CHARLES UNIVERSITY IN PRAGUE
FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY

Metabolism of and DNA Adduct Formation by Carcinogenic *o*-Anisidine and its Metabolite *N*-(2-Methoxyphenyl)hydroxylamine

Summary of Ph.D. Thesis

RNDr. Karel Naiman

Supervisor: Prof. RNDr. Marie Stiborová, DrSc.

PRAGUE 2010

INTRODUCTION

Aromatic amines are potent toxic or carcinogenic compounds, presenting a considerable danger to the human population (NTP, 1978; IARC, 1982; Garner *et al.*, 1984). They are widely distributed environmental pollutants found in workplaces (e.g. in chemical industry), in emissions from diesel and gasoline engines and on the surface of ambient air particulate matter (NTP, 1978; IARC, 1982), where they add to local and regional pollution (car exhausts, technological spills). Their toxicity and carcinogenicity has been widely examined, but the knowledge in metabolism of several aromatic amines and their physiological effects in humans are still incomplete. This is also the case of *o*-anisidine.

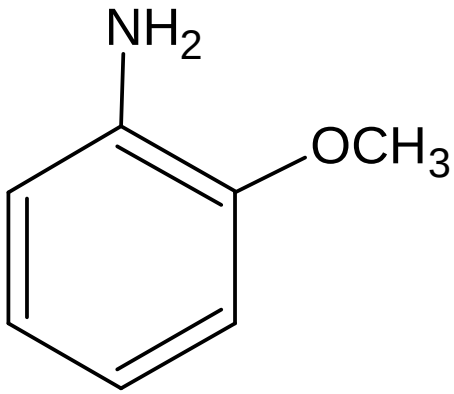


Figure 1: *o-anisidine*

2-Methoxyaniline (*o*-anisidine, Fig. 1) is a potent carcinogen, causing tumours of the urinary bladder in both genders of F344 rats and B6C3F1 mice (NTP, 1978; IARC, 1982). The International Agency for Research on Cancer (IARC) has classified *o*-anisidine as a group 2B carcinogen (IARC, 1982), which is possibly carcinogenic to humans. Besides its carcinogenicity it exhibits other toxic effects, including haematological changes, anaemia and nephrotoxicity (NTP, 1978; IARC, 1982). *o*-Anisidine is used as an intermediate in the manufacturing of a number of azo and naphthol pigments and dyes, which are used for printing (90%) and for paper (3%) and textile (7%) dyeing (NTP, 1978; Garner *et al.*, 1984). This carcinogen is also a constituent of cigarette smoke (IARC, 1982; Stabbert *et*

al., 2003). Moreover, *o*-anisidine was detected in the samples of Rhine (Köln, Düsseldorf) and Elbe Rivers (Reifferscheid and Grummt, 2000). This finding suggests that *o*-anisidine ranks not only among occupational pollutants produced in the manufacturing of chemicals, but also among environmental pollutants. It can be assumed that human exposure is widespread. Indeed, *o*-anisidine was found in human urine samples in the general population, in concentrations of 0.22 µg/l (median) (Weiss and Angerer, 2003). In addition, haemoglobin adducts of *o*-anisidine were detected in blood samples of persons living in urban or rural areas of Germany (Falter *et al.*, 1994; Branner *et al.*, 1998; Richter *et al*., 2001; Kütting *et al*., 2009). The haemoglobin adducts as well as *o*-anisidine in urine might originate not only from the sources mentioned above, but also from a possible *o*-anisidine precursor, 2-methoxynitrobenzene (2-nitroanisole). This chemical was released into the environment in the course of an accident in a German chemical plant, causing subsequently local and regional contamination (Hauthal, 1993; Falter *et al.*, 1994; Traupe *et al.*, 1997). 2-Nitroanisole exhibits strong carcinogenic activity, causing neoplastic transformation in the urinary bladder, and to a lesser extent, in the spleen, liver and kidneys in rodents (NTP 1993). It is also a toxic compound, causing anaemia (NTP 1993).

Recently, it has been found that *o*-anisidine is oxidatively activated by cytochromes P450 to species binding to DNA *in vitro* (Rýdlová *et al*., 2005; Stiborová *et al.*, 2005; Naiman *et al.*, 2008a, b, 2010). It was confirmed that *o*-anisidine also forms DNA adducts *in vivo*. The same adducts as found in DNA incubated with *o*-anisidine and human microsomes *in vitro* were detected in urinary bladder, the target organ, and to a lesser extent, in liver, kidney and spleen of rats treated with *o*-anisidine (Stiborová *et al.*, 2005). The *o*-anisidine-derived DNA adducts were identified as deoxyguanosine adducts formed from a metabolite of *o*-anisidine, *N*-(2-methoxyphenyl)hydroxylamine (Fig. 2; p. 4), which is

generated by oxidation of *o*-anisidine with human, rabbit and rat hepatic microsomes (Rýdlová *et al.*, 2005; Stiborová *et al.*, 2005; Naiman *et al.*, 2008a, 2008b). The same deoxyguanosine adducts were also detected in DNA of the urinary bladder, kidney, liver and spleen of rats treated with 2-nitroanisole (Stiborová *et al.*, 2004), an oxidized counterpart of *o*-anisidine, and in DNA incubated with 2-nitroanisole *in vitro* with human or rat hepatic cytosolic enzymes or xanthine oxidase (Stiborová *et al.*, 1998; 2004). These enzymatic systems were found to produce *N*-(2-methoxyphenyl)hydroxylamine during 2-nitroanisole reduction (Mikšanová *et al.*, 2004). The data indicate that formation of *N*-(2-methoxyphenyl)hydroxylamine, the reactive metabolite of both carcinogens, is critical for generation of DNA lesions in target organs (Stiborová *et al.*, 2009). Therefore, it is clear that *N*-(2-methoxyphenyl)hydroxylamine formation and its further conversion, as well as the enzymes participating in such processes, play a key role in carcinogenic effects of both carcinogens.

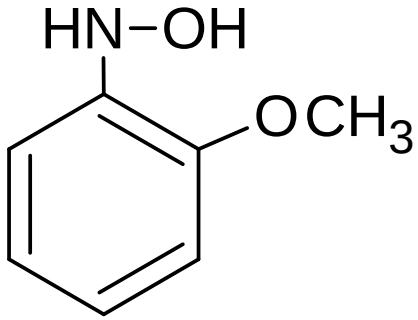


Figure 2: *N-(2-methoxyfenyl)hydroxylamine*

AIMS OF THE THESIS

The aim of the thesis was to contribute to explanation of activation metabolism of *o*-anisidine and its reactive intermediate, *N*-(2-methoxypfenyl)hydroxylamine. Further work was targeted to propose the mechanism of *o*-anisidine carcinogenicity on the molecular level. In order to fulfil the research objectives, it was necessary to accomplish following partial goals:

- To identify rat and rabbit CYPs participating in the metabolism of *o*-anisidine and *N*-(2-methoxypfenyl)hydroxylamine, we used specific inhibitors of individual CYPs and hepatic microsomes from animals pre-treated with specific inducers of CYPs. Additionally, oxidation and reduction of these compounds by rat recombinant CYPs were investigated.
- To evaluate participation of human CYPs participating in metabolism of *N*-(2-methoxypfenyl)hydroxylamine, human recombinant enzymes were used.
- To characterize the metabolites formed during *o*-anisidine and *N*-(2-methoxypfenyl)hydroxylamine metabolism by CYPs, mass spectrometry and nuclear magnetic resonance analyses were utilized.
- To characterize structures of covalent DNA adducts formed by *o*-anisidine oxidation with CYPs, mass spectrometry and/or nuclear magnetic resonance analyses were utilized.
- To evaluate persistence of the DNA adducts formed by *o*-anisidine *in vivo*, rats were used as model for this study.

Another target of this work was to partially characterize deoxyguanosine adduct formed by ellipticine metabolite, 13-hydroxyellipticine.

RESULTS and DISCUSSION

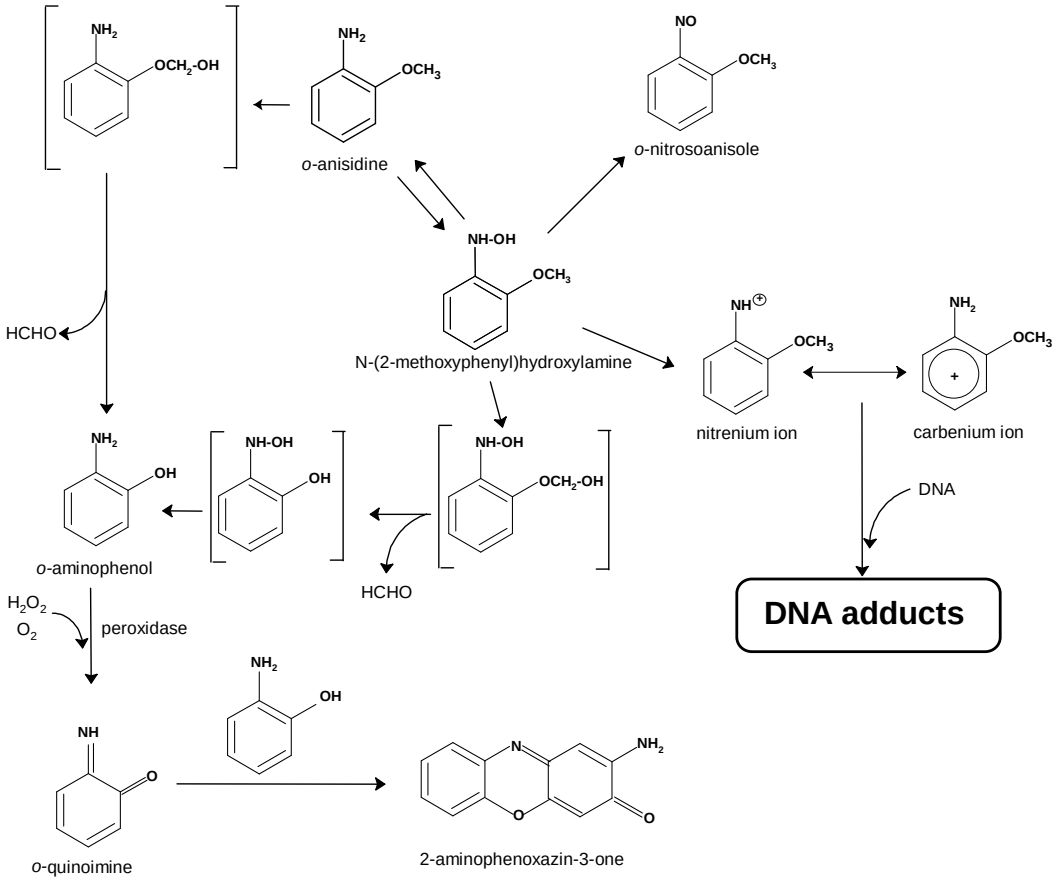
The present study has contributed to increase our knowledge on *o*-anisidine induced carcinogenesis and activation metabolism mediated by *N*-(2-methoxypfenyl)hydroxylamine. This reactive compound is the proximate carcinogen formed not only from *o*-anisidine but also from its oxidative counterpart, 2-nitroanisole. The main conclusions followed from the results found in this thesis are as follows:

o-Anisidine and *N*-(2-methoxyphenyl)hydroxylamine Metabolisms by Rat and Human CYPs

The results of our study demonstrate that *N*-(2-methoxyphenyl)hydroxylamine, a reactive metabolite of carcinogenic *o*-anisidine and *o*-nitroanisole can be metabolised by rat and rabbit hepatic microsomes. The hepatic microsomal CYP enzymes of rabbit and rat catalyze both *O*-demethylation of *o*-anisidine to form *o*-aminophenol and its *N*-hydroxylation to form a reactive metabolite, *N*-(2-methoxyphenyl)hydroxylamine, which was found previously to generate deoxyguanosine adducts in DNA *in vitro* (Stiborová *et al.*, 2005). The data indicate that the reactive *N*-(2-methoxyphenyl)hydroxylamine formed as a metabolite of both carcinogens is critical for the formation of DNA adducts in target organs.

The results show that *o*-anisidine is a subject of complex redox cycling reactions. It is primarily converted to *o*-aminophenol and *N*-(2-methoxyphenyl)hydroxylamine and metabolite M1 (Scheme 1; p. 8). *N*-(2-methoxyphenyl)hydroxylamine is additionally converted to

the nitrenium/carbenium ion and/or a product whose mass spectrum corresponds to this ion. Furthermore, *N*-(2-methoxyphenyl)hydroxylamine is metabolised by hepatic microsomal CYP enzymes not only to *o*-aminophenol but also to two metabolites (M1 and M2) and, particularly, to parental *o*-anisidine.



Scheme 1: *Pathways of o-anisidine, N-(2-methoxyphenyl)hydroxylamine and o-aminophenol metabolism showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions.*

The results of this study demonstrate that metabolism of *o*-aminophenol by hepatic microsomes or peroxidases did not lead to formation of covalent DNA adducts. This oxidation leads to formation of an *o*-quinoimine metabolite (Akazawa

et al., 2000; Puiu *et al.*, 2008), but this intermediate seems not to be reactive enough to react with DNA. Formation of 2-aminophenoxazine-3-one seems to be a final metabolite of the *o*-aminophenol oxidation. Furthermore, *o*-aminophenol is a suitable substrate for major human hepatic sulfotransferase, SULT1A1 (Riches *et al.*, 2007) and after conjugation might be easily excreted from body. All these findings suggest that *o*-aminophenol and its metabolism by CYPs and peroxidases are not included into genotoxic processes occurring during carcinogenesis caused by *o*-anisidine.

The results of the study show that reduction of *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine is essentially not mediated by NADPH:CYP reductase, but it is catalyzed by other enzymes present in hepatic microsomes. These enzymes were identified to be CYPs.

The role of specific microsomal CYP enzymes in the metabolism of *o*-anisidine was identified. The results indicate that *o*-anisidine is a promiscuous substrate of rat and rabbit hepatic CYPs. CYPs of 1A, 2B, 2E, and 3A subfamilies metabolise *o*-anisidine in hepatic microsomes of studied species. Using purified enzymes (CYP1A1, 1A2, 2B2, 2B4, 2E1, and 3A6) reconstituted with NADPH:CYP reductase, their ability to metabolise *o*-anisidine was confirmed. In the reconstituted CYP system, rabbit CYP2E1 was the most efficient enzyme metabolising *o*-anisidine.

The role of CYP enzymes in the metabolism of *N*-(2-methoxyphenyl)hydroxylamine was also characterized in the present study. The results indicate that *N*-(2-methoxyphenyl)hydroxylamine is also a promiscuous substrate of CYP enzymes, being metabolised by rat hepatic CYP1A1, 1A2, 2B1, 2B2, 2C, 2D, 2E1 and 3A. The results also show that rat CYP2E1 was the most efficient enzyme metabolising this compound. The role

of these CYPs was confirmed by recombinantly expressed rat CYPs. The most efficient enzymes reducing *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine were CYP2E1 and 2C11, followed by CYP2A2, 2D2, 2A2, 2C12, 2D1, 3A1, and 3A2.

o-Anisidine (Stiborová *et al.*, 2005) and *N*-(2-methoxyphenyl)hydroxylamine were found to be suitable substrates for human CYP2E1 and of several other human CYPs, namely CYP1A1, 1A2, 2A6, 2B6, 2D6, and 3A4. Thus orthologous human and animal CYP enzymes are responsible for the metabolism of these compounds. This conclusion might be one of the criteria indicating that rodents might be suitable models to predict human metabolic susceptibility to *o*-anisidine. It might be important in the evaluation of *o*-anisidine carcinogenicity as a carcinogenic risk factor for humans.

Formation and Persistence of DNA adducts of o-Anisidine and Structural Characterization of the Major Adduct

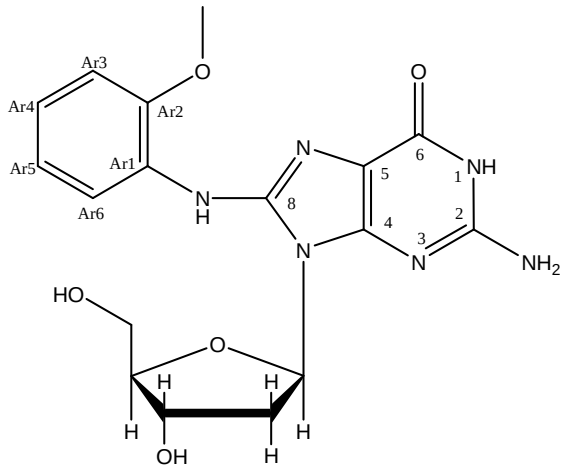
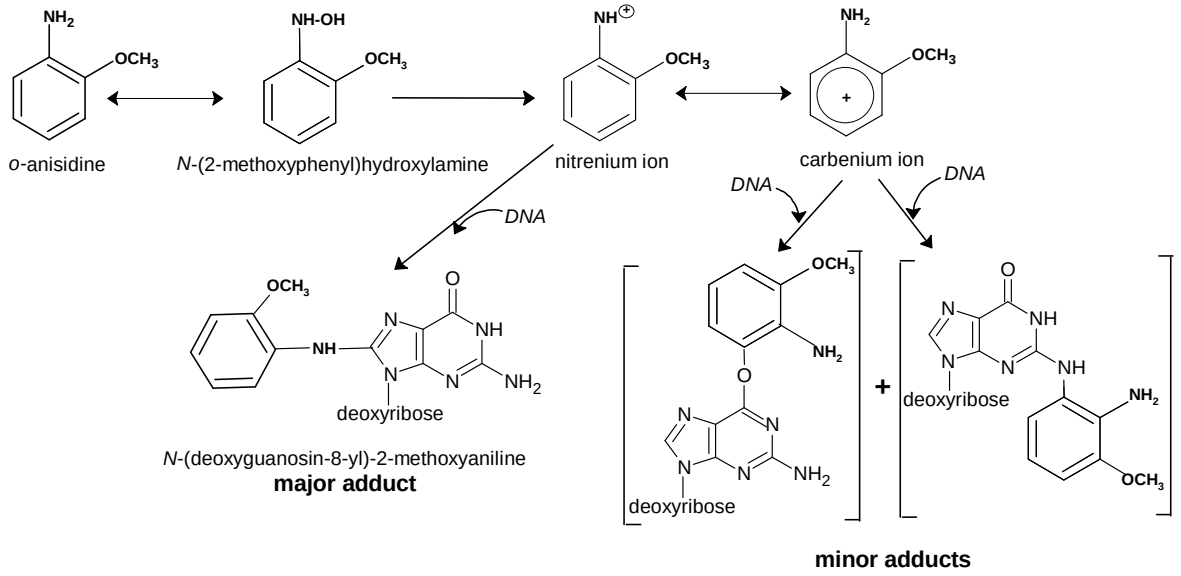


Figure 3: *The structure of the major deoxyguanosine adduct, N-(deoxyguanosin-8-yl)-2-methoxyaniline*

The major adduct formed by reaction of deoxyguanosine with *N*-(2-methoxyphenyl)hydroxylamine under the acidic conditions is the same as the major adduct found in rats *in vivo*. This major adduct was characterized both by mass spectrometry and nuclear magnetic resonance, and identified to be *N*-(deoxyguanosin-8-yl)-2-methoxyaniline (Fig. 3). The other two

adducts detected by the ^{32}P-postlabelling technique and found to be formed by the reaction of deoxyguanosine with *N*-(2-methoxyphenyl)hydroxylamine are adducts with mass corresponding to deoxyguanosine adducts formed from nitrenium/carbenium ions of *o*-anisidine. These two adducts might be N^2- and O^6-substituted deoxyguanosine adducts, with the N^2-substituted being the most prominent (Scheme 2). On positive and negative ion MS, all adducts gave molecular ions at *m/z* 388.4 and 387.0, respectively, corresponding to adducts of deoxyguanosine with nitrenium/carbenium ion of *o*-anisidine.



Scheme 2: *Pathways of o-anisidine activation showing the characterized activation metabolites and DNA adducts. Characterization of compounds shown in brackets by MS indicate to be adducts of deoxyguanosine with nitrenium/carbenium ions, but their structures were not exactly identified under the experimental conditions.*

The results found in this and previous (Stiborova *et al.,* 2005) studies, showing formation of covalent DNA adducts by *o*-anisidine, demonstrate a genotoxic mechanism of *o*-anisidine carcinogenicity. The highest level of DNA adducts was always found in the urinary bladder, the target organ for tumour development by *o*-anisidine. DNA adduct formation was also seen in liver, kidney and spleen,

but at levels more than one order of magnitude lower than in the urinary bladder. The levels of DNA adducts was 4 adducts per 10^6 nucleotides in DNA of the urinary bladder.

The adducts found in DNA of liver, kidney and spleen did not persist in these organs; no *o*-anisidine-derived DNA adducts were detectable in these tissues 10 weeks after the treatment. In spleen, no DNA adducts were even found 13 days after *o*-anisidine administration. On the contrary, in the urinary bladder levels of the *o*-anisidine-derived DNA adduct decreased over time, but considerable amount of DNA adducts persisted even after 36 weeks after *o*-anisidine administration.

Formation and Partial Characterization of Deoxyguanosine Adduct Formed by 13-Hydroxyellipticine

Formation of the major DNA adduct by anticancer drug, ellipticine, *in vitro* and *in vivo*, 13-hydroxyellipticine-derived DNA adduct, is increased by an increase in pH of the incubation mixture. The formation of the DNA adduct was also significantly increased by conjugation of 13-hydroxyellipticine to the sulfate and acetate esters catalyzed by sulfotransferases and *N*-acetyltransferases, which were found to be expressed in the target tumours for ellipticine action (Williams and Phillips, 2000). Therefore, by stimulation of the formation of the DNA adduct, by 13-hydroxyellipticine conjugation to sulfate and acetate esters, the pharmacological efficiency of ellipticine could be increased. Moreover**,** it follows from the results found in this work that it will be possible to prepare sufficient amount of deoxyguanosine adduct with 13-hydroxyellipticine for further structural characterization.

Elucidation of the carcinogenic effect of *o*-anisidine shed more light also on the activation metabolism of its oxidative counterpart, 2-nitroanisole. Both carcinogens are activated to the same proximate carcinogenic metabolite, *N*-(2-methoxyphenyl)hydroxylamine, which is responsible for DNA adduct formation. Further piece of scientific information was added to the mosaic of complex metabolism and carcinogenic effect of *o*-anisidine and its oxidized counterpart, 2-nitroanisole. Thereby, our study contributes to the evaluation of *o*-anisidine and 2-nitroanisole carcinogenicity as a carcinogenic risk factor for humans.

The study was supported by Grant Agency of Charles University (grant 7418/2007), Grant Agency of the Czech Republic (grant 303/09/0472) and the Ministry of Education of the Czech Republic (grants MSM0021620808 and 1M0505)