducts as well [4, 26]. In comparison with 3-NBA, the ability of 2-NBA-DNA adducts formation is very low, therefore, the 2-NBA is regarded as slightly toxic compound [4, 38]. Despite this fact, high concentrations of 2-NBA in the air may pose substantial health risk to human population.

AIM OF THE STUDY

The aim of this study is to extend our knowledge on the metabolism of the carcinogenic aromatic nitro compounds, specifically industrial pollutant 2-nitroanisole and two environmental pollutants 2-nitrobenzanthrone and 3-nitrobenzanthrone.

The objectives of this work are as follows:

- To prepare and characterize 2,5-dihydroxynitrobenzene as a metabolite of 2-nitroanisole that is formed from this compound besides 2-nitrophenol and 2,6-dihydroxynitrobenzene
- To evaluate metabolism of 2-nitroanisole by cytochromes P450 in rat microsomes
- To investigate metabolism of 2-nitroanisole by rat and human cytochromes P450
- To examine 2-nitrophenol metabolism by rat and human cytochromes P450
- To study kinetics of 2-nitrophenol oxidation by cytochromes P450 2E1
- To elucidate metabolism and activation of environmental pollutants 2-nitrobenzanthrone and 3-nitrobenzanthrone

RESULTS AND DISCUSSION

The results shown in this study extend our knowledge on the metabolism of carcinogenic aromatic nitro compounds, especially industrial pollutant 2-nitroanisole and two environmental pollutants 2-nitrobenzanthrone and 3-nitrobenzanthrone endangering the human population. The main results of this work are summarized as follows:

2,5-Dihydroxynitrobenzene was identified as a metabolite of 2-nitroanisole that is formed from this compound besides 2-nitrophenol and 2,6-dihydroxynitrobenzene

On the basis of comparison of chromatographic properties of the unknown 2-NA metabolite and the synthetics standard and with the use of co-chromatography with incubation mixture this metabolite was characterized as 2,5-dihydroxynitrobenzene (*figure 4*).

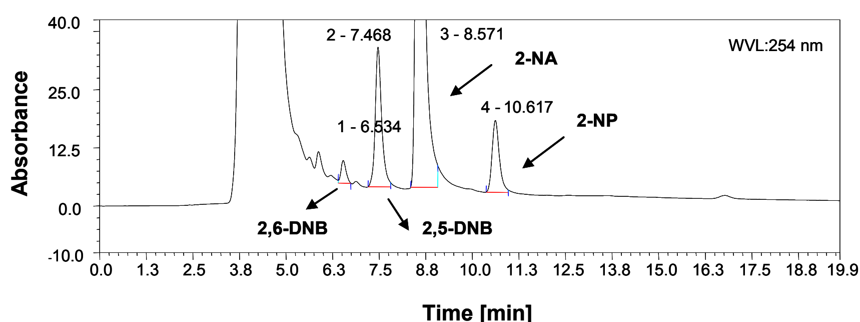

Figure 4 The separation of 2-NA metabolites formed by rabbit hepatic microsomes with added 2,5-DNB by means of HPLC.

Individual cytochromes P450 responsible for generation of individual 2-nitroanisole metabolites were identified

In order to identify the role of specific CYP enzymes in the oxidation pathway of 2-nitroanisole to its metabolites we used hepatic microsomal fractions of rat pretreated with known inductors of individual cytochromes P450 and recombinant rat and human cytochromes P450 in Supersomes^{TM}. The results that involved rat enzymes were compared with those found for 2-nitroanisole oxidation by human CYPs. The same metabolites are

formed in both biological species (*figure 5*, pg. 6). The CYP 2E1 and 1A1 enzymes were the most efficient in metabolism of 2-NA to 2-NP. Among other CYPs tested in this study, CYP2B, 2C and 2D were also capable of oxidizing 2-NA, but to a lesser extent (*figure 5*).

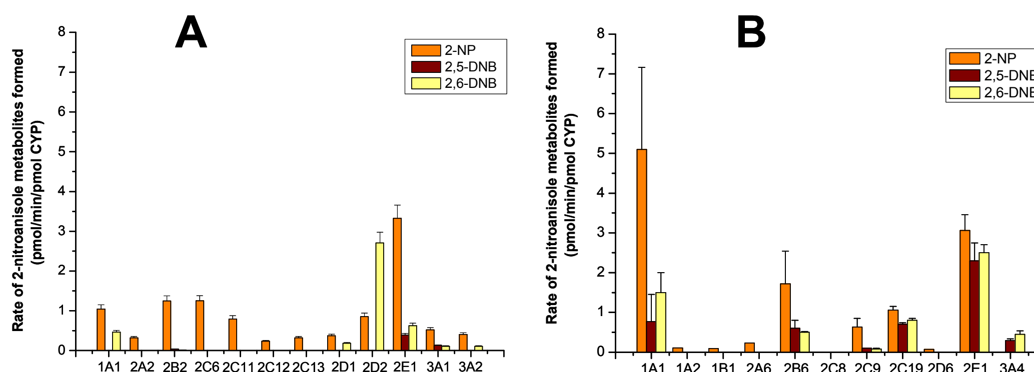

Figure 5 Metabolism of 2-nitroanisole by recombinant rat (A) and human (B) cytochromes P450. The values are averages and standard deviations of triplicate incubations.

2-Nitrophenol is oxidized by microsomal cyochromes P450 only to 2,5-dihydroxynitrobenzene

2-Nitrophenol is oxidized only to one metabolite, 2,5-dihydroxynitrobenzene. The efficiency of cytochromes P450 of individual animal species (human, rat, rabbit and mouse) to oxidize 2-NP was compared (*figure 6, pg. 7*). Among tested animal species human CYPs followed by rat CYPs were the most efficient in 2-NP oxidation.

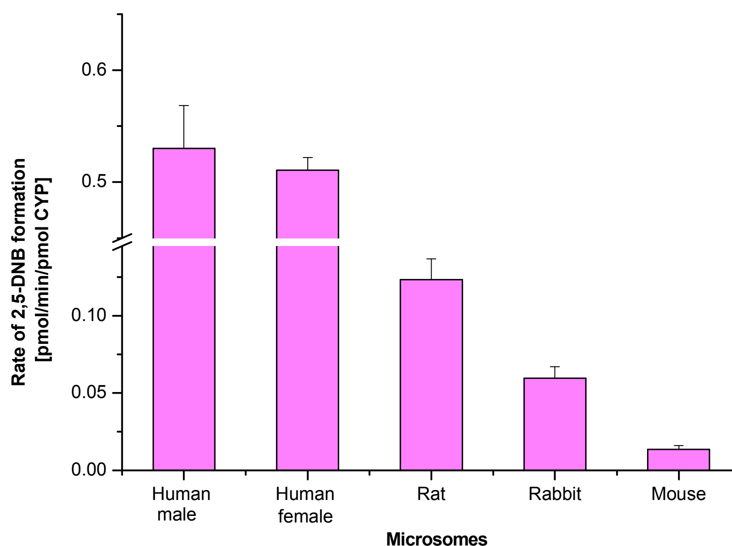

Figure 6 Rate of 2,5-DNB formation from 1 mM 2-NP by human, rat, rabbit and mouse hepatic microsomes. The values are averages and standard deviations of triplicate incubation.

Identification of individual cytochromes P450 involved in 2-nitrophenol oxidation

In order to resolve which human cytochromes P450 are able to oxidize 2-nitrophenol, three experimental approaches were employed: (i) microsomes of rat pretreated with known inductors of individual CYPs, (ii) heterologous expression systems (Supersomes^{TM}) and (iii) selective inhibition of CYPs. The results with rat enzymes were again compared to those found for 2-NP oxidation by human CYPs. The same metabolite is, as in the case of 2-NA, formed in both biological species. CYP2E1 followed by CYPs of 3A, 2A, 2C and 2D subfamilies were the most efficient to oxidize 2-NP (*figure 7* and *figure 8*, pg. 8).

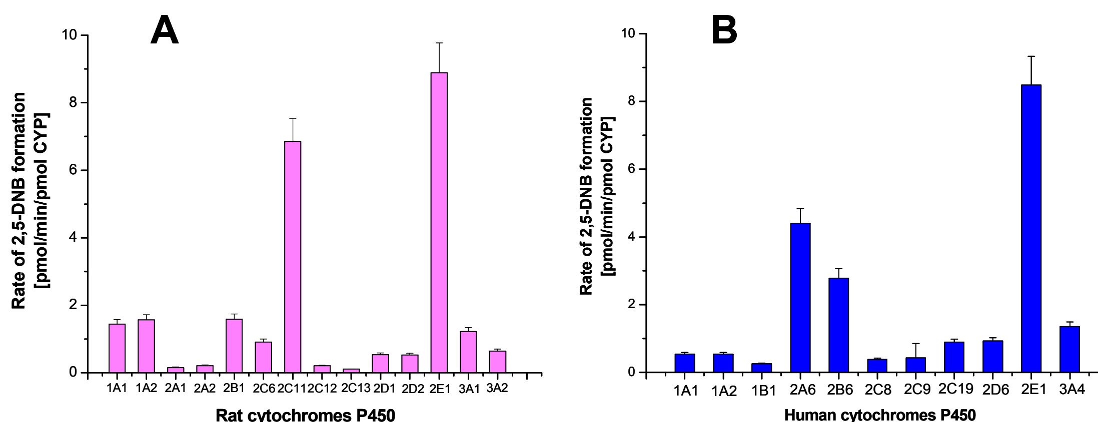

Figure 7 Oxidation of 2-NP to 2,5-DNB by recombinant rat (A) and human (B) cytochromes P450. The values are averages and standard deviations of triplicate incubation.

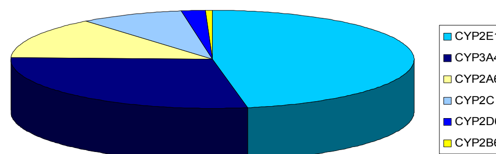

Figure 8 The contributions of individual human CYPs to 2-NP oxidation in human hepatic microsomes

2-Nitrophenol is a detoxication metabolite of carcinogenic 2-nitroanisole

Nitroreduction of 2-NP to *N*-(2-hydroxyphenyl)hydroxylamin or 2-aminofenol was not detected in human, rat, rabbit and mouse hepatic microsomes even under aerobic and anaerobic conditions. No reactive species binding to DNA generated during the 2-NP metabolism by human hepatic microsomes and cytosols after incubation with DNA were detected (*figure 9*).

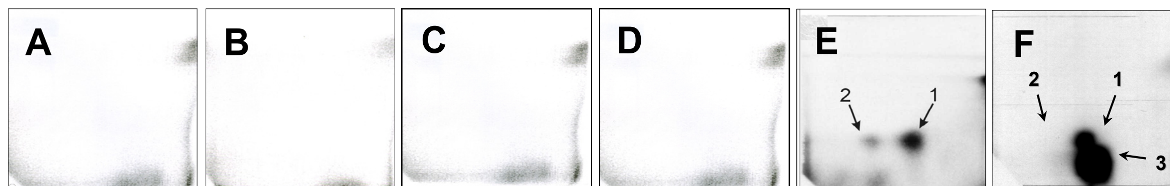

Figure 9 Autoradiographic profiles of ^{32}P-labeled DNA adducts in calf thymus DNA formed by 2-NP activated with human hepatic microsomes under anaerobic conditions (A, B), with human

hepatic cytosol under the same conditions (C, D), in DNA of urinary bladder of rats treated with 2-NA (E) and in dGp reacted with *N*-(2-methoxyphenyl)hydroxylamine (F). Samples E and F were used as positive controls. The nuclease P1 version of the ^{32}P-postlabeling assay was used for analysis shown in panels (A, C and E), the standard procedure under ATP-deficient conditions for that in panels (B, D and F).

Kinetics of 2-nitrophenol oxidation by rat and human cytochrome P450 is identical

Oxidation of 2-NP to 2,5-DNB by CYP2E1 exhibits the Michaelis-Menten kinetics in both biological species. The values of Michaelis constant (K_m) and maximum reaction rate (V_{max}) were similar as well (*table 1*).

Table 1 The values of V_{max} a K_m for 2-NP oxidation by rat and human CYP2E1

	V_{max} [pmol/min/pmol CYP]	K_m [mM]
rat CYP2E1	16,2	0,35
human CYP2E1	29,5	0,21

Activation of 3-nitrobenzanthrone by studied enzymatic systems to species forming DNA adducts

Using the ^{32}P-postlabeling method we detected the DNA adducts formed after activation of 3-NBA by human hepatic cytosols, human hepatic microsomes, human NQO1, butter milk xanthinoxidase and human NADPH:CYP reductase. In contrast to the results with 3-NBA, no 2-NBA-derived DNA adducts were identified under the same experimental conditions with any of these enzymatic systems (*figure 10*, pg. 10).

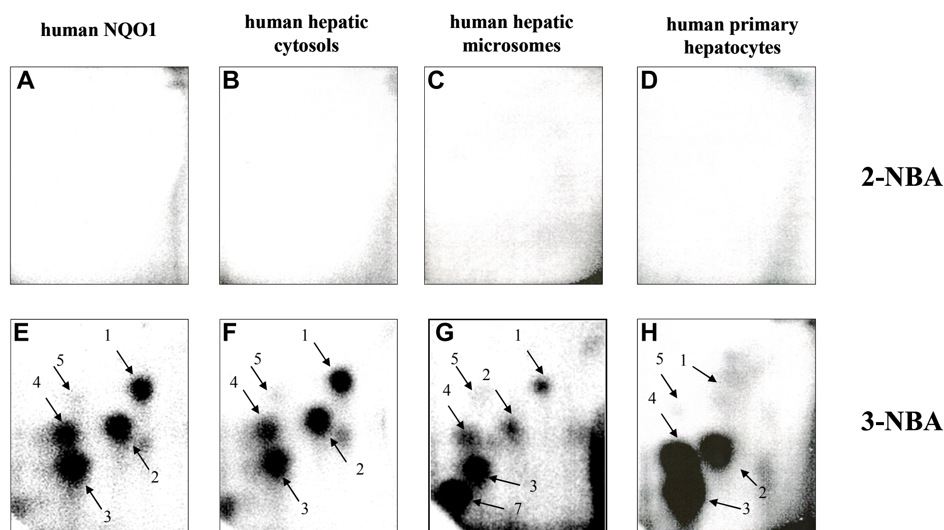

Figure 10 Autoradiographic profiles of DNA adducts generated by 100 μM 2-NBA (A-C) and 100 μM 3-NBA (E-G) after activation with recombinant human NQO1 (A, E), human hepatic cytosols (B, F), human hepatic microsomes (C, G) and in primary cultures of human hepatocytes (HL 21 hepatocyte sample) treated with 1 μM 2-NBA (D) or 3-NBA (H) using ^{32}P-postlabeling. Spot 1, dA-*N*^{6}-3-ABA; spot 3, dG-*N*^{2}-3-ABA; spots 4/5, dG-C8-*N*-3-ABA.

3-Nitrobenzanthrone is reduced by human NQO1 to 3-aminobenzanthrone

3-NBA is metabolized by human NQO1 to one reductive metabolite, 3-ABA. The reduction of 3-NBA depended on concentration of NQO1 and 3-NBA and was time-dependent as well. The reaction exhibited Michaelis-Menten kinetics (*figure 11*). In contrast, no 2-ABA was found to be generated by human NQO1.

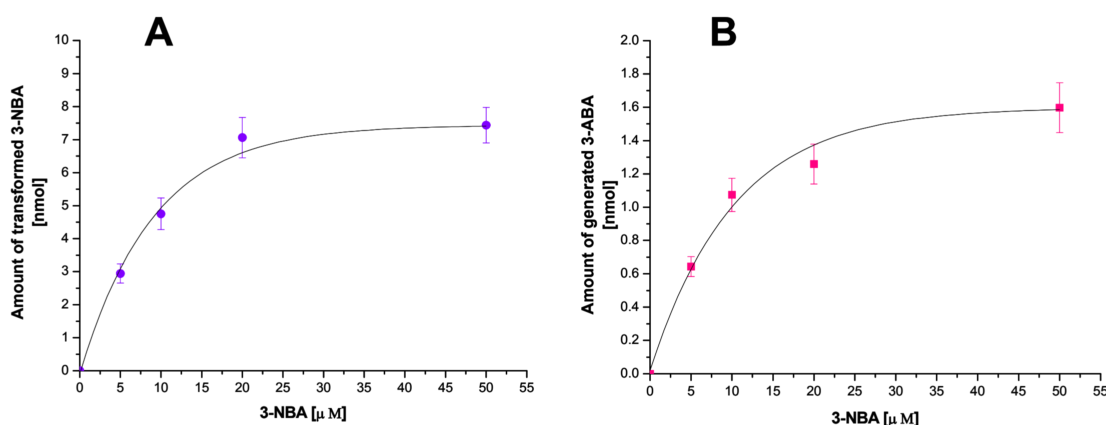

Figure 11 The dependence of 3-NBA reduction (A) and production of 3-ABA (B) by recombinant human NQO1 on 3-NBA concentration. The values are averages and standard deviations of triplicate incubation.

The different orientation of 2-nitrobenzanthrone and 3-nitrobenzanthrone in the active site of NQO1 dictates their reduction

Both izomers of nitrobenzanthrone bind to the active site of NQO1 with the similar binding affinities. The orientations of the two NBA isomers in the active site are different (*figure 12*), however, resulting in a greater distance of the nitro group of 2-NBA to the hydrogen on N5 of the isoalloxazine ring of FAD than that of 3-NBA. These spatial arrangements favor a hydride transfer to the nitro group of 3-NBA but not to that of 2-NBA.

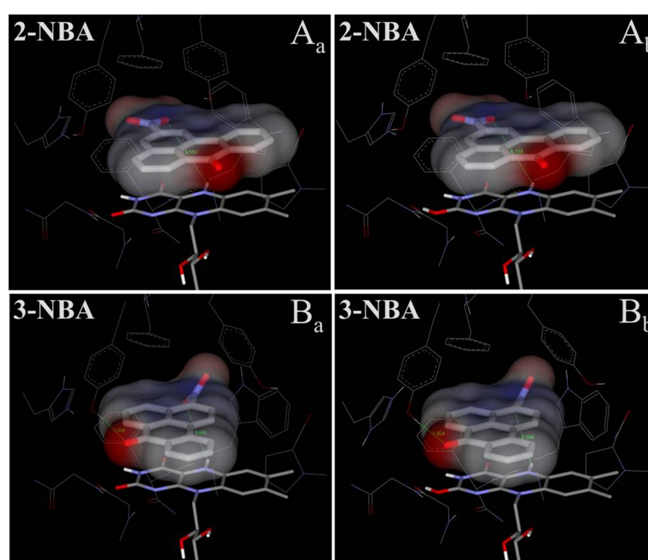

Figure 12 The binding orientations resulting from molecular docking calculations enabling the hydrogen transfer from N5 of isoalloxazine ring of FAD to the nitro group of 3-NBA (B_a,B_b) but less to that of 2-NBA (A_a,A_b) are shown docked to the active site of human NQO1. The two possible forms of reduced isoalloxazine ring – ionized enolate and protonated enol - bound to the NQO1 active site are labeled with subscript *a* and *b*, respectively. Both NBA ligands are positioned parallel to the flavin prosthetic group. 2-NBA or 3-NBA, FAD cofactor and amino acids residues within 5.5 Å from ligand are rendered as bold sticks, and sticks and lines, respectively.

2-Nitrobenzanthrone inhibits formation of DNA adducts generated by 3-nitrobenzanthrone

2-NBA inhibited the formation of the 3-NBA-DNA adducts (*figure 13*, pg. 12). This result indicates that 2-NBA competes with 3-NBA for binding to NQO1, thereby decreasing the metabolite activation of 3-NBA to DNA adduct forming intermediates.

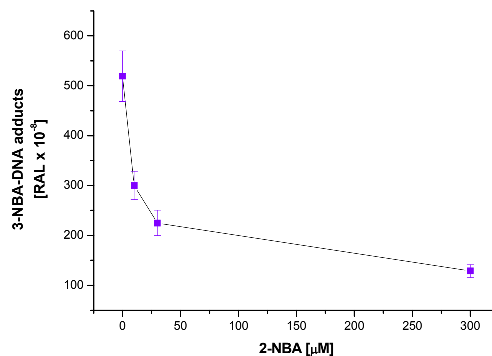

Figure 13 Effect of 2-NBA (10, 30, 300 μM) on DNA adduct formation by 30 μM 3-NBA activated with recombinant human NQO1. The values are averages and standard deviations of triplicate incubation.

A part of results of this PhD. thesis has already been published in scientific journals (see papers 1-7 in List of publications).

This work was supported by the Grant Agency of the Czech Republic (grant 303/09/0472) and Ministry of Education of the Czech Republic (grants MSM 0021620808 a 1M0505).