

L01**HEALTH RISKS OF HARMFUL COMPOUNDS FIXED ON PARTICULATE MATTER (PM_{2.5}) IN URBAN AIR****VLADIMÍR ADAMEC, ROMAN LIČBINSKÝ,
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Introduction

Amount of gases and solid pollutants emitted from anthropogenic activities to the atmosphere continually increase and demonstrably have adverse effects on human health and environmental ecosystems. These unfavourable conditions are evident especially in large cities where transport becomes the dominant source of pollution. 347,900 European died in the year 2000 in accordance with the newest EU study¹ due to air pollution. These results thus testified former epidemiological studies that indicate an early death of hundreds of thousands people in Europe² exposed to these pollutants and life shortening³ of a couple of years because of cardiovascular⁴ and respiratory⁵ diseases. Last studies actually advert to cancer origin⁶. Particulate matter (PM) and some non limited pollutants such as polyaromatic hydrocarbons (PAH), benzene, 1,3-butadiene become an actual part of research not only due to increasing concentrations in the ambient air and primarily because of their negative effects on human health (respiratory and cardiovascular diseases). Possible human health effects of PM are associated with particle diameter and of course with chemical composition because even particle diameter is the dominant factor determining whether it can be inhaled and in which part of the respiratory system it can be deposited. PM smaller than 2.5 µm and pollutants adsorbed on their surface can penetrate into the bronchioles. Particles with diameter less than 1 µm penetrate into pulmonary alveoli where can be accumulated or ultrafine particles enter the cardiovascular system and are transported to different organism tissues³. The most life-threatening group of people are those living near the streets with intensive road transport and first of all children⁷.

Described facts confirming recency of solving of this issue there through was realized air quality monitoring with respect to NV 350/2002 Collection of Law as amended by later documents in period from 2005 till 2006. The goal was to clarify long term trends in PM_{2.5} concentrations and composition on selected localities including assessment of possible negative impacts on population health state.

Experimental Part

Localities in Brno city with high traffic intensity and different morphology of surroundings were chosen for taking air samples. Locality Arboretum representing wide opened site with a lot of trees is situated near the I. transport city circular near the crossroads Drobného, Lesnické, Provazníkové and tř. generála Píky streets. Locality Kotlářská is situated also near the I. transport city circular but in contrast to the first locality in the surroundings stand five floor residential buildings and this street could be considered “street canyon”.

Middle volume samplers LECKEL MVS6 (Sven Leckel Ingenierbüro, Germany) were used for taking ambient air samples, two on each locality – one equipped with nitrocellulose filter for PM_{2.5} concentrations determination, second with quartz filter for analyses of PAH adsorbed on the surface of this PM fraction. Gravimetric analysis on Mettler-Toledo MX5/A balance (Mettler-Toledo GmbH, Switzerland) of each exposed filter was used for PM_{2.5} concentrations determination. 112 samples from both localities were taken and analysed during seven days campaigns in 24 hours intervals. Vertical distribution and continual measurements of PM_{1.0}, PM_{2.5} a PM₁₀ fractions were performed by using EnvironCheck 107 (Grimm Aerosol Technik GmbH, Germany).

PAH extraction from quartz filters (IKA, IKA – Werke GmbH, Germany) and disturbing compounds separation on silica gel column were done before proper analysis. Gas chromatograph with mass detector SHIMADZU QP 2010 (SHIMADZU, Japan) equipped with autosampler was used for PAH concentration analysis. Helium was the carrier gas in the analysis. Internal standard analysis (*p*-terfenyl) with calibration with standard mixture of 16 PAH from US EPA MIX 63 and coronen (Dr. Ehrenstorfer, Germany) was used for PAH concentrations determination. Chromatograms evaluation was done by peak manual integration and data were expressed as concentration of PAH fixed to PM_{2.5} for unit air volume.

On the basis of experimentally determined annual mean concentration of pollutants that were count over using toxic equivalent factors (TEF) to benzo(*a*)pyrene concentration⁸ was assessed long term (chronic) inhalation health risk of people living near the chosen localities. Health risk assessment was done for simplicity as a risk for ideal average population it means for individual with body weight 70 kg without exposition data splitting between men and woman. Basic exposition scenario considering 3 hours exposition to the ambient air, age 70 years, exposition duration 30 years, exposition frequentation 350 days per year and inhalation rate 0.83 m³ h⁻¹. The same scenario was used for health risk assessment for children with modification of age to 14 years, body weight 15 kg,

exposition duration 14 years and inhalation rate $3.2 \text{ m}^3 \text{ h}^{-1}$.

Results and Discussion

The highest $\text{PM}_{2.5}$ concentrations were determined in autumn (turn of November and December) whereas the lowest in summer (turn of June and July) (fig. 1). Monitoring results indicate strong correlation of $\text{PM}_{2.5}$ and temperature. By the lowest measured temperatures were the highest $\text{PM}_{2.5}$ concentrations, by the high temperatures the concentrations were lower. This effect could be either due to loss of volatile PM compounds (such as ammonium salt) in warmer period that coagulate in low temperatures and can be captured on filters or it could have connection to vertical stability of the atmosphere. Better ventilation in warmer season (convection) amuses pollutants further from the sources whereas in colder season ventilation is limited (inversion) and PM cumulate in the baser parts of the atmosphere near the source. Local furnace that heat surrounding houses could be another important source of these particles in colder period. Comparison of the PM concentrations in the range of diameter 2.5–10 μm , 1–2.5 μm a 0–1 μm measured in different seasons shows the significant dependence of separate PM fractions on the season. The share of fine fraction $\text{PM}_{2.5}$ on total PM_{10} concentration is higher in colder period when it is 92.9 % from PM_{10} and in warmer period the same share is 59 % respectively. This fact confirm the higher burden of people respiratory system in colder seasons.

Very important group of harmful compounds often fixed to PM are PAH originating especially during the incomplete combustion of fossil fuels. Obtained results show that concentrations of selected PAH indicate significant seasonal correlation such as $\text{PM}_{2.5}$ and exist some specific context with temperature. Two PAH groups were identified on the basis of multidimensional analysis, “volatile PAH” containing naftalene, acenaftylene, fluorene, fenantrene a antracene, “non volatile PAH” containing fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)

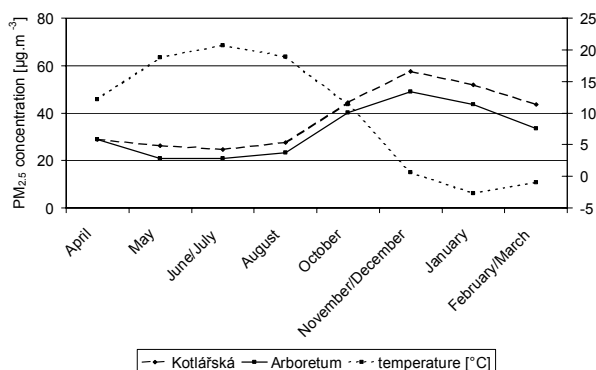


Fig. 1. $\text{PM}_{2.5}$ concentration and temperature progress during the observed period

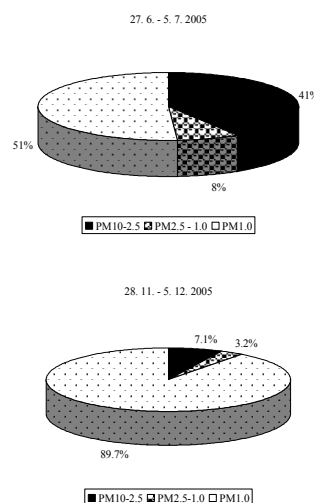


Fig. 2. PM_{10} , $\text{PM}_{2.5}$ and $\text{PM}_{1.0}$ distribution comparison

pyrene, indeno(1,2,3-*cd*)pyrene, benzo(*ghi*)perylene, coronene. PAH concentrations fixed to $\text{PM}_{2.5}$ have different presence during the warm season from April till August and in colder season from October till April. Principal component analysis Varimax for two factors was used to possible sources identification. This spend 89 % of total variability of results that can be considered sufficient for describing problem. According to factor loadings values benzo(*ghi*)perylene and coronene represent traffic sources and benzo(*a*)pyrene other sources than traffic. The other PAH are significantly produced by both sources. Comparing the ratios of coronene – benzo(*a*)pyrene and benzo(*ghi*)perylene – benzo(*a*)pyrene show traffic as the dominant source of air pollution in the warmer season (May – August) with better ventilation conditions. Other sources than traffic become important in March, January and February and become dominant in turn of November and December.

PAH concentrations were and number of people living near the chosen localities were used as input data for carcinogenic risk assessment due to inhalation of these compounds. Possibility of tumor diseases occurrence due to PAH inhalation for adult individual is acceptable on both localities, this risk for children individual is on the edge of acceptance (Table I). Inclusion the population living near the chosen localities to the assessment show that the possibility of tumor diseases is for both adults population and especially children population higher than the barrier of socially assumed point of risk for tumor diseases occurrence $1 \cdot 10^{-6}$.

Conclusion

Measured annual mean $\text{PM}_{2.5}$ concentrations confirm the higher burden of people on locality Kotlářská ($38.1 \mu\text{g m}^{-3}$) than on locality Arboretum ($32.5 \mu\text{g m}^{-3}$). These values are in both cases higher than limits ($25 \mu\text{g m}^{-3}$)

Table I
Individual and population risk due to PAH inhalation

Locality	ILCR – individual lifetime cancer risk		APCR - population lifetime cancer risk	
	adult	children	adult	children
Kotlářská	1.38E-07	5.78E-06	1.30E-04	2.72E-03
Arboretum	1.24E-07	5.21E-06	9.02E-05	1.90E-03

that are being prepared in the new European Union directive (Directive of the European Parliament and council on ambient air quality and cleaner air for Europe). Obtained results thus confirm conclusions with dependence of PM and PAH concentrations⁹ on temperature when the highest concentrations were also by the lowest measured temperatures. We can also hypothesize that the source of air pollution can be also other sources than traffic especially by lower temperatures near zero. Limit for benzo(a)pyrene settled in NV 350/2002 Collection of Law was not exceeded on both localities. Measured PM and PAH concentrations stand with the concentrations measured on localities with mean traffic intensity^{10,11} and industry¹². Possibility of tumor diseases occurrence due to PAH inhalation for adult individual is acceptable on both localities, but not for children individual (Table I). Possibility of tumor diseases is not acceptable for both adults population and especially children population. Results unambiguously show that already PAH inhalation is potentially harmful for exposed population living near both localities.

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L02

POSSIBILITY OF PHARMACOLOGICAL PROPHYLAXIS AGAINST HIGHLY TOXIC ORGANOPHOSPHATES

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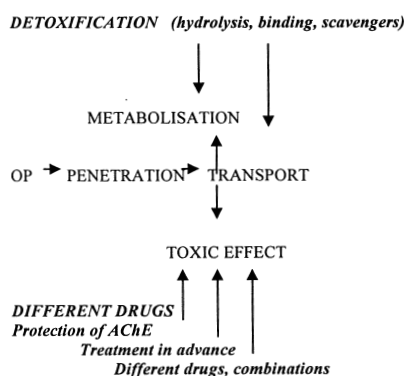
Inhibitors of cholinesterases are very important chemicals in the group of organophosphates (OP). These compounds are used in industry, in veterinary or human medicine and, last but not least, these compounds are, unfortunately, usable (and used) for military purposes as chemical warfare agents (nerve agents) and as poisons used by terrorists as it was documented in the Tokyo subway. The broadest spectrum of these compounds is used as pesticides (insecticides), acaricides etc. According to the World Health Organization, more than one million serious accidental and two million suicidal poisonings with insecticides occur worldwide every year, and of these approximately 200,000 die, mostly in developing countries¹⁻³. The mechanism of their action, prophylaxis and treatment of intoxications with OP is a very hot topic at present. Basic mechanism of action of OP is based on their ability to inhibit the enzyme acetylcholinesterase (EC, 3.1.1.7) at cholinergic peripheral and central synapses². The term prophylaxis used in this article is limited to medical countermeasures applied relatively shortly before penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic drug. In general, the prophylaxis can be focused on protection of AChE against the

inhibition using reversible cholinesterase inhibitors. The diminishing the level of OP using enzymes hydrolyzing these agents or enzymes binding the agents (to specific proteins or to antibodies) and thus reducing the OP level and inhibition of cholinesterases – AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8) (scavenger effect) in the organism can be described as detoxification. Another approach to prophylaxis is based on using present antidotes and other drugs^{2,4} (Scheme 1).

Protection of AChE against inhibition

Keeping AChE intact is a basic requirement for effective prophylaxis. This can be achieved by using reversible inhibitors, which are able to inhibit AChE reversibly and after spontaneous recovery of the activity, normal AChE serves as a source of the active enzyme. The ability of some carbamates to protect an organism poisoned with OP has been known for many years. Physostigmine and neostigmine have been used to protect animals against DFP. Nevertheless, the protective effect of physostigmine, aminostigmine, pyridostigmine and others against AChE inhibition caused by different OP (mostly soman) has been demonstrated^{2,4}. From the results published (and unpublished), it appears that pyridostigmine is the most promising prophylactic drug especially against soman poisoning. On the basis of these results, pyridostigmine was introduced into some armies as a prophylactic antidote against nerve agents. Its prophylactic effect (like the effects of other carbamates) is limited by its dose. With a higher dose, a higher efficacy was observed, but the side effects were more expressed, too (for review, see^{2,4}). This problem can be solved by the adding of pyridostigmine antagonizing drugs – anticholinergics. The prophylactic combination of pyridostigmine with trihexyphenidyle and benactyzine called PANPAL was introduced into the Czech Army. The presence of these two anticholinergics allowed us to increase the pyridostigmine dose and to increase its prophylactic efficacy. This combination (including follow-up therapy) is not limited to soman, sarin and VX poisoning but its high efficacy against tabun, GV and cyclosarin^{5,6} intoxications was observed. The prophylactic antidotal combination PANPAL has not side effects as it has been demonstrated on volunteers: no statistically different changes in the actual psychic state as well as no negative changes in the dysfunction time and heart function and blood pressure were observed⁷. On the basis of the results with the prophylactic efficacy of other different carbamates, aminostigmine was chosen as the most effective. All the results dealing with reversible AChE inhibitors as prophylactics against nerve agents including initial historical papers were summarized^{2,4}.

Other carbamates also have a good prophylactic effi-



Scheme 1. **Four basic reactions of OP in the organism (in CAPITALS) and possible targets for prophylaxis (in italics, bold)**

cacy, especially physostigmine (due to its central effect on the contrary to pyridostigmine)^{8,9}. Human study with transdermal physostigmine suggests a serious interest in the prophylactic use of this drug¹⁰. Structurally different inhibitors from the carbamate and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, 7-methoxytacrine (7-MEOTA) and huperzine A were considered and experimentally studied with respect to prophylaxis *in vitro* and *in vivo*^{4,11}. The most interesting results were obtained with huperzine A. Huperzine A was tested as a potential candidate against OP for its long-lasting efficacy and relatively low toxicity^{11,12}. However, the results obtained do not support replacement of pyridostigmine by these drugs.

Detoxification

This principle involves two different approaches: administration of enzymes splitting the OP or specific enzymes which bind the OP (cholinesterases). OP is bound to the exogenously administered enzyme and thus the OP level in the organism is decreased (it acts as “scavenger”). The use of scavengers as prophylactics either as enzymes binding^{13,14} or hydrolysing^{15,16}. OP was tested many years ago. A lot of studies have been made with cholinesterases as scavengers. BuChE and AChE were observed to be very effective in protection against OP intoxication^{17–22}. The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects¹⁷. All of these features are of great interest and they are yielding practical results – isolation of the enzyme, examination for lack of and auto immune response and establishment of pharmacokinetic and pharmacodynamic properties^{17,20}. Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain parts following low level sarin inhalation exposure^{21,22}. Given our increasing knowledge in bioengineering and biotechnology, the connection between these two enzymes will be possible with the aim of obtaining a modified enzyme splitting OP and simultaneously reacting with AChE as a scavenger²³. Antibodies against OP are in the stage of research and they are more focused on the detection of OP²⁴.

Standard antidotes as prophylactics

The antidotes currently used for the treatment of OP poisoning can be tested as prophylactics. The aim of this approach is very simple – to achieve sufficient level of antidotes in the blood vessel before intoxication. Standard antidotes were studied in this respect, i.e. anticholinergics, reactivators, anticonvulsants and others^{2,4}. The problem with their use is the timing and duration and achievement of sufficient levels of these antidotes after administration. However, the prophylactic efficacy is good as it has been demonstrated in treatment studies but administration of these antidotes mostly takes place very shortly (minutes)

after the intoxication. The prolongation of the duration of the antidote effects by achievement of their sufficient level in the blood by oral administration is not possible (especially reactivators) and therefore it is excluded. It was a reason for searching for other routes of administration. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach^{2,4,25}. The final result was the new prophylactic transdermal antidote called TRANSANT. This preparation was clinically tested (including dermal sensitivity) without any harmful effects and field testing was also successful²⁵. The final reports were finished and TRANSANT has been introduced into the Czech Army. The prophylactic efficacy of other drugs was studied. As anticonvulsant drugs, benzodiazepines (diazepam, midazolam, alprazolam, triazolam, clonazepam) were studied, but isolated prophylactic administration had not very good effects^{2,4,26}.

Prophylaxis with other drugs

Prophylactic administration of different drugs (alone or in combination) against intoxication with OP were studied. Calcium antagonists (nimodipine), neuromuscular blockers (tubocurarine), adamantanes (memantine), and the opiate antagonist meptazinol^{27,28} were also tested with different results but they were not very useful for practical use. On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (antimuscarinic, antinicotinic and the anti-NMDA receptor drug)²⁹. However, all these studies are experimental ones and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as pre-treatment and following treatment with different antidotes. Administration of pyridostigmine (or other inhibitors) prior to intoxication and treatment with different drugs is a typical example^{8,30–32}. There are other combinations such as the administration of triesterase^{32,33}, procyclidine^{8,29}, clonidine³⁴, sustained release of physostigmine and scopolamine³⁵. The results are very dependent on experimental conditions but this approach – administration of different drugs - has yielded some good results though up to now they have been on an experimental level. Only one prophylactic mixture has been introduced into the army – PANPAL composed of pyridostigmine, benactyzine and trihexyphenidyle. When PANPAL⁷ and TRANSANT²⁵ are administered simultaneously, their combination represents the best prophylaxis against nerve agents at present.

Conclusions

There are many drugs tested for their prophylactic efficacy against nerve agent intoxication. However, only three prophylactics (pyridostigmine alone, PANPAL and TRANSANT) were introduced into the military medical practice. Perspective approach seems to be the use of purified enzymes, especially butyrylcholinesterase or acetyl-

cholinesterase produced by biotechnology.

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L03

HAIR ANALYSES FOR DRUG ABUSE AND FORENSIC APPLICATIONS

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Key words: hair analysis, drugs of abuse

Introduction

Many years hair analysis for drugs has been gaining increased attention in toxicology and several comprehensive reviews were published on this topics^{1–6}. Hair differs from other materials analyzed in toxicological laboratories due to its relative stability and due to its ability to store foreign substances for long time (months to years) in relation to their temporal appearance in blood or urine. Toxicological hair analyses can be useful above all to confirm or exclude chronic intentional or unintentional poisonings or alcohol or drug abuse in various contexts. The development of analytical technologies, especially mass spectrometry, enabling to improve method sensitivity and selectivity, will expand the field of hair analyses for additional drugs and poisons in trace concentrations and will expand the boundaries of rational interpretation with potential serious impacts to individual.

Hair analyses have potential applications to human performance toxicology as preemployee or employee drug screening, or driving ability examination. Hair analyses are used to gestational drug exposure detection, diagnosis of chronic intoxication or pollutant exposition. The results from hair analyses can elucidate the backgrounds of investigated drug-facilitated crimes. Hair analyses are used in divorce proceedings to assess the parents' reliability for children custody in context of parents' potential alcohol or drug addiction. In postmortem toxicology, they can contribute to explanation of pathological autopsy findings. There is only limited relevance of hair in therapy compliance control. Hair can not substitute blood in therapeutic blood monitoring in quantitative sense.

Hair analysis for drugs is not a simple routine procedure. It is necessary to follow substantial scientific guidelines^{7,8} starting from sample collection with respect to the individual dignity, case history and aim of investigation.

Hair grows in cycles: the anagen (active growing stage), the catagen (transitional stage) and telogen (resting stage). The individual length of hair depends on the mutual duration of these stages and on the growth rate. Average

values for the anagen stage in human are 4–8 years, the catagen a few weeks, and the telogen stage 4–6 months. The scalp hair growth rate is reported to be in the range 0.6–1.4 cm per month in general^{1,3}. There are significant differences both in the proportions anagen/telogen hair and both in the growth rate between hairs from various anatomical part of the body^{1,3}. The both parameters are dependent on race, sex, age, health conditions. On the scalp of an adult, the approximately 85 % of the hair is in the growing phase (anagen) and the remaining 15 % is in a resting phase (telogen)^{1,3}. The consequence of the cyclic hair growth is the nonhomogeneity of the hair bunch at the horizontal level, at a certain distance from the skin.

Incorporation and elimination of drugs in hair

The ideal model assumes that drugs or chemicals enter hair by passive diffusion from blood capillaries into the growing cells at the base of hair follicle. However, experimental data indicate that drugs may enter hair in different locations and in different times from different sources by various mechanisms. The drugs can be transported from blood and also from deep skin compartments not only into hair growing cells but with some time delay also into keratogenous zone during hair shaft formation. The other mechanisms are diffusion from sweat or sebum secretions. A contamination of the hair surface from external environment needs to be diminished by washing. The scheme of possible sources of drug incorporation and elimination is demonstrated in fig. 1.

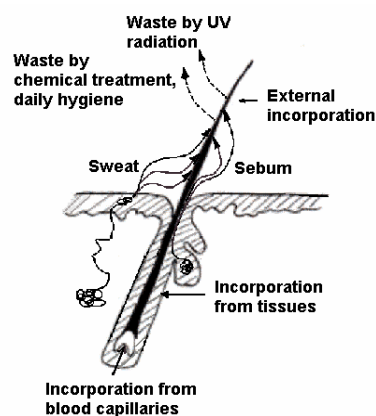


Fig. 1. The scheme of incorporation of drugs in hair and their potential elimination

The three key factors which influence the drug incorporation into hair are melanin content in a hair, lipophilicity and basicity of a drug substance. The physicochemical

properties of drugs, lipophilicity and basicity related to molecular structure clearly affect the drug incorporation into hair and on the other side, hair structure and its color plays a very important role too. The pH of melanocytes is between 3 and 5 and significant melanin affinity for basic drugs has been demonstrated in several experimental studies⁹. It was confirmed that drug concentration in pigmented hair was much higher than in blond or grey hair after the same dosage^{10,11}.

The second important factor is the polarity of a drug or its metabolite. It has been many times documented that more polar metabolites as benzoylecgonine or amphetamine enter the hair in a lesser extent than their more lipophilic precursors cocaine or methamphetamine. The acidity or basicity of a drug is the third important factor. The matrix of hair is more acidic than blood, therefore the resulting pH gradient is more convenient to transfer basic compounds. For example the acidic carboxy metabolite of delta-9-tetra-hydrocannabinol enters the hair only in tiny traces¹².

The retention and stability of drugs in hair is considered to be good, nevertheless it can be affected by cosmetic treatment as bleaching or dyeing and permanent wave application. The long term effects of weather (sunshine, rain, wind) may cause the damage of hair shaft with impacts to changes of concentration in hair. In case of long hair, above all the structure of distal part could be damaged and its analysis should be avoided.

The practical steps in hair analysis

The meaningful hair analysis needs the complex attitude from sampling to interpretation including the adequate information about the aim of investigation. The main steps of hair analysis include: 1) Information about the case and aim of investigation; 2) Hair sampling and documentation; 3) Washing the external surface; 4) Segmentation if appropriate; 5) Opening the inner hair space by grinding or cutting to small strips; 6) Digestion of hair matrix; 7) Clean up the hair digest; 8) Sensitive and specific hair extract analysis (GC-MS or LC/MS); 9) Interpretation of results, conclusions.

The interpretation of results related to time may be complicated from various reasons:

- Incorrect sampling, mutual shift of individual hair fibers in a sampled bunch
- Nonhomogenous hair fibers growth, individual ratio anagen/telogen, variation in hair growth rate
- Incorporation from other sources than blood (sweat, sebum, delayed incorporation of deposits from tissues)
- Potential waste by environmental effects, daily hygiene, chemical treatment
- Potential longitudinal diffusion

The schemes of various models of drug distribution along the hair tuft after a month consumption a year ago are outlined in the fig. 2.

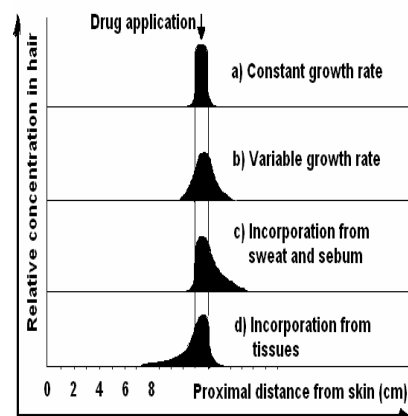


Fig 2. Models of drug distribution along the hair tuft after continuous one month consumption a year ago

The information about the assumed period of previous drug consumption in a specific case is very important. When testing for previous drug use about the certain date, the approximate localization of a drug in hair must be estimated. The analysis must be performed at least in 3 segments with one covering the date of the event and two other adjacent segments. Nevertheless, the temporal information gained may be uncertain or insufficient. The width of a segment and their total number selected to cover the time scale of interest are often the compromise between the number of segments and the cost of examination. However, the broader width of a segment can cause the lower concentration of a drug in hair or the concentration below the limit of detection, see fig. 3.

Cases of application

Postmortem toxicology

Segmental hair analysis can provide a retrospective calendar of individual's drug use, period of abstinence, or

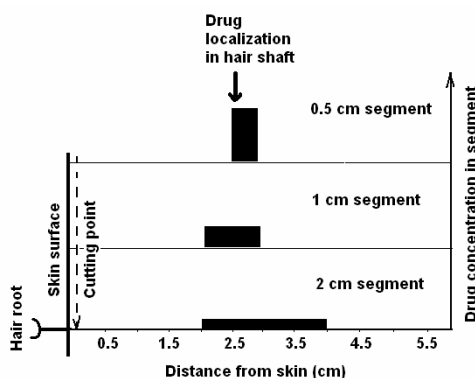


Fig 3. Drug position in hair and various concentrations in sample segments of various width prepared for analysis

the evidence of switching from one drug to another or of mixing various drugs e. g. heroin, dihydrocodeine, hydrocodone¹³. A significant factor in interpretation postmortem blood concentration of opioids is chronic or single consumption and the degree of tolerance at the time of death. Only hair results can indicate whether the deceased subject was long term opioid addict and the determined periferic blood concentration was fatal or not, as we have presented earlier in case of a young heroin addict⁵.

The extensive pathological autopsy findings in a man, 38 years old, were also in compliance with the toxicological evidence of the drug mixture in hair (methamphetamine, amphetamine, ephedrine, norephedrine, 6-acetylmorphine, morphine, buprenorphine, norbuprenorphine) confirming the chronic and variable drug addiction.

Long term drug abuse or chronic poisoning can gradually induce certain harmful effects on the human organism and can exacerbate some preexisting diseases. For example chronic abuse of methamphetamine is known to be associated with cardiovascular diseases. During autopsy certain types of morphological alterations are found in the hearts of stimulant addicts. The rapid increase in blood pressure after an intravenous methamphetamine dose can be risky for addicts with arteriosclerosis which can develop after long term abuse. However, the information on the life style of a deceased person need not be available to explain the pathological cardiovascular alterations and to classify the cause of death correctly. The findings in hair segments may be useful in this respect. The results in four 2 cm segments and in the fifth distal 7 cm segment were used to explain pathomorphological observation during autopsy of a methamphetamine addict with bleeding into cerebellum. The results provided clear evidence that the man was methamphetamine addict for more than 8 months¹⁴.

Drug facilitated crimes

A case of 13 years old boy belongs to this category. The boy's tutor was under the suspicion of child sexual abuse and of prohibited methamphetamine administration. In December 2005 the boy was transferred to another children institute. The hairs were sampled in the middle of January 2006. After washing, five 1 cm segments and the resting 2–3 cm distal segment were prepared for GC-MS targeted analysis. Methamphetamine presence was confirmed in all segments except for the proximal segment. The absence of methamphetamine in the proximal segment corresponded to the period after cutting off the contact with the tutor. By hair analyses it was proved that the prohibited drug administration to the infant covered the time window July to November 2005, with the maximum in the third segment roughly corresponding to October at supposed hair rate growth 1 cm month⁻¹. The results of this hair analysis are demonstrated in fig. 4.

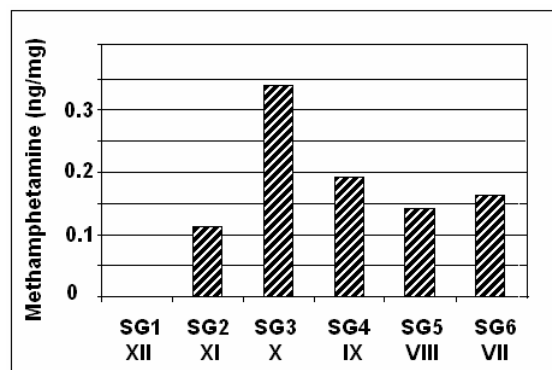


Fig. 4. Methamphetamine in hair of 13 years old boy. The drug absence in proximal segment SG1 corresponded to the time after removing the infant from his tutor

Human performance toxicology

Many companies have established their own policy for drug testing which is based usually on urine testing where the detection is restricted only to recent dose. In Germany and Italy, the hair testing is allowed in monitoring chronic drug or alcohol abuse in problematic drivers in regrating driver's licence. In majority of European countries the hair tests for drugs are limited to criminal investigations. As an example from our practice, it is a case of a driver (man 32 years old) who caused a fatal traffic accident. After positive urine drug results for cannabis and amphetamines, the driver admitted the chronic consumption of marijuana and admitted only one dose of methamphetamine after the accident. The hair was sampled 2.5 months after the crash. The hair length was 3.5 cm (covering the approximate period of 4 months of hair growth). Two equal segments were prepared and analyzed. Methamphetamine was confirmed only in the distal segment (0.15 ng mg⁻¹), which corresponded to longer term drug consumption before the accident or to several high doses around the accident time.

Children custody

Hair testing for drugs can be ordered by a judge in divorce proceedings involving parents mutually accused for alcohol or drug addiction. There is the problem to decide whom to charge as a more reliable person for child custody in this situation. The results in hair of a mother or father or even the child can serve as objective evidence supporting the correct decision of the court. We have had the opportunity to provide such evidence in several cases.

Conclusion

The properties of basic drugs as cocaine, amphetamines, opiates are convenient for their efficient incorporation into hair from blood. The transport of acidic or neutral compounds as cannabinoids from blood into hair is less

effective. Therefore to prove their low amount in hair, very sensitive methods are needed. Hair is a unique and stable material for retrospective investigation of a person's life style, to prove or exclude abstinence from alcohol or drugs in a specific time window, to ascertain decreasing or increasing trends in drug abuse. There is no direct relationship between drug concentration in hair and the dose. The relationship between drug concentration in blood and hair is strictly individual and can not be generalized.

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L04**IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY EFFECT OF DERMAL EXPOSURE TO COAL TAR AND UV-RADIATION**

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Key words: coal tar, polycyclic aromatic hydrocarbons, UV-radiation, Goeckerman regimen, immunosuppressive effect

Introduction

Goeckerman regimen (GR) combines dermal application of therapeutic coal tar (containing polycyclic aromatic hydrocarbons – PAHs)¹ and UV-radiation². GR is often used as the first option for topical treatment of psoriasis^{3–8}. PAHs and UV-radiation are carcinogenic (mutagenic) agents¹ with suspected effect to immune system^{9–15}. The aim of presented study was to evaluate the changes of selected cellular (lymphocyte subpopulations CD3, CD4, CD8) and humoral immunological parameters (immunoglobulin IgG, IgA, IgM, IgE, β_2 -microglobulin, α_2 -macroglobulin, transferrin, C3 complement, orosomucoid, prealbumin, haptoglobin, neopterin) in a group of 56 patients with psoriasis, treated by GR.

Research design and methods**Subjects, therapy and sampling**

A group of 56 patients (average age 37 years, range 18–77 years), diagnosed with psoriasis and undergoing GR were selected. The group consisted of 34 males and 22 females, 38 % of the patients were smokers. A questionnaire was administered to each subject to determine previous exposures to coal tar (or to other mixtures containing PAHs) and UV-radiation. Patients with positive exposure history were excluded from this study. The efficiency of GR was assessed with respect to erythema, infiltration and desquamation by means of a Psoriasis Area and Severity Index (PASI) score¹⁶.

The GR was applied individually according to disease activity (duration from 8 to 30 days; average time of GR 17 days). Coal tar (CT) ointment (containing 5 % of CT-Pix Litantracis) was applied daily to affected areas (32–77 %

of the patient's body surface). Concurrently, the patients were irradiated daily by ultraviolet radiation-A (UV-A) and ultraviolet radiation-B (UV-B). Radiation was applied individually in relation to the disease activity in intervals from 1 to 20 min. Density of UV-B radiation ($134.45 \mu\text{W cm}^{-2}$) and UV-A radiation ($245.60 \text{ mW cm}^{-2}$) was controlled by a spectroradiometer Sola-Scope 2000 (Solatell Ltd., United Kingdom).

Immunological findings

Blood samples were collected before and after GR. Selected parameters of cell-mediated immunity (lymphocyte subpopulations CD3, CD4, CD8) were determined in heparin-treated blood and the immunophenotyping analysis was performed on a flow cytometer Coulter Epics XL (Coulter-Beckmen, USA). Blood serum was used to estimate the parameters of humoral immunity (immunoglobulin IgG, IgA, IgM, IgE, β_2 -microglobulin, α_2 -macroglobulin, transferrin, C3 complement, orosomucoid, prealbumin, haptoglobin, neopterin). The levels of serum proteins α_2 -macroglobulin, IgG, IgA, IgM, C3 complement, orosomucoid, prealbumin, haptoglobin and transferrin were determined using rate nephelometry (Beckman, USA). IgE and β_2 -microglobulin were measured by chemiluminiscent immunoassay (DPC, USA). Levels of neopterin were evaluated using ELISA technique (Brahm, Germany).

Statistical analysis

The data were statistically processed in software "Statistica", version 6.1 (USA). Since the Shapiro-Wilk W test for normality rejected hypothesis of normal distribution, for all followed values we used the nonparametric tests. To assess the effect of treatment, dependent data before and after the GR were compared by the Wilcoxon matched pairs test. The associations between selected parameters were evaluated by Pearson's correlation coefficient. The statistic significance was determined on a probability level less than 0.05 in all calculations.

Results

The levels of IgG, IgM, IgE, transferrin and neopterin were significantly decreased after GR (Table I). The changes of other immunological findings were not significant (lymphocyte subpopulations CD3, CD4, CD8, IgA, β_2 -microglobulin, α_2 -macroglobulin, C3 complement, orosomucoid, prealbumin, haptoglobin). The levels of PASI score were significantly decreased after GR (22.2 ± 6.9 ; 6.3 ± 3.7 ; $P < 0.001$).

Table I
Significantly changed immunological parameters in serum

Units	Before GR					After GR					
	Parameters	n	MED	LQ	UQ	Parameters	n	MED	LQ	UQ	P
[g l ⁻¹]	IgE	56	48.5	30.0	200.0	IgE	56	41.3	25.1	170.0	<0.01
[g l ⁻¹]	IgM	56	1.2	0.9	1.6	IgM	56	1.1	0.8	1.5	<0.001
[g l ⁻¹]	IgG	56	11.4	9.0	13.6	IgG	56	10.5	9.1	12.8	<0.001
[nmol l ⁻¹]	Neo	56	6.2	4.4	8.5	Neo	56	5.3	4.0	7.1	<0.01
[g l ⁻¹]	Trf	56	2.6	3.60	5.20	Trf	56	2.5	2.1	2.8	<0.01

IgE, IgM, IgG = Immunoglobulins, Neo = Neopterin, Trf = Transferrin, MED = median, LQ = lower quartile, UQ = upper quartile, n = number of patients

Discussion

The skin, as an interface between internal and external “environment” plays an important role in protecting and supporting of the organism⁷. Deregulation of skin's immune system, however, frequently occurs and can result into undesirable inflammatory skin processes. A typical example of an undesirable inflammation skin process is a chronic inflammatory skin disease – psoriasis¹⁷.

Psoriasis is a T-cell driven immunologically mediated systemic skin disease (with altered Th1/Th2 cytokine balance)¹⁸, afflicting up to 2.5 % of the world's population^{19,20}. Pathogenesis of psoriasis is associated with variable provoking factors of both environmental²¹ and endogenous origin in genetically predisposed individuals, e.g. certain HLA (Human Leukocyte Antigens) haplotype (HLA-Cw*0602)¹⁸. In a case of psoriasis, the complex of interaction between T-lymphocytes, antigen-presenting cells, keratinocytes, pro-inflammatory cytokines and chemokines is disturbed^{17,20,22}.

Many biological and chemical agents used in the therapy of psoriasis are able to alter the immune functions in human. For example, in one of our previous work we concluded that chemokine pathway of IL-8 and TNF-alpha could be significantly modulated by GR (mainly PAHs)⁴. In another work we found that GR significantly alleviated angiogenic potential, which is in patients with psoriasis usually abnormally increased¹.

In a good agreement with our previous results⁵, serum concentrations of IgM and IgG, which are recognized as positive reactants of inflammation, were significantly decreased after GR. It is very likely associated with diminished activity of immunopathological inflammation in treated patients. Also production of IgE class of immunoglobulins significantly decreased in psoriatic patients after GR.

In spite of the fact, that transferrin is a typical negative marker of inflammation, its serum level was significantly decreased after GR. At this moment we are not able seriously explain to this fact, nevertheless, given reality is

in accordance with our previous findings⁵.

Neopterin is a non-specific marker of activation of cell-mediated immunity²³. In presented study the serum level of neopterin was significantly decreased, immediately after GR. In agreement with other authors, these findings confirmed that serum neopterin concentration reflects disease activity in psoriasis^{20,23,24}.

According to the PASI score, our study confirms effectiveness of GR for treatment of chronic psoriasis. The level of PASI was significantly decreased after GR ($P < 0.001$). It was in a good compliance with our previous results⁵ and with the results of other authors^{6–8}.

Conclusions

The results of the study indicate that combine dermal exposure to therapeutic coal tar and UV-radiation (GR) induced significant immunosuppressive and significant anti-inflammatory effects.

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L05

SKIN SENSITISATION AND APPROACHES FOR THE DEVELOPMENT OF CELL-BASED TESTING METHODS

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Key words: skin sensitization, dendritic cells, Langerhans cells

Introduction – mechanisms

This paper is focused specifically on the impact of chemical exposure on immune target cells and the potential application of derived information in the development of cell-based assay for the assessment of skin sensitization potential of chemicals *in vitro*. The possibilities of discrimination between irritating and sensitizing action of chemicals on *in vitro* level are also discussed.

The development of skin sensitization is associated with, and requires, the activation and clonal expansion of allergen responsive T lymphocytes (T-cells). These cells orchestrate the cutaneous allergic reaction. In recent years, much has been learned of the characteristics of immune responses to skin sensitizing chemicals and of the roles played by dendritic cells (DC), cytokines and chemokines. Langerhans cells (LC), the principal DC, are settled in the epidermis. The LC form a network designed to „trap“ foreign antigens that have entered the skin, including chemical allergens¹.

One of the characteristics of a chemical allergen, which has been used for the assessment of skin sensitization potential is its ability to react with proteins prior to the induction of skin sensitization². In order to determine if reactivity correlates with sensitization potential, 38 chemicals representing allergens and nonsensitizers were evaluated for their ability to react with glutathione or three synthetic peptides containing either cysteine, lysine, or histidine. UV detection was used to monitor the depletion of glutathione or the peptide following reaction. The results show that a significant correlation exists between allergenic potency and the depletion of glutathione, lysine, and cysteine³.

Methods for the detection of skin sensitization

A variety of animal test methods are available for the identification of chemicals that have the potential to cause skin sensitization and allergic contact dermatitis. Originally, guinea pigs represented the species of choice for skin sensitization predictive tests and two methods using this species, the guinea pig maximization test⁴ and the occluded patch test of Buehler⁵, have found wide application. In the early 1980s the mouse ear swelling test (MEST) was developed to provide a lower cost, shorter and objectively graded alternative to the existing guinea pig tests for assessment of skin sensitization. Initially the MEST was employed to evaluate sensitization potential for industrial chemicals only⁶. It has since found much wider utility, particularly in those areas where a cost effective screen for strong irritants and sensitizers is required (such as for finished fabrics and medical devices)⁷. Further method using mouse, the popliteal lymph node assay (PLNA), has been proposed as a screening test for detecting chemicals with potential of inducing allergic reactions in humans⁸. More recently, similar method in the mouse has been developed, the murine local lymph node assay (LLNA)^{9,10}, having been endorsed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for the evaluation of skin sensitizing potential¹¹. However, even with the significant animal welfare benefits provided by the LLNA, there is still interest in the development of nonanimal test methods for skin sensitization testing using *in vitro* approaches.

Discrimination of irritant and sensitizing agents

Cutaneous toxicity may have several forms – those of greatest prevalence being allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD)¹². Although the skin elicited reactions are usually indistinguishable with respect to macroscopic or histopathological appearance, the mechanistic bases for ACD and ICD are clearly very different, with the former, but not the latter, being dependent upon the initiation of a primary cutaneous immune response and skin sensitization¹³. It is well established that antigen presentation by antigen presenting cells to the T-cells are essential in the mechanism of ACD. In contrast ICD is believed to activate the immune cascade independent of the antigen presentation pathway, by stimulating release of proinflammatory mediators and cytokines that directly recruit and activate T cells. The precise mechanism of skin irritancy is still unclear¹.

Increases in lymph node cellularity were observed in both allergen- and irritant-treated mice compared to naive or vehicle-treated animals. Mice treated with allergens

showed a preferential increase in the percentage of B220⁺ (specific cell marker) B cells compared with irritant-treated mice. Treatment with allergens, but not irritants, resulted in a selective increase in the percentages of CD4⁺ (marker of T-cell help) and CD8⁺ (marker of cytotoxic T-cell) cells expressing the T-cell activation/memory phenotype CD62L^{lo}CD44^{hi}. Flow cytometric analysis of cell phenotype and measuring of expression of T-cell activation/memory markers may provide important information for differentiating allergen- and irritant-induced proliferative responses in the draining lymph nodes (DLNs) of chemically treated mice^{14,15}. Coquette et al. published that divergent *in vitro* interleukine (IL) IL-1 α and IL-8 release profiles characterize the reconstructed human epidermis response to irritants and sensitizers. For the first time, it was demonstrated that the combination of dose-dependent cell viability measurements, with IL-1 α and IL-8 quantification, could provide enough information to allow, in a single assay, *in vitro* detection and discrimination of irritant and sensitizing agents. Whether these initial and early molecular responses of the keratinocytes are sufficient to detect and discriminate all types of compounds, which are sensitising and/or irritating to skin remains to be determined in further studies¹⁶.

Despite these differences, there is evidence that in response to both ACD and ICD Langerhans cells are mobilized and induced to migrate from the skin via afferent lymphatics to DLNs the site of contact¹⁷. With a growing appreciation of the cellular and molecular events that initiate and regulate immune responses to chemical allergens and the induction of sensitization, there have emerged new opportunities to design *in vitro* models. The induction phase of skin sensitization is dependent on the activity of cutaneous DC and, in particular, of epidermal LC. Topical exposure of mice (or humans) to skin sensitizing chemicals causes the mobilization of LC and their directed movement from the skin. These cells, many of which bear high levels of antigen, move via afferent lymphatics to skin DLNs where they accumulate in the paracortical regions as immunostimulatory DC¹⁸.

DC and LC – markers for sensitization pathways

The induction of tyrosine phosphorylation following stimulation with contact sensitizers has been examined in both human¹⁹ and murine²⁰ LC. Whereas freshly isolated human LC failed to demonstrate changes in phosphotyrosine (p-tyr) following exposure to the strong hapten 5-chloro-2-methyl-isothiazolinone plus 2-methylisothiazolinone (MCI/MI), 24-h cultured LC demonstrated a significant increase in p-tyr by flow cytometric quantitation¹⁹. Neisius et al. reported similar increase in p-tyr in murine LC following *in vitro* stimulation with the strong contact sensitizers trinitrochlorobenzene (TNCB) and MCI/MI but not with the irritants SLS or benzoic acid. Although poorly defined, the mechanisms of signalling pathways in LC during hapten-mediated activation may serve as a basis for the development of an *in vitro* test system²⁰.

The potential for changes in expression by DC of messenger RNA (mRNA) for other cytokines or chemokines to serve as useful markers for sensitization testing has recently been explored by Verheyen et al.²¹. Following exposure of CD34⁺-progenitor derived DC to allergens or irritants, mRNA expression for IL-1 β , IL-6 and IL-8, and the chemokines (CCL) CCL2, CCL3, CCL3L1, and CCL4 was examined by real-time polymerase chain reaction (RT-PCR.) Significant interindividual variations in mRNA expression in response to chemical treatment were observed. Based on these results, it was concluded, as did Pichowski et al.²², that allergen-induced IL-1 β mRNA expression in DC was not an appropriate indicator of sensitizing potential. Neither IL-6 nor IL-8 was able to discriminate clearly allergens from irritants. However, at the 24-h time point, mRNA levels for CCL2, CCL3, and CCL4 displayed a two-fold or greater increase relative to control for the allergens, but not for the irritants. The authors suggest that further investigation of these chemokine genes is warranted. Using LC-like DC generated from CD34⁺ cord blood cells, Rougier et al.²³ consistently observed increased expression of cell markers HLA-DR, CD83, and CD86, and decreased levels of E-cadherin (cell adhesion protein) following treatment with a strong allergen but not with an irritant. Tuschl and Kovac²⁴ examined CD86, CD54, and HLA-DR cell surface expression in parallel with the induction of intracellular expression of IL-1 β in peripheral blood mononuclear cells (PBMC)-derived DC. An up regulation of these surface markers was observed in the majority of donors following culture with allergen but not with irritant. However, no clear results were obtained for the induction of intracellular IL-1 β .

Searching for novel markers by holistic expression profiling

Microarrays are the most frequently used technology for transcript profiling allowing detection of thousands of mRNAs in cells or tissue. They have been used to study various aspects of DC biology and function such as differentiation, maturation, and migration²⁵, as well as to elucidate DC intracellular signalling pathways for application to potential immunomodulation strategies²⁶. While expression profiles induced by various stimuli such as viral and bacterial pathogens²⁷, lipopolysaccharide²⁸, and cytokines²⁹ have been monitored; there are limited reports of genome-wide analysis of the changes induced in human DC upon contact with skin sensitizers.

In addition the genes not previously associated with skin sensitization or DC biology, such as AKR1C2, DUSP6, and QPCT, were identified and their role in those mechanisms was established, therefore widening the pool from which potential markers can be selected. It is anticipated that similar genomics studies using LC surrogates with different allergens and irritants may provide additional gene targets not previously discovered. However, genes that are selected as markers for skin sensitization must fit the criterion for dynamic range, robustness, sensi-

tivity, and selectivity for a predictive model and cover a range of chemical classes³⁰.

PBMCs-DCs were treated for 24h with various concentrations of chemicals and in each instance the expression of up to 60 genes was examined by RT-PCR analysis. Consistent allergen-induced changes in the expression of many genes were observed and further prioritization of the targets was conducted by analysis of the same genes in DCs treated with non-sensitizing chemicals to determine their specificity for skin sensitization. RT-PCR analyses of multiple chemical allergens, irritants, and non-sensitizers have identified 10 genes that demonstrate reproducibly high levels of selectivity, specificity, and dynamic range consistent with providing the basis for robust and sensitive alternative approaches for the identification of skin sensitizing chemicals³¹.

Conclusion

There is currently some enthusiasm for the application of microarray technology in identifying new gene candidates which expression could serve as potential markers of sensitization effect of chemicals. The ideal candidate in this context would be a gene that displays dramatic increase in expression following encounter of DC with a chemical allergen, fails to show similar changes in expression in response to other stimuli (irritants), and codes for a protein that correlates mechanistically and quantitatively with the acquisition of skin sensitization.

It remains to be seen whether the novel genes will be identified that provide the transcriptional dynamic range and selectivity required for use in *in vitro* prediction models.

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L06

HEALTH RISK EVALUATION OF EXPOSURE TO MIXTURES OF CHEMICALS IN OCCUPATIONAL AIR

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Key words: occupational environment, chemical mixtures, biological effects, interactions, risk evaluation

Introduction

Workers are regularly exposed to multiple chemical agents¹. The health criteria relating to these exposures in workers generally do not take into account the possibility of interactions between these contaminants, which may result in a change in toxicity. However, priority should be given to this factor². Regarding general environment, both the U.S. Environmental Protection Agency³, and the Agency for Toxic Substances and Disease Registry⁴ published guidance documents on this topic in recent years.

The central question involves the nature of the possible interactions. Regulations and common industrial practices address the question of interactions by hypothesizing, that toxic effects are additive. In some cases, this hypothesis may lead to an underestimation or an overestimation of the actual risk⁵. As in the ACGIH approach⁶ (American Conference of Governmental Industrial Hygienists), the Czech regulation prescribes that when two or more hazardous substances are present in workplaces and have similar effects on the same organ of the human body, their effects should be considered additive, unless established otherwise. The mechanism of toxic action is not taken in account.

The power of additive effect is estimated according following formulae:

$$Rm = K_1/PEL_1 + K_2/PEL_2 + \dots + K_n/PEL_n$$

where Rm is the mixture exposure index (the sum of the K/PEL ratios from the individual components of the mixture), K the measured concentration of a substance at a workplace, PEL the permissible time-weighted average exposure value in accordance with Czech regulation, $1,2,\dots,n$ the indices designating the various individual sub-

stances in the mixture. If Rm is greater than one, the time-weighted average exposure value of the mixture of the substances is exceeded.

Presented work is a part of wider research project. The project is oriented on completion and validation of user-friendly Czech toxicological database (CTD) for identification the possible additive effects in mixtures presented in Czech occupational environments and listed in Czech occupational regulation.

Methodology

The Quebec toxicological database (QTD) was used as a starting point for formation of CTD^{7,8}. Totally of 350 important substances were selected from QTD. For those selected compounds which were listed in QTD but not in Czech regulation, the ACGIH limits were accepted. Both databases include information on toxicokinetics and target organs. This information was taken mainly from secondary sources: ACGIH⁶, OSHA⁹, “Proctor and Hughes’ chemical hazards of the workplace”¹⁰, “Toxicologie industrielle et intoxications professionnelles”¹¹, NIOSH², and WHO/IPCS/ILO¹². To evaluate carcinogenic properties, the data from IARC¹³ and from the DFG¹⁴ were taken into account. The “CSST’s Service du répertoire toxicologique”, a toxicological on-line database developed and maintained by the Quebec Occupational Health and Safety Commission (CSST)¹⁵ was consulted as well.

In relation with QTD, the types of effects on the organs and the mechanisms of toxicity were also determined⁷. The target organs in QTD were identified in relation to the exposure concentration of the substances in the air, when this information was available. In humans, the target organs and effects were determined only for realistic exposure concentrations corresponding, as a maximum, to the STEL (where the “Short-Term Exposure Limit” is the maximum concentration to which workers can be exposed for a period of 15 minutes), to the ceiling value (CV), or to 5 times the TWA (time weighted average, which represents the average concentration of a given chemical to which workers can be exposed for normal 8-hour work-days, 5 days a week). Animal data were used when no human data were available. In this case, the target organs and the effects were determined only for exposure concentrations corresponding to a maximum 100 times the TWA or the CV (factor of 10 for extrapolation of the LOEL [lowest observed adverse effect level] towards the NOAEL [no observed adverse effect level] and 10 for the differences between the species)⁷. For CTD, all data mentioned above were modified according guidelines for Czech regulation, e.g. for PEL (TWA) and NPK (CV).

To facilitate the assessment of the possibility of toxicokinetic interactions, the data on the main metabolites

(with their CAS number when available), on the absorption and elimination rates, on the distribution, and on the accumulation of the substances in the organs were included. According to its toxicological characteristics, each substance was assigned to one or more of the 32 classes of similar biological effects⁷. This concept of QTD was necessary to accept because it constitutes the basis for the additivity rule in calculating the R_m .

Computer program was developed for creating work sheets as well as data sheets for the user. The software used for CTD was Microsoft® Access 2003 and Microsoft® Internet Explorer 6. The database disposes of several tables: names of substance, CAS numbers and toxicokinetic characteristics, effects of substances on the body and target organs or systems, as well as intermediate tables that link the main tables. All information in CTD is in both, Czech and English language. By choosing the database, as well as its structure as a tool, easy export is possible in an Internet-adaptable format. Eventually, it will be possible to consult the database via the Internet.

Results and discussion

Since relevant human toxicological data are rare, it is not surprising that the procedures adopted by different countries to define the admissible exposure values (AEVs) differ. This is why the Canadian authors and consequently the Czech authors took the toxicological data from several secondary references, despite the fact that the majority of Czech AEVs are based on the ACGIH's TLVs (ref.⁶).

The mechanism of non-carcinogenic effects for many substances is rarely described in the secondary references. In addition, this description is quite often rather general. Since one of specific objectives of databases was to determine the classes of similar effects from the list of selected effects, more general effects (for example, damage to the lower respiratory tract) had to be combined with more specific effects (for example, pulmonary edema) since the authors could not disregard any possibility of interaction between two substances for the simple reason that they do not currently know a given substance's specific effect on an organ. For evaluation of carcinogenic effects primarily the information from ACGIH and IARC were used.

From the list of selected effects (overall 83 effects) the list of 32 classes of similar biological effects was constructed. The effects retained do not all have the same degree of specificity, and the list contains rather specific effects, or rather general ones. Furthermore, the general effects often encompass effects that are more specific. As a precaution, the definition of the similarity of effects in the organs must therefore be broad enough to cover, for example, a possible interaction between a substance for which detailed human toxicological data are available, and another substance for which even animal toxicological data is scarce and for which the TLV was estimated by physico-chemical analogy with another substance. This is why CTD have defined all effects on an organ or a system as being similar, irrespective of the degree of specificity of

this effect. According to its toxicological characteristics, each substance is assigned to one or more of the 32 classes of similar biological effects.

Specific attention has been given to Class no. 32, which contains all the carcinogenic and mutagenic effects. This is because for the majority of carcinogens, information is lacking on the human organs affected, since data on their carcinogenicity come from animal studies. It is difficult to predict which organs may be affected in humans based on results of animal studies. In the majority of cases, tumors are observed in multiple sites in animals. When multiple sites exist, we simply identified the generic effects as *CANCER*. The additional problem that is raised involves additivity between the carcinogenic and non-carcinogenic effects on a given organ or system. The calculation of an R_m in this case could suggest that the concentrations of any of the substances could be reduced in the workplace in order to reach an R_m value of less than 1 and thus comply with the Czech regulation. A specific efforts must, however, always be made to reduce exposure to carcinogens. Consequently, QTD/CTD did not consider it appropriate to allow the software to calculate an R_m for a mixture of two or more carcinogens or for a mixture of carcinogens and non-carcinogens affecting the same anatomic site. When the user queries the computer system about such mixtures, a warning is displayed indicating that one or more of the substances in the mixture are carcinogenic. At the same time, the tool is deactivated for calculating the R_m .

The information entered in the different tables in the database was organized to create a separate data sheet for each of selected substance. The application essentially consists of a series of HTML pages. The main page is the search form. There are twelve drop-down zones on this page, and from each, a substance can be selected. Once a selection has been made, the application shows the PEL (TWA) level or the NPK (CV, if applicable), as well as the list of classes for each of the substances. Finally, the exposure value measured in the workplace is entered. In this way, the application not only determines whether the additivity rule should apply or not, but also calculates all the possible combinations of R_m in relation to the chosen substances. Hyperlinks also provide very easy access to the list of classes as well as to the individual toxicity data sheets for the substances.

Conclusion

The database is intended for practitioners working in branches of industrial hygiene, industrial toxicology and occupational medicine. The database provides basic toxicological characteristics, predicts the potential additivity among components of particular mixture and enables identification of substances for which R_m formula (mixture exposure index) should be applied. If applicable, the database calculates correspondent R_m . Prediction of possible additive effects can markedly improve assessment of occupational health risks.

To the future we assume to extent the number of substances (approximately 200 other compounds) and specify the type of interaction for mixtures most likely to be found in the occupational environment, for which primary literature data are available. In addition the usefulness of the tool will be tested in selected industrial settings.

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L07

CHROMOSOMAL ABERRATIONS AND CHROMIUM BLOOD LEVELS IN RELATION TO POLYMORPHISMS OF *GSTM1*, *GSTT1* AND *GSTP1* GENES IN WELDERS

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Key words: chromium exposure, chromosomal aberrations, and polymorphisms in *GSTM1*, *GSTT1* and *GSTP1* genes

Introduction

Welders are exposed to chromium (III) and (VI) and to a lesser extent to polycyclic aromatic hydrocarbons (PAHs). Hexavalent form is used in many industrial processes, including corrosion inhibition, chrome plating, stainless steel production and metal welding^{1,2}. Experience with excessive exposure at the work place has shown that Cr can act as an acute irritant, as an allergen and as a carcinogen^{2,3}. In our previous study⁴ we focused on lung cancer frequency in relation to occupational and environmental chromium exposure and we found out that chromium increases the lung cancer occurrence especially in chromate workers. Cr(VI) produces DNA strand breaks, DNA-DNA and DNA-protein cross-links and modifies nucleotides, such as 8-hydroxyguanine. The latter mode of action indicates oxygen radical formation^{5,6}. Highly reactive intermediates such as Cr (V) and Cr (IV) formed due to cellular Cr (VI) reduction are primarily responsible for the observed genotoxicity⁷. Cellular reducing agents that may be important for Cr (VI) reduction include ascorbate and sulfhydryl compounds such as cysteine and glutathione⁸⁻¹². Glutathione transferases catalyze the reaction of glutathione with a wide variety of compounds and this reaction is a first step in a detoxification process. Some

studies report relationship between level of chromosomal aberrations and polymorphisms of glutathione S-transferase (*GST*) genes. *GSTT1*-null genotype was associated with significantly higher aberration frequencies¹³.

Genetic changes are considered as one of the most important steps in carcinogenesis¹⁴. Cytogenetic analysis of peripheral lymphocytes is traditionally used to evaluate an exposure to clastogens¹⁵ and increased levels of cytogenetic parameters such as chromosomal aberrations (CA) may reflect an increased risk of cancer¹⁶. The purpose of this study was to conduct a biomonitoring study in welders by employing CAs in peripheral blood lymphocytes as a marker of genotoxic effect and the blood levels of chromium as a marker of internal exposure. Association with genetic polymorphisms in genes encoding metabolising enzymes GSTs as biomarkers of individual susceptibility to procarcinogens was assessed as well.

Material and methods

Subjects and sampling

The study was performed on a population of 31 welders and 31 control individuals that were not exposed to any known carcinogens or mutagens. The detailed characteristics of members of observed groups are shown in Table I. Each person included into the study signed informed consent. Ethical approval based on the Declaration of Helsinki was provided by the Institute of Medical Biology, Jessenius Faculty of Medicine in Martin, Slovak Republic.

Chromosomal aberrations assay

CAs were analyzed in peripheral blood lymphocytes in two separate tubes using previously described method¹⁷. Structural CAs include chromosomal breaks and exchanges visible in arrested metaphase-stage cells and they were divided into chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs).

Determination of chromium in the blood

Chromium analysis in the blood was performed using the atomic absorption spectrophotometer AAS Varian Spectr. AA 30 P. All samples were assessed in duplicate or in triplicate, if significant differences occurred.

Assessment of genetic polymorphisms in genes encoding GSTs

The *GSTM1* (deletion), *GSTP1* (alleles ile/val in codon 105 of exon 5) and *GSTT1* (deletion) polymorphisms were analysed by the multiplex PCR method¹⁸.

Table I
Characteristics of observed groups

	Exposed group (N=31)	Control group (N=31)
Age±S.D. (years)	39.83±2.07	37.72±2.71
Employment±S.D. (years)	9.33±1.66	–
Gender M	31	31
Smoking S/NS	19/12	19/12

M – males, S – smokers, NS – non-smokers, S.D. – standard deviation

Statistical analysis

Statistical analysis was carried out with the use of Statgraphics program, version 7 (LEAD Technologies, Manugistics, Cambridge, MA). For data without normal distribution Mann-Whitney U-test were used to test the differences between the groups. Spearman correlation analysis was employed to calculate the correlation between individual parameters. Kruskal-Wallis test was performed to evaluate associations between biomarkers and specific genotypes. Heterozygous (ile/val) and variant (val/val) genotypes were considered as one group because of low number of variant (val/val) individuals.

Results

There was no significant difference ($P>0.05$) in the number of aberrant cell between the exposed $1.93\pm 0.17\%$ (\pm SE) and control $1.54\pm 0.12\%$ groups. Chromatide type - aberrations (CTA) were higher in the control group $1.03\pm 0.10\%$ in comparison with exposed one $0.70\pm 0.13\%$. The difference was not significant ($P>0.05$). Biologically more serious chromosome type - aberrations (CHSA) were significantly higher ($P<0.005$) in the exposed group $1.22\pm 0.20\%$ versus control group $0.54\pm 0.12\%$. Frequencies of CAs, CTAs and CAs total observed in exposed and control groups are presented in the Table II.

Frequencies of CSA in individuals with wild-type ile/ile GSTP1 genotype were significantly lower ($p<0.05$) than in those with variant val/val and heterozygous ile/val genotypes ($1.4\%\pm 0.13$ versus 1.88 ± 0.20) (Table III).

Polymorphisms in *GSTM1* and *GSTT1* did not modulate the frequencies of CAs, CTA and CSA. We did not find any correlation between chromium level in the blood

and chromosomal aberrations.

Discussion

Our previous studies^{4,12} as well as many other epidemiological studies^{19,20} showed that workers in ferrochromium industry exhibit an excessive risk for chromosomal injury and lung cancer. Other studies relate this ability to evidenced genotoxicity of chromium and one mode of genotoxic action may proceed via reactive oxygen species that are formed during chromium activation²¹. Glutathione may act as an antioxidant, participating in reduction of chromium species and in diminishing formation of oxygen radicals. The aim of this study was to conduct a biomonitoring study in welders by employing CAs in peripheral blood lymphocytes as a marker of genotoxic effect in relation to genetic polymorphisms of genes encoding principal metabolizing enzymes *GSTM1*, *GSTT1* and *GSTP1* as biomarkers of individual susceptibility to procarcinogens.

Our results showed no significant difference in frequency of CAs, CTA and CSA among different genotypes of *GSTM1* and *GSTT1*. These findings are in agreement with previous conclusions of Lee et al.²². They studied the relationship between differences in plasma antioxidant capacity, and genetic polymorphisms in detoxification (*GSTM1*, *GSTT1*, and *NQO1*) and DNA repair (*OGG1*, *XRCC1*) genes and levels of sodium dichromate-induced DNA damage. In their hands *GSTM1* and *GSTT1* genotypes had no significant effect on levels of either background or dichromate-induced DNA damage suggesting that neither *GSTM1* nor *GSTT1* play a rate-limiting role in metabolic detoxification or activation of sodium dichromate. Alternatively compensatory mechanisms may exist to overcome GST deficiency, and their study cannot com-

Table II
Types of structural chromosomal aberrations and chromium level in the blood in studied populations

	CAs±SEM [%]	CTA±SEM [%]	CSA±SEM [%]	Chromium±S.D. [$\mu\text{mol l}^{-1}$]
Exposed group (N=31)	1.93±0.17	0.71±0.13	1.22±0.2**	0.05±0.04
Control group (N=31)	1.54±0.12	1.33±0.11	0.54±0.12	0.03±0.02

CAs – chromosomal aberrations (total), CTA – chromatide-type aberrations, CSA – chromosome-type aberrations, ** ($P<0.005$), SEM – standard error of mean, S.D. – standard deviation

Table III

Total chromosomal aberrations, chromatide-type and chromosome-type aberrations stratified for polymorphism of *GSTM1*, *GSTT1* and *GSTP1* genes

	Exposed group		
	CAs±SEM [%]	CTA±SEM [%]	CSA±SEM [%]
<i>GSTM1 plus</i>	2.01±0.27	0.81±0.21	1.19±0.28
<i>null</i>	1.93±0.26	0.61±0.16	1.46±0.31
<i>GSTT1 plus</i>	2.03±0.19	0.78±0.15	1.22±0.23
<i>null</i>	1.50±0.29	0.25±0.25	1.25 ±0.25
<i>GSTP1 ile/ile</i>	1.87±0.27	0.47±0.31	1.40±0.13*
<i>ile/val, val/val</i>	1.99±0.24	0.86±0.23	1.88±0.20

SEM – standard error of mean, * ($P < 0.05$)

pletely exclude a role for *GSTM1* and *GSTT1* in the metabolic activation of Cr(VI).

On the other hand we found that frequencies of CSA in individuals with wild-type *ile/ile* *GSTP1* genotype were significantly lower than in those with variant *val/val* and heterozygous *ile/val* genotypes. It could indicate the possible role of *GSTP1* in detoxification of oxidative stress products. Correlation between genetic polymorphism of *GSTP1* and oxidative stress markers was published by Vibhuti²³ and complies with our findings.

The goal of most studies dealing with polymorphism of detoxification and DNA repair genes is to look for the tools for monitoring of individual susceptibility to mutagens and carcinogens in order to protect susceptible individuals. However, functional consequences of the studied polymorphisms have not yet been explored. Our study represents a piece in the puzzle of knowledge needed for developing of optimal and effective monitoring of environmental and occupational exposure in respect to individual characteristics.

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L08

RISK - PROTECTION OF VITAMINS

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Key words: vitamin, micronutrient, recommendation, daily intake, deficiency

Introduction

Vitamins are organic substances, which the organism needs for its function. They are needful for optimal enzymatic activity. Many of them are indispensable activators of enzymatic systems; they are part of enzymes or directly react in metabolic pathways. Vitamins have essential importance for appropriate development and function of organism. Another use of vitamins is in protection before harmful effect of free radicals, in enhancement organism resistance towards stress and also towards infections. Intake of vitamins could influence health condition of population. Incidence of “civilization disease” has close relationship to nutrition, its quantitative and qualitative aspect¹.

Variety of names for recommendation intake are used such as Recommended Daily Amounts, Recommended Daily Allowances (RDA), Recommended Daily Intakes, Recommended Dietary Intakes and Recommended Nutrient Intakes (RNI). The RNI is the intake level sufficient to meet the daily nutrient requirements of almost all (97.5 %) apparently healthy individuals in a specific life-stage and gender group. RNI does not include recommendation for condition with increasing demand such as illness physical and psychical stress, but it is recommendation for homogeneous group of population whereas really demand of individual could vary from average. It is based on an estimated average nutrient requirement (EAR) plus two standard deviations above the mean: $RNI = EAR + 2SD_{EAR}$ ²

Water-Soluble Vitamins³⁻⁷

To group of water-soluble vitamins belong B-complex vitamins and vitamin C. By the toxicity water-soluble vitamins are safe.

B-complex vitamins

There are Vitamin B₁ – *Thiamin*, Vitamin B₂ – *Riboflavin*, Vitamin B₃ – *Niacin*, Vitamin B₅ – *Pantothenate*,

Vitamin B₆ – *Pyridoxine*, Vitamin B₁₁ – *Folate*, Vitamin B₁₂ – *Cyanocobalamin* and Vitamin H – *Biotin* in this group of B-vitamins. All of these vitamins have important function in metabolic pathways.

All of vitamins B-complex has important function in metabolic pathways. Classical deficient syndromes, for examples Beri-Beri (thiamin deficiency) or Pellagra (niacin deficiency) is relatively rare in developed countries. But there is evidence of widespread sub-clinical deficiency of these vitamins including these symptoms: fatigue, apathy, nausea, diarrhea, cheilosis, angular stomatitis, glossitis, seborrhea dermatitis, muscular pain, anemia, peripheral neuritis, depression, and convulsion. High doses of folate may cause moderate gastrointestinal disorders. Chronic high doses of niacin (therapeutically useful in lowering serum cholesterol) can lead to hepatotoxicity as well as dermatologic manifestations. So it was determine upper limit of 35 mg day⁻¹. Niacin could be synthesized from dietary tryptophan. About 60 mg dietary tryptophan is equivalent to 1 mg preformed niacin.

Vitamin C – Ascorbic Acid

Vitamin C involved in oxidation and reduction reactions in metabolic pathways of carbohydrates, amino acids, fatty acids, prostaglandins, steroid hormones and metals. Classical deficient syndromes, scurvy is relatively rare in developed countries. A common feature of vitamin C deficiency is anaemia, due impaired iron and perhaps folate metabolism. A lesser degree of hypovitaminosis C can manifest in symptoms such as weakness, lassitude, fatigue, increased susceptibility to infections. Intoxication is extremely rare. High doses (10–20 g day⁻¹) may cause at some individuals disquiet, sleep disturbances and oxalate kidney stones³.

Fat-Soluble Vitamins³⁻⁷

Absorption of these vitamins is depending on absorption of lipids. Occurrence of fat-soluble vitamins deficiency is rather as a consequence of malabsorption (for example: gliadin intolerance, gastrointestinal I¹ inflammatory, obstructive icterus) than their insufficiency in food.

Vitamin A – Retinol

Vitamin A is an essential nutrient needed in small amounts by humans for the normal functioning of the visual system, growth and development, maintenance of epithelial cellular integrity, immune function and reproduction. These dietary needs for vitamin A are normally provided for as preformed retinol and provitamin and carotenoids. Efficiency is expressed as Retinol equivalent (RE): 1 µg RE = 1 µg retinol = 0.6 µg β-carotenoids = 3.3 IU.

Table I
Recommended Nutrient Intakes for water-soluble vitamins part 1 *

Recommendation of FAO/WHO		Thiamin [mg]	Riboflavin [mg]	Niacin [mg] (a)	Pyridoxine [mg]	Pantothenate [mg]
Infants	0–6 months	0.2	0.3	2 (b)	0.1	1.7
	7–11 months	0.3	0.4	4	0.3	1.8
Children	1–3 years	0.5	0.5	6	0.5	2
	4–6 years	0.6	0.6	8	0.6	3
	7–9 years	0.9	0.9	12	1.0	4
Adolescents	10–18 y. Males	1.2	1.3	16	1.3	5
	10–18 y. Females	1.1	1.0	16	1.2	5
Adults	19–65 y. Males	1.2	1.3	16	1.3/1.7(b)	5
	19–50 y. Females	1.1	1.1	14	1.3	5
	51–65 y. Females	1.1	1.1	14	1.5	5
Older adults	65+ Males	1.2	1.3	16	1.7	5
	65+ Females	1.1	1.1	14	1.5	5
	Pregnancy	1.4	1.4	18	1.9	6
	Lactation	1.5	1.6	17	2.0	7

(a) niacin equivalent NE : 1 mg NE = 60 mg dietary tryptophan = 1 mg preformed niacin, (b) 1.3 mg for 19–50 years and 1.7 mg for 50+ years

Table II
Recommended Nutrient Intakes for water-soluble vitamins part 2 *

Recommendation of FAO/WHO		Biotin [μ g]	Folate [μ g]	Vit. B ₁₂ [μ g]	Vit. C [mg]
Infants	0–6 months	5	80	0.4	25
	7–11 months	6	80	0.5	30
Children	1–3 years	8	160	0.9	30
	4–6 years	12	200	1.2	30
	7–9 years	20	300	1.8	35
Adolescents	10–18 y. Males	25	400	2.4	40
	10–18 y. Females	25	400	2.4	40
Adults	19–65 y. Males	30	400	2.4	45
	19–50 y. Females	30	400	2.4	45
	51–65 y. Females	30	400	2.4	45
Older adults	65+ Males	–	400	2.4	45
	65+ Females	–	400	2.4	45
	Pregnancy	30	600	2.6	55
	Lactation	35	500	2.8	70

* For the purpose of these composite tables of RNI values, the body weights used was derived from the 50th percentile of NCHS data until adult weights of 55 kg for females and 65 kg for males were reached. The weights used are the following: 0–6mo = 6 kg, 7–12mo = 8.9 kg, 1–3ys = 12.1 kg, 4–6ys = 18.2 kg, 7–9ys = 25.2 kg, 10–11ys M = 33.4 kg, F = 34.8 kg, 12–18ys M = 55.1 kg, F = 50.6 kg, 10–18ys M = 55.1 kg, F = 50.6 kg, 19–65ys M = 65 kg, F = 55 kg

Hypovitaminosis A is often expressed as only nausea. Other typical symptoms like night blindness, xerophthalmia, hyperkeratosis, hepatosplenomegaly, metaplasia of mucous membrane and anaemia are rare in developed coun-

tries. Vitamin A as well as vitamin D is the only vitamin which may cause a hypervitaminosis. High doses of vitamin A are toxic and cause visual disorders; they are teratogenic (dosage of 7500 μ g day⁻¹). Upper limit is 2800–

Table III
Recommended Nutrient Intakes for fat-soluble vitamins *

Recommendation of FAO/WHO		Vit. A(a) [μg] RE	Vit. D [μg]	Vit. E [mg] α -TE	Vit. K(d) [μg]
Infants	0–6 months	375	5	2.7 (c)	5
	7–11 months	400	5	2.7 (c)	10
Children	1–3 years	400	5	5	15
	4–6 years	450	5	5	20
	7–9 years	500	5	7	25
Adolescents	10–18 y. Males	600	5	10	35–65
	10–18 y. Females	60	5	7.5	35–65
Adults	19–65 y. Males	60	5/10 (b)	10	65
	19–50 y. Females	500	5	7.5	55
	51–65 y. Females	500	10	7.5	55
Older adults	65+ Males	600	15	10	65
	65+ Females	600	15	7.5	55
	Pregnancy	800	5	(c)	55
	Lactation	850	5	(c)	55

(a) Vitamin A values are “recommended safe intakes” instead of RNIs. Recommended safe intake as μg RE/day; 1 μg retinol = 1 μg RE = 0.6 mg β -carotenoids = 3.3 IU, (b) 5 μg for 19–50 years and 10 μg for 50+ years, (c) For pregnancy and lactation there is no evidence of requirements for vitamin, (d) The RNI for each group is based on a daily intake of 1 μg kg^{-1} of phyloquinone

Table IV
Referential range for vitamins C, A, E, D - upper tolerable intake level

	Vitamin C – UL [g day^{-1}]	Vitamin D – UL [$\mu\text{g day}^{-1}$]	Vitamin E – UL [$\mu\text{g day}^{-1}$]	Vitamin A – UL [$\mu\text{g day}^{-1}$]
Children to 3 years	400	50	200	600
Children to 8 years	650	50	300	900
Children to 13 years	1200	50	600	1700
Adolescents	1800	50	800	2800
Pregnancy	1800	50	800	2800-3000
Adults	2000	50	1000	3000

3000 $\mu\text{g day}^{-1}$.

Vitamin D – Cholecalciferol

Vitamin D is required to maintain normal blood levels of calcium and phosphate that are in turn needed for the normal mineralization of bone, muscle contraction, nerve conduction and general cellular function in all cells of the body.

The early signs of vitamin D deficiency are decreased serum concentration of calcium resulting from depressed absorption of calcium from the intestine leading to depletion of calcium from bones. Later signs are inadequate skeletal mineralization (rickets or osteomalacia), bone pain, severe bone deformities and alterations in muscle metabolism and respiratory function. Symptoms of acute and chronic vitamin D intoxication include nausea, diarrhea, polyuria, weight loss, hypercalcemia, hypercalciuria

and eventually nephrocalcinosis, decreased renal function or calcification of soft tissues. Upper limit for vitamin D intakes is 50 $\mu\text{g day}^{-1}$.

Vitamin E – Tocopherol

The biological action of vitamin E results principally from its antioxidant properties, it prevents propagation of the oxidation of unsaturated fatty acids by trapping free radicals. Vitamin E also has anti-inflammatory effect and modulates immunity response.

Vitamin E deficiency causes oxidation of PUFA, which leads to structural and functional damage to cellular membranes. Vitamin E has very low toxicity.

Vitamin K – Fylochinon

Vitamin K is essential cofactor for synthesis blood

clotting factors. The most common type of deficiency is from malabsorption or using antibiotics or anti-vitamin K drugs (warfarin). Deficiency results in a bleeding syndrome. Clinical features may range from mild bruising to severe life-threatening haemorrhage. No adverse signs have ever been reported from large oral doses of vitamin K.

Conclusion

Vitamins are essential nutritional elements which affect different biochemical and metabolic processes in the human organism. Adequate concentrations of individual vitamins in biological tissues are a necessary pre-requisite for metabolism of basic nutrients – glucose, lipids and proteins and for energetic metabolism. In addition they influence many physiological functions and intervened pathobiochemical processes. Besides positive projective vitamin effects on health, possible health risks of overwhelming consumption of some vitamins must be emphasized.

A recommended vitamin intake in the Czech Republic, EU as well as the US is provided in recommended dietary allowances (RDA). A definition of upper tolerable intake level (LA), NOAEL and LOAEL allowances are included into the recommended vitamins intake (Tables I–IV).

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L09

USE OF PHOTOAFFINITY LABELING FOR STUDY OF CYTOCHROMES P450, TOXICOLOGICALLY IMPORTANT ENZYMES

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Key words: Cytochrome P450, photoaffinity probe, metabolism, access channel, three dimensional structure

Introduction

Cytochromes P450 (CYPs) comprise a superfamily of b-type heme proteins, characterized by their cysteinyl-heme ligation. CYPs belong to the most extensively studied enzymes involved in the metabolism of foreign compounds (xenobiotics) e.g. drugs, pollutants, dyes, carcinogens, as well as endogenous compounds (steroid hormones, fatty acids, prostaglandins)¹. Because of the clinically important role of CYPs in the human body, understanding of the principle of CYP substrate binding is crucial. Moreover, as the carcinogen activation is mediated by CYPs, the research of CYP structure-function relationships is important to reveal the first stage of the carcinogenesis process at the molecular level. Recent knowledge of this process is limited to three-dimensional (3D) structures for mammalian CYPs derived from X-ray crystallography of truncated chimeric CYP protein constructs. Therefore, the possibility exists that in the CYP crystal the CYP native structure is perturbed by the protein changes prior the microsomal CYP crystallization (e.g. elimination of N-terminal transmembrane domain, addition of C-terminal 4xHis tag) and/or caused by the process of crystallization itself (e.g. CYP dimerization). Hence, it is necessary to utilize other approaches to validate structure-function data of mammalian enzymes.

Photoaffinity labeling, a chemical modification technique, was proved to be useful for structure study of membrane-bound proteins, difficult to examine by other techniques. It makes use of the highly reactive intermediates generated by photolysis from photolabile substrate analogues. Azides, diazirines, and benzophenones are most frequently introduced into the substrate molecule, providing the photoaffinity probe. Several photolabile substrates have been used in the past as effective photoaffinity ligands of mammalian and bacterial CYPs². Recently,

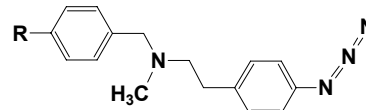


Fig. 1. Structure of desmethylbenzphetamine bifunctional photolabile probe. Probe *I* - R = -NH₂; probe *II* - R = -N₃

CYP2B4 putative access channel was identified by a heterobifunctional photoaffinity probe, *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-aminophenethylamine (*I*) (for structure see fig. 1) and the 3D model of this CYP verified³. In the present study, we examined *I* as photolabile probe for labeling of the access channel region of CYP3A6, the CYP that serves as a model of highly homologous human microsomal cytochrome CYP3A4. In the human body, this CYP is involved in metabolism of a majority of drugs and a large number of carcinogens. Although crystals of N-terminal modified CYP3A4 chimeras were prepared, and 3D structure models developed⁴, the problem, how the substrate reaches the active center deeply buried in the CYP macromolecule remains to be solved.

Materials and methods

Cytochrome P450A6 preparations

Liver microsomal (Ms) samples were prepared from rabbits treated with rifampicin (RIF). Cytochrome CYP3A6 was purified from the microsomal fraction basically as described by Haugen and Coon⁵. The final electrophoretically homogeneous preparation had a specific content of 7.6 nmol of CYP/mg protein as determined on the basis of the absorbance of reduced CYP complex with CO at 450 nm (ref.⁶).

Cytochrome P450 difference spectra

CYP difference spectra with photolabile probes⁷, *I* and *II* (see fig. 1), were recorded on a Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany). CYP3A6 samples were diluted with the phosphate buffer (0.1 M K/PO₄, pH 7.4, with 20% glycerol) to 2 μM. The contents of the sample cuvette was treated with a gradually increasing amount of the probe tested (final concentration 2–700 μM), while the same volume of solvent (water) was added to the reference cuvette. The difference spectra were recorded from 350 to 500 nm.

Inhibition studies

The inhibitory effect of the probe **I** (10–400 μM) on CYP3A6 specific metabolic activity: *O*-dealkylation of dibenzylfluorescein (DBF)⁸, *O*-dealkylation of 7-benzyl-oxy-4-trifluoromethylcoumarin (BFC)⁸ and 6 β -hydroxylation of testosterone⁹ was determined with Ms-RIF and purified CYP3A6 in reconstituted system. Samples were diluted with the phosphate buffer (0.1 M K/PO₄, pH 7.2 with 20% glycerol) to the final CYP concentration of 10–200 nM. The reaction mixtures containing up to 600 nM DBF, 93 μM BFC or 50 μM testosterone (final concentrations) were incubated at 37 °C for 30, 10 or 15 minutes, respectively. Reaction products of BFC and DBF assays were analyzed using a Perkin–Elmer LS-5B spectrofluorimeter. The amount of 6 β -hydroxytestosterone produced from testosterone was determined on a C18-HPLC column (Nucleosil 100-5, Macherey-Nagel) using 70% (v/v) methanol as a mobile phase.

Homology modeling and docking

The 3D structure of rabbit CYP3A6 was built based on the crystal structures of CYP3A4 (PDB code – 1TQN) using Modeller 6.2 and Clustal X software³. Probe **I** was docked into the CYP3A6 model as described previously³. Briefly, for the docking Autodock 3.05 software has been employed, using the genetic algorithm method, with 27 000 000 generations and 200 populations, with 20 runs for the heme anchored probe **I**. The conformation having the lowest energy has been chosen as the result.

Results and discussion

Our previous study proved that the heterobifunctional desmethylbenzphetamine probe **I**, containing azido- and amino-groups on the opposite end of the molecule, is use-

ful for mapping the substrate access channel region of CYP2B4 (ref.³). The probe **I** is designed to be anchored via its amino-group to the heme while its azido-group is able (after photoactivation) to modify amino acids in a defined distance from the heme. To use this probe **I** for CYP3A6 structural studies, the active center binding, as well as the heme ligation of the probe, should be examined.

In order to estimate the probe ability to enter the active site of the CYP3A6, its inhibitory potential towards three marker catalytic activities of the CYP3A subfamily was examined. The assays were performed with the microsomal fraction as well as with a reconstituted system consisting of purified CYP3A6 and NADPH:CYP reductase. All assays were conducted with the probe **I** and ketoconazole, a CYP3A selective inhibitor. The inhibitory potential of tested compounds was expressed as IC₅₀ values (see Table I). All assayed systems with exception of DBF dealkylation in microsomes had low IC₅₀ values (in nM range) for ketoconazole. Thus, they are specific for CYP3A6 activity. The probe **I** showed a concentration dependent inhibition of all CYP3A6 specific activities. As judged from its IC₅₀ values, the probe **I** is a moderate CYP3A6 inhibitor. The detected inhibitory ability of the probe **I** suggests its binding in the active center of CYP3A6. Moreover, these experiments supported ketoconazole to be a proper competing compound to expel the probe from the binding site of CYP3A6 for competition studies.

To further characterize the interaction of the probes **I** with CYP3A6, experiments using the difference spectroscopy were carried out. The binding of probes **I** and **II** (aminoazide and diazide) in RIF-induced liver microsomes was examined. From the difference spectra (not shown) it is clear that both probes interact with CYP present in microsomal sample. While probe **II**, used for comparison, elicited formation of a typical Type I substrate spectrum

Table I
Data of inhibition experiments

CYP assay	CYP sample	Tested compound	CYP [nM]	Substrate [μM]	IC ₅₀ [μM]
DBF dealkylation	Ms	ketoconazole	10	0.20	10.80
DBF dealkylation	Ms	probe I	10	0.20	30.40
DBF dealkylation	RS	ketoconazole	40	0.60	0.04
DBF dealkylation	RS	probe I	40	0.60	31.10
BFC dealkylation	Ms	ketoconazole	50	27.00	0.03
BFC dealkylation	Ms	probe I	50	27.00	105.00
BFC dealkylation	RS	ketoconazole	120	93.00	0.90
BFC dealkylation	RS	probe I	120	93.00	n.d.
Testosterone hydroxylation	Ms	ketoconazole	200	50.00	0.09
Testosterone hydroxylation	Ms	probe I	200	50.00	110.30

BFC – 7-benzyl-oxy-4-trifluoromethylcoumarin; CYP – cytochrome P450; DBF – dibenzylfluorescein; IC₅₀ – inhibitor concentration eliciting 50 % inhibition; Ms – microsomal fraction; RS – reconstituted system; n.d. – not determined

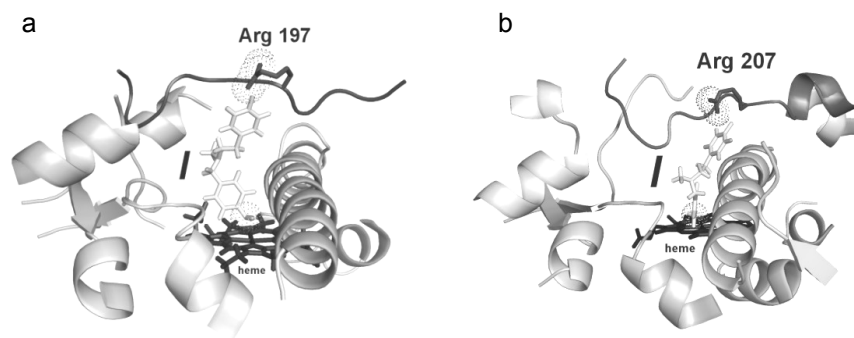


Fig. 2. **Docking of probe I to model structures of CYP2B4 (A) and CYP3A6 (B).** In both structures the probe I is anchored via its amino-group to the heme iron. Arg residues of CYP2B4 (already detected as modified) and of CYP3A6 (predicted in a close vicinity to reactive probe nitrene) are highlighted

(absorbance maximum at 387 nm and minimum at 422 nm), probe I showed the ligand Type II spectrum with an absorbance maximum at 438 nm and minimum at 418 nm. Thus, the probe I proved its amino-group to provide the heme iron ligation. In other words, the probe I, in contrast to probe II, is oriented in the CYP active center in a desired orientation, necessary for photoaffinity experiments.

As the probe nitrene was previously shown to specifically modify Lys and Arg residues¹⁰, the presence of these residues within the probe I reaction radius in the CYP3A6 structure should be examined. The experimentally defined binding and orientation of the probe I in the CYP3A6 active center was compared with results of docking computational experiments. At first, the 3D molecular model of rabbit CYP3A6 was constructed by homology modeling based on the known crystal structure of highly related CYP3A4. The CYP2B4 structure with the probe I in an Arg 197 adduct productive orientation as well as the probe I docked to the putative access channel of the CYP3A6 model is presented in fig. 2. Of the amino acid residues close to the probe I nitrene, Arg in the sequence position 207 is likely to be modified with the photoactivated probe.

Conclusion

The *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-aminophenethylamine (I) heterobifunctional compound was examined as a potential photolabile probe for mapping the access region of CYP3A6. Metabolic inhibitory studies with various CYP3A6 specific substrates and difference spectroscopy measurements revealed the probe I to be bound in the CYP active center in the orientation allowing the heme iron ligation. In addition, docking experiments with CYP3A6 structure predict Arg in the sequence position 207 to be a potential target of the probe I modification.

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and MSM 0021620808 and LC 7017 from the Czech Ministry of Education is highly acknowledged.

Abbreviations

BFC	7-benzyloxy-4-trifluoromethylcoumarin
CYP	cytochrome P450
DBF	dibenzylfluorescein
IC ₅₀	inhibitor concentration eliciting 50% inhibition
Ms	microsomal fraction
RIF	rifampicin
3D	three-dimensional

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L10**EFFECTS OF BORNEOL, A BICYCLIC TERPENE, ON HUMAN CELLS OF DIFFERENT ORIGIN****EVA HORVÁTHOVÁ, DARINA SLAMEŇOVÁ**

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Key words: borneol; comet assay; hydrogen peroxide; human cells HepG2, VH10, Caco-2

Introduction

Various industries are now looking for alternative, more natural and environmentally friendly antimicrobials, antibiotics, antioxidant and crop protection agents as well as an alternative route for the substitution of synthetic chemicals, side effects of which are always in question. The possibility of utilizing volatile oils is now being investigated as, although their biological activity has been known for centuries, their mode of action was not fully understood. For this, the essential oils and the extracts of many plants have been prepared and screened for their activities. Borneol belongs to monoterpenoid alcohols that represent a class of natural compounds that are widely present in the environment as component of plants and are often occurring in the production of oils, perfumes, and foods. In addition, borneol has been reported to have an antimalarial¹, antimicrobial and antifungal activities^{1–3}. Interestingly, borneol has been used as a medicine by many Asian cultures. Some investigators suggested that borneol had the potential to be used as an ophthalmic⁴ and nasal⁵ penetration enhancer. Borneol is also used in folk remedies for various purposes, such as the treatment of abdominal pain, particularly stomachache⁶. Our study compares cytotoxic, DNA-damaging and possible DNA-protective effects of borneol on human cells of different origin.

Material and methods

Cell lines: HepG2 and Caco-2 cells were obtained from Prof. A.R. Collins (University of Oslo, Norway) and VH10 cells from Dr. A. Kolman (Laboratory of Radiobiology, University of Stockholm, Sweden). The cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂ in William's medium (HepG2) or Dulbecco's modified minimum essential medium (VH10, Caco-2) supplemented with 10 % fetal calf serum and antibiotics (penicillin 200 U ml⁻¹, streptomycin and kanamycin 100 µg ml⁻¹).

Borneol (Sigma-Aldrich Co., Steinheim, Germany) were kept at room temperature, dissolved in 96% ethanol (1 mol l⁻¹) and diluted in complete culture medium to the concentrations 0.1–6 mmol l⁻¹ immediately before use.

Chemicals: Hydrogen peroxide – H₂O₂ (Sigma-Aldrich Co., Steinheim, Germany) stock solution (10 mol l⁻¹) was kept at 4 °C and diluted immediately before use in Ca²⁺- and Mg²⁺-free phosphate buffered saline (PBS) at 4 °C. Control cells or cells pre-treated with borneol were embedded in 0.75 % LMP agarose and treated by 250 µmol l⁻¹ H₂O₂ for 5 min on ice in the dark.

Cytotoxicity testing

Cytotoxic effects of borneol were in all human cells evaluated by the trypan blue exclusion technique. Cells were exposed to different concentrations of borneol for 24 h. Control cells were kept in a fresh complete culture medium containing 0.1% ethanol for 24 h. The assayed cells were washed with PBS (Ca²⁺- and Mg²⁺- free), trypsinized, stained by 0.4% trypan blue and the number of viable and dead cells were scored.

Single cell gel electrophoresis (comet assay)

The procedure of Singh et al.⁷ was followed with modifications of Slameňová et al.⁸ and Gábelová et al.⁹. Briefly: The assayed cells embedded in 0.75 % LMP agarose were immersed in ice-cold lysis mixture (2.5 mol l⁻¹ NaCl, 0.1 mol l⁻¹ Na₂EDTA, 0.01 mol l⁻¹ Tris-HCl, pH 10, 1 % Triton X-100) for 1 h. The slides were then transferred to an electrophoresis solution (0.3 mol l⁻¹ NaOH, 1 mmol l⁻¹ Na₂EDTA, pH>13) for 40 min at 4 °C. A current of 25 V (0.3 A) was then applied for 30 min. The slides were neutralized with 0.4 mol l⁻¹ Tris-HCl (pH 7.5) and stained with ethidium bromide (5 µg ml⁻¹). For each sample, 100 "comets" were evaluated and scored with an Olympus fluorescence microscope and computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of % DNA in tail.

Statistics

The results represent a mean of two or three independent experiments ± standard deviation (SD). The significance of differences between samples was assessed by Student's *t*-test.

Results and discussion

Cytotoxicity and genotoxicity testing on human cells

The trypan blue exclusion assay showed that human fibroblastoid VH10 cells were the most sensitive to the toxic effects of a 24 h treatment with borneol (0.1–3 mmol l⁻¹) among all human cell lines studied. HepG2 and Caco-2 cells reacted on a 24 h treatment with borneol (0.15–6 mmol l⁻¹) similarly (figs. 1A,B,C-insets). Measuring of DNA strand breaks induction in human cells of all three types treated for 24 h with different concentrations of borneol (figs. 1A,B,C-black columns) by the comet assay showed that at concentrations <IC₅₀ borneol induced no or only slight increase in DNA damage. Our results on assessing genotoxicity of borneol correlate with the findings of Azizan et al. who observed no mutagenic activity of short-term (20 min) or 120 min borneol-treatment in Ames Salmonella/S9 microsomal assay¹⁰.

DNA-protective effects of borneol

The comet assay was used also for evaluation of possible protective effects of borneol against DNA-damaging effects of H₂O₂ (fig. 1 – stripped bars). As borneol reduced significantly the level of H₂O₂-induced DNA strand breaks in human hepatoma HepG2 cells and slightly but significantly in human fibroblastoid VH10 cells, it is evident that borneol protects some types of cells against the DNA-damaging effect of H₂O₂. On the other side borneol had no DNA-protective effects against DNA damage induced by

H₂O₂ in Caco-2 cells (fig. 1C – stripped bars). These distinctions among cytotoxic, genotoxic and mainly DNA-protective effects of borneol in different human cells are at present unclear, but we cannot exclude that they could be connected with different antagonistic action of borneol on signal transduction systems of cells of different origin^{11,12}.

Conclusion

The cytotoxic, genotoxic and DNA-protective effects of borneol were studied in human cells of different origin (human hepatoma HepG2, human fibroblastoid VH10 and human colon carcinoma Caco-2 cells). Cytotoxicity testing was secured by the trypan blue exclusion technique. The levels of DNA damage were determined using alkaline single cell gel electrophoresis (comet assay). The trypan blue exclusion technique showed that borneol was cytotoxic in increasing concentrations on all cells tested, VH10 cells being the most sensitive. Borneol itself was slightly genotoxic at concentrations <IC₅₀ only on HepG2 and Caco-2 cells. Borneol protected human hepatoma HepG2 cells and partially human fibroblasts VH10 against H₂O₂-induced DNA damage at concentrations <IC₅₀, but it manifested no effect on human colon carcinoma Caco-2 cells. These differences in effectiveness of borneol found in cytotoxicity, genotoxicity and DNA-protectivity testing among human cells of different origin are not explained at present.

This study was supported by the Science and Technology Assistance Agency APVV 51-015404.

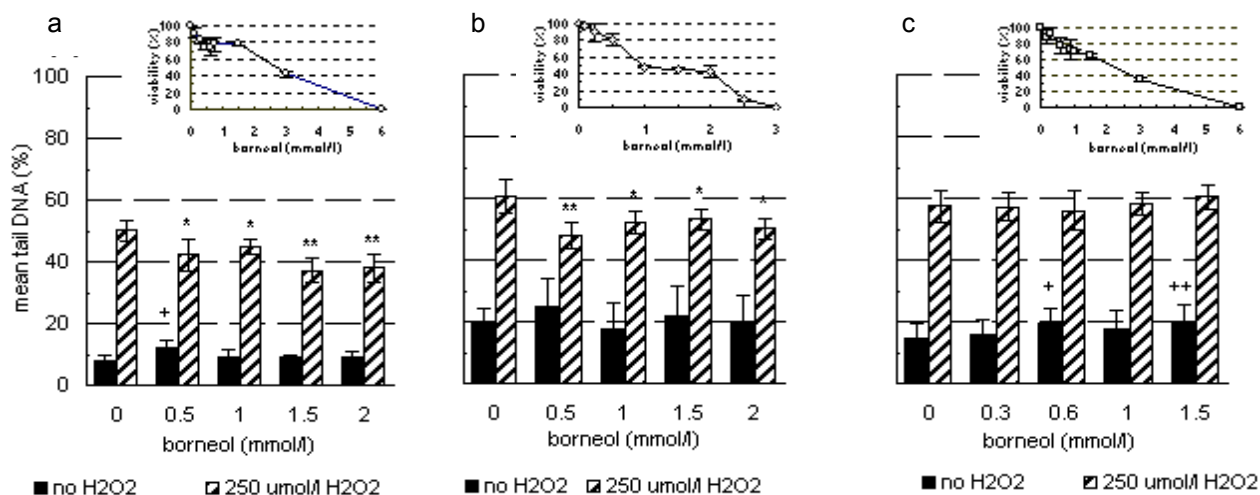


Fig. 1. Levels of DNA damage in HepG2 (A), VH10 (B) or Caco-2 (C) cells treated with borneol for 24 h, with 250 μM H₂O₂ for 5 min or pre-treated with borneol and then treated with H₂O₂. Data represents three independent experiments with three replicate samples ± SD. Statistically different from control treated with 0.1% ethanol or from value for hydrogen peroxide +; * *P*<0.05; ++; ** *P*<0.01; +++; *** *P*<0.001. Insets: Viability of HepG2 (A-open circles), VH10 (B-open diamonds) and Caco-2 (C-open squares) treated for 24 h with different concentrations of borneol. Data represent means of two determinations (with two parallels each) ± SD

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L11

POTENTIAL ANTIMUTAGENIC EFFECT OF POLYSACCHARIDE *N*-(2-CARBOXYETHYL) CHITOSAN

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Key words: DNA-topology, plasmid DNA, *Salmonella typhimurium*, DPPH radicals, OH radicals, carboxyethyl chitosan

Introduction

Every year some new natural substances have been isolated. These compounds can positively influence human health, and due to their potential antigenotoxic and antioxidant effects they could be used in the cancer prevention^{1,2}.

Chitosan is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin. Chitin is contained in the cell wall of fungi, exoskeletons of insects and crustaceans. Over the past decades, chitin and chitosan have found many applications in agriculture, textile and paper industry, cosmetics, waste treatment, and food processing. Due to their biodegradability, non-toxicity, and antioxidant properties the chitin derivatives have been attracted attention for their possible application in human medicine and pharmacy as a biological material, with unique biological activities, which include antitumor, immuno-enhancing effect, and antibacterial activities^{3–6}. The applications of chitosan are limited because of the insolubility in water at neutral or high pH region. One of the most popular way to provide a hydrophilic character to chitosan is carboxyethylation⁷.

In this work antimutagenic/bio-protective and antioxidant effects of CECh were evaluated with a special emphasis on the study of its potential mechanism of action. The possible antimutagenic activity of carboxyethyl chitosan was assessed in the Ames assay on histidine-deficient bacterial *Salmonella typhimurium* strain TA100. In the DNA-topology assay bio-protective activity of CECh on pBR322 plasmid DNA was evaluated. The potential antioxidant activity of CECh was evaluated in the DPPH assay and the hydroxyl radicals scavenging assay.

Material and methods

N-(2-carboxyethyl) chitosan (CECh) was obtained by reaction of a low molecular weight chitosan with a low degree of acetylation and 3-halopropionic acids under mild alkaline media (pH 8–9, NaHCO₃) at 60 °C. It is the derivative of chitin, synthesized by Skorik et al.⁷ from coarse ground crab. CECh with molecular weight about 70 kDa, and 1.5 degree of substitution was used⁷.

Salmonella typhimurium mutagenicity/antimutagenicity (Ames) assay

For mutagenicity/antimutagenicity of *N*-(2-carboxyethyl) chitosan (CECh) the Ames pre-incubation assay with 1 hour pre-incubation was performed. The *Salmonella typhimurium* tester strain TA100 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic). As a positive control direct mutagen sodium azide (NaN₃) was used.

The assay was conducted in test tubes containing 0.1 ml of the overnight bacterial culture (approximately 10⁸ cells ml⁻¹, cultivated in 50 ml of LB medium), sterile water and the tested CECh (750, 500, 250 µl of 1 mg ml⁻¹ per plate). Test tubes containing CECh were incubated for 1 h with bacteria. After 1-hour-incubation direct-acting mutagen and the top agar were supplemented. The content was mixed, and plated on minimal bottom agar plates. His⁺ revertants were counted after 72 h of incubation at 37 °C in Biotran III Colony Counter (New Brunswick Scientific Co.). The data were analysed using the Student's *t*-test.

DPPH radical scavenging assay

The potential antioxidant activity of CECh was measured in terms of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging ability, with slight modifications⁸. Ascorbic acid was used as the reference compound. The highest concentration of ascorbic acid was considered as 100 % of scavenging activity. A methanolic solution of CECh (50 µl) at different concentrations was placed in a cuvette and 1 ml of 23.7 µg ml⁻¹ methanolic solution of DPPH radical was added followed by 30 min incubation. The decrease in absorbance at 517 nm was determined with the Spekol 221 spectrophotometer. All determinations were performed in three replicates.

Hydroxyl radical scavenging assay

The hydroxyl radicals (•OH) were generated in an L-

ascorbic acid/CuSO₄ system by reduction and were assayed by the oxidation of cytochrome c⁹. In this experiment, the •OH were generated in 1 ml of 7.5 mM sodium phosphate buffer (pH 7.4) containing 5 mM L-ascorbic acid, 5 mM CuSO₄, 0.6 mM cytochrome c, and the samples of CECh to be tested at different concentrations (0.01 mg ml⁻¹, 0.025 mg ml⁻¹, 0.05 mg ml⁻¹, 0.1 mg ml⁻¹). The mixture was incubated at 25 °C for 15 min. Change in transmittance caused by a color change of cytochrome c was measured at 550 nm using Spekol 221.

DNA-topology assay

The plasmid pBR322 was purchased from Advanced Biotechnologies Ltd. (Units B1-B2 Longmead Business Centre, Blenheim Road, Epsom, Surrey KT 199QQ, U.K.) The method of electrophoretically monitored DNA damage and DNA protectivity was described in detail by Rauko et al.¹⁰. Briefly, the reaction mixture (final volume 20 ml) contained 200 ng plasmid DNA in buffer. The plasmid DNA was exposed either to Fe²⁺ (10 μM), or H₂O₂ (1 mM) alone or in combination with CECh (1 mg ml⁻¹, 0.75 mg ml⁻¹ a 0.5 mg ml⁻¹).

DNA single-strand breaks were assayed by measuring the conversion of supercoiled DNA (form I) to relaxed circular DNA (form II). Topological changes of DNA molecules correspond with the electrophoretic mobility of DNA topoisomers. Analysis of DNA modifications was made by agarose gel electrophoresis (1.5 % agarose, 45 min/60 V). The DNA was made visible by staining with ethidium bromide (1 mg ml⁻¹, Sigma, USA) and UV illumination (Ultra-Lum Electronic UV Transilluminator, USA). Percentages of supercoiled, relaxed and linear DNA forms were calculated by a computer program (Uthesa, Image Tool for Windows, Version 1.27).

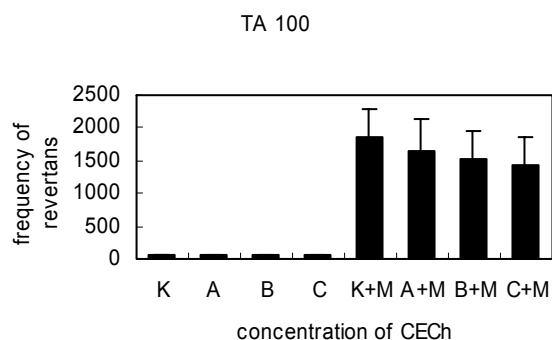


Fig. 1. Effect of CECh on NaN₃-induced mutagenicity in *S. typhimurium* strain TA100 using the Ames 1 h pre-incubation assay; C = control, A = 0.75 mg ml⁻¹, B = 0.5 mg ml⁻¹, C = 0.25 mg ml⁻¹, M = mutagen (NaN₃)

Results and discussion

In this paper investigation of the potential anti-genotoxic and antioxidant properties of newly synthesized carboxyethyl chitosan were presented.

In the Ames assay no mutagenic effect was found, and also antimutagenic effect of CECh against mutagenicity induced by NaN₃ on the *S. typhimurium* strain TA100 was not statistically significant (fig. 1).

The results of the DNA-topology assay suggest that polysaccharide CECh did not induce single- and double-strand DNA breaks in the plasmid DNA. In the presence of Fe²⁺ CECh reduced the plasmid DNA relaxing in the concentration-dependent manner. In the presence of H₂O₂, CECh stimulated the plasmid DNA relaxation in the concentration-dependent manner. In the simultaneous incubation CECh with oxidative agent Fe²⁺, CECh exerts DNA protective activities, while in combination with H₂O₂, polysaccharide CECh increased damage to the plasmid DNA (fig. 2 and 3).

The antioxidant activity of the CECh was assessed using two different methods. The first method used was the DPPH assay. On the basis of the results obtained we can conclude that due to the high molecular weight of CECh used (70 kDa) it did not show any antioxidant effect (fig. 4). Also the results of other researchers documented that the scavenging activity of CECh is depending on the molecular weight¹¹. While, the 30 kDa chitosan exhibited a strong scavenging activity, antioxidant activity of 120 kDa chitosan was considerably low.

The second method for antioxidant detection used was the hydroxyl radical scavenging assay. The scavenging rate of CECh increased in the concentration range from 0.01 mg ml⁻¹ to 0.1 mg ml⁻¹ (fig. 5). The scavenging activity may be attributed to its metal-bonding capacity also Xue et al.¹², reported that water-soluble chitosan may che-

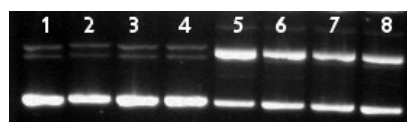


Fig. 2. Electrophoretic monitoring of the DNA-topology of the plasmid DNA treated with CECh line 2–4, and CECh with Fe²⁺ line 6–8

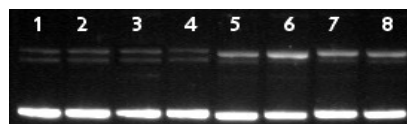


Fig. 3. Electrophoretic monitoring of the DNA-topology of the plasmid DNA treated with CECh line 2–4, and CECh with H₂O₂ line 6–8

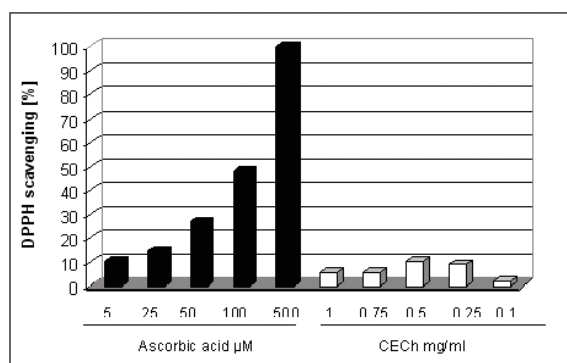


Fig. 4. Antioxidant activity of CECh determined by the DPPH radical scavenging assay; The values represent the data from three independent experiments (S.D.< 5 %). Ascorbic acid was used as a reference compound

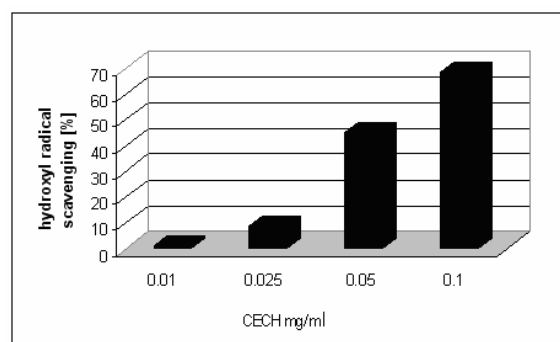


Fig. 5. Antioxidant activity of CECh determined by the OH radical scavenging assay; 25 μg ml⁻¹ of thiourea = 100 % of scavenging activity

late metals.

In conclusion, the results suggest that antimutagenic and antioxidant effects of this polysaccharide are dependent on concentration, way of application and method used. Comparing two procedures used for the CECh antioxidant activity assessment we can conclude that owing to molecular weight of CECh used in our experiments, the hydroxyl radical scavenging assay was more convenient and sensitive than DPPH scavenging assay.

Conclusion

Our research was aimed at the CECh mutagenicity exclusion, and potential antimutagenic effect assessment, using the Salmonella/microsome assay. We also aimed at the evaluation of CECh DNA protective activity, using the DNA-topology assay, and at the antioxidant properties of CECh, using the DPPH assay and the hydroxyl radical scavenging assay. On the basis of results obtained we can conclude that CECh may be applied in the biomedicine and due to its antioxidant properties it may be useful in cancer prevention.

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L12

DOSE-VARIABLE EFFECT OF NITRENDIPINE ON THE DISTRIBUTION OF GALANTHAMINE INTO THE BRAIN OF THE LABORATORY RAT

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Key words: galanthamine, nitrendipine, pharmacokinetic interactions

Aim

Effect on the concentration of the cholinomimetic agent (galanthamine) in the target sites of the central nervous system by means of pharmacokinetic interaction with nitrendipine.

Introduction

In the present therapeutic strategy of cognitive deficient conditions (e.g., senile dementia of Alzheimer type – SDAT), pharmacological stimulation of the central cholinergic transmission /e.g.,^{1/} outlasts. Galanthamine (GAL), a parasymphomimetic agent with a dual mechanism of effect, ranks among the second generation of SDAT drugs. In previous experiments², the present authors succeeded to partially strengthen the cerebral anticholinesterase activity of GAL by means of L-carnitine (an agent influencing transport mechanisms). Hypothetic strengthening of GAL-efficiency by means of nitrendipine (NITR) is based on other types of pharmacological interactions: the inhibitory action of NITR on calcium channels (and a subsequent cascade of reactions resulting in an influence on cytosol release of calcium) and the intervention of NITR into the activity of P-glycoprotein transporters (which influence the kinetics of a number of xenobiotics by the efflux mechanism).

In order to determine suitable doses and suitable time intervals, in the introductory experiment the animals were administered a single dose of GAL (based on previous studies²) and subsequently the kinetics of plasma concentrations of GAL and its three metabolites, *O*-desmethylgalanthamine (ODMGAL), *N*-desmethylgalanthamine (NDMGAL) and chirally converted epigalanthamine (EPIGAL) was determined.

The proper study consisted in repeated medication with two different doses of NITR, subsequent single-dose administration of GAL, and determination of GAL concentrations (in its steady state of the pharmacokinetic

phase) in purposefully selected biological samples: in the brain tissue (= the pharmacologically targeted tissue), in the hypophysis (= lying outside the hematoencephalic barrier), in the liver tissue (= representing the biotransformational and peripheral pharmacokinetic compartments), in blood plasma (= the central distributional compartment). For the supplementing verification of the relationship of the nitrendipine dose and the degree of influence on galanthamine biodistribution, NITR levels were determined in identical biological samples.

Methods

Experimental animals: rats – Wistar males, weight range 210–255 g, six individuals in each experimental group.

Drug administration: galanthamine hydrobromide (Janssen Pharmaceutica, Belgium): 10 mg kg⁻¹ i. m.; nitrendipine (European Pharmacopoeia Catalogue): 5 mg kg⁻¹ or 20 mg kg⁻¹ p. o. (vehicle: polyethylene glycol : water = 1 : 1).

Bioanalytical techniques: determination of galanthamine and its metabolites: HPLC + detection: fluorescence, mass-spectrometric, and on the photodiode field³; nitrendipine determination: GC + ECD detection⁴.

Design of the experiment for the determination of the time course of plasma levels of galanthamine and its metabolites: single-dose i. m. administration of 10 mg kg⁻¹ GAL, withdrawals of samples of blood plasma from the 5th to the 120th minute from the incannulated *v. jugularis*.

Design of the biodistribution study of GAL under the influence of nitrendipine: repeated administration of NITR (5 mg kg⁻¹ or 20 mg kg⁻¹) for two days; on the third day, administration of another dose of NITR (5 mg kg⁻¹ or 20 mg kg⁻¹), after 30 minutes i. m. administration of galanthamine (10 mg kg⁻¹), and after another 30 minutes withdrawals of biological samples (plasma, brain, hypophysis, liver) from animals under general anesthesia; in a portion of samples determination of GAL concentration, in another portion NITR concentration.

Results

- 1) Demonstration of T_{max} plasma levels of GAL between the 10th and 30th minute (fig. 1) made it possible to determine the steady state phase and the withdrawal biodistribution interval for further experiments at 30 minutes after GAL administration. As the levels of GAL metabolites were low they were not examined in further experiments.
- 2) A comparison of inter-organ distribution ratios of GAL (fig. 2) clearly shows higher levels in the tissues against plasma levels (in the hypophysis ca. 6.5 times,

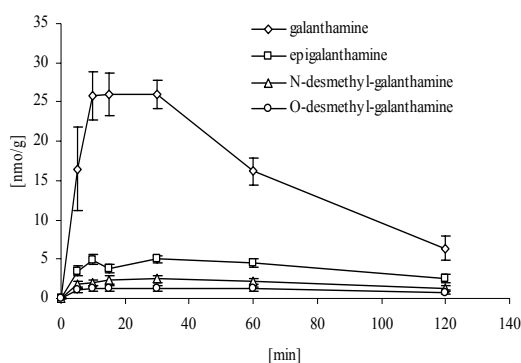


Fig. 1. Rat: plasma levels of GAL + its metabolites (after single – dose 10 mg kg^{-1} i.m. administration of GAL)

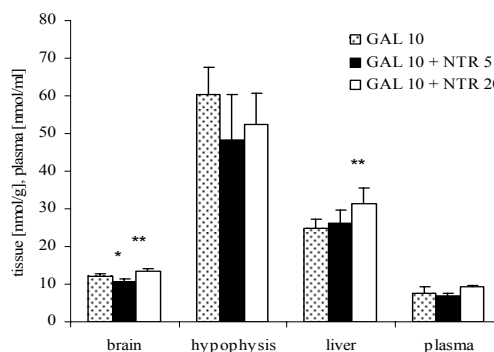


Fig. 2. Rat: effect of repeated p.o. doses of NITR (5 mg kg^{-1} or 20 mg kg^{-1}) on the biodistribution of GAL (after 10 mg kg^{-1} i.m. administration)

Table

Rat: effect of NITR-dose (5 mg kg^{-1} or 20 mg kg^{-1}) on its organ distribution

NITR-dose [mg kg^{-1}]	Nitrendipine conc. [ng ml^{-1}]		
	plasma	brain	liver
5	88.75 ± 57.51	105.65 ± 57.62	225.95 ± 67.02
20	203.72 ± 85.71	175.07 ± 69.45	1208.05 ± 103.70

in the liver 3.2 times, in the brain 1.4 times).

- 3) Effect of nitrendipine on the biodistribution of GAL is dose-dependent (fig. 2):
 - in the brain tissue after doses of 5 mg kg^{-1} of NITR there occurs a statistically significant decrease in GAL levels, after doses of 20 mg kg^{-1} of NITR there is a statistically significant increase in GAL levels,
 - in the hypophysis, GAL levels insignificant decrease by the action of NITR,
 - in the liver tissue, after doses of 5 mg kg^{-1} NITR, GAL levels are unchanged, after doses of 20 mg kg^{-1} NITR, GAL levels are increased in a statistically significant manner,
 - in blood plasma there is a statistically insignificant similar trend in the shifts of GAL levels as in the brain.
- 4) Nitrendipine levels in dependence on its dose change in a nonlinear manner (Table).

Discussion

The finding of a relatively intensive extravasal “outflow” of GAL and its different distribution ratios between the brain tissue and hypophysis (and also the liver) confirms the importance of hematoencephalic barrier for this distribution.

Dose-dependent interactions of NITR and GAL can be interpreted as follows:

- in the case of lower doses of NITR by the predominance of its vasodilatory action and a subsequent probable increase in the supply of GAL towards bioelimination compartments,
- in the case of higher doses of NITR by the predominance of its effect on the efflux carrier mechanism in the distribution of GAL into the brain (or the liver).

This interpretation is also based on a probable direct biodistribution interaction between NITR and GAL, i.e., from the variable tissue distribution of NITR in dependence on its dose. Whereas the ratio of the NITR-doses is 1 : 4, the levels in the brain are higher only ca. 1 : 1.4, in plasma only 1 : 2.3, but in the liver 1 : 5.4.

Conclusions

- In the steady state of the pharmacokinetic phase, galanthamine levels are higher in the tissues than in blood plasma, and mutual tissue biodistribution ratios suggest an effect of the hematoencephalic barrier on the penetration of GAL into the brain.
- Nitrendipine in a repeated dose of 5 mg kg^{-1} decreases the distribution of GAL into the brain (influence of nitrendipine vasodilatory effect?), in a repeated dose of 20 mg kg^{-1} it increases the distri-

bution of GAL into the brain and the liver
(nitrendipine effect on P-glycoprotein efflux?).

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