

QUANTITATIVE STRUCTURE-HEPATOTOXICITY ASSESSMENT OF SERIES ARYLPIPERAZINE-N¹-SUBSTITUTED THEOBROMINE DERIVATIVES

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Abstract

In this work series new theobromine derivatives containing an arylpiperazine moiety at N¹ with established antioxidant and antiproliferative activities were evaluated for their hepatotoxic effects on cellular and sub-cellular level. On isolated rat hepatocytes, **3c** and **4c** expressed lowest toxicity, while **3f** and **4f** showed highest toxicity. The same compounds **3f** and **4f** showed the most evident pro-oxidant effect in a lipid peroxidation model on rat liver microsomes, followed by **3b** and **4b**, while the other compounds didn't reveal statistically significant pro-oxidant effects. The performed quantitative structure-toxicity relationship (QSTR) analysis show that increased lipophilicity of the tested compounds positively correlates to their hepatotoxicity. Opposite, the presence in the structure of highly positive H-atoms and strongly negative oxygen, possibly originating from hydrogen bond donor groups, are associated with reduced hepatotoxicity.

Rezumat

În această lucrare, noi derivați de teobromină care conțin o grupare arilpiperazină la N¹ cu activități antioxidante și antiproliferative consacrate, au fost evaluați pentru efectele lor hepatotoxice la nivel celular și subcelular. Asupra hepatocitelor izolate de șobolan, **3c** și **4c** au exprimat cea mai mică toxicitate, în timp ce **3f** și **4f** au prezentat cea mai mare toxicitate. Aceiași compuși **3f** și **4f** au arătat cel mai evident efect pro-oxidant într-un model de peroxidare lipidică asupra microzomilor hepatici proveniți de la șobolan, urmați de **3b** și **4b**, în timp ce ceilalți compuși nu au arătat efecte pro-oxidante semnificative statistic. Analiza cantitativă a relației structură-toxicitate (QSTR) a arătat că lipofilia crescută a compușilor testați se corelează pozitiv cu hepatotoxicitatea lor. În mod contrar, prezența în structură a atomilor de H foarte pozitivi și a celor de oxigen puternic negativi, este asociată cu o hepatotoxicitate redusă.

Keywords: theobromine, arylpiperazine, hepatotoxicity, QSTR

Introduction

Methylxanthines are purine derivatives which exhibit many pharmacological effects like bronchodilation, anti-inflammatory, immunomodulating, vasodilating, psychostimulating, diuretic, antiviral, antioxidizing and antineoplastic [4]. Their effects result from complex mechanisms including inhibition of phosphodiesterases (PDEs) [7, 10, 22] and adenosine receptors [6] without selective action on their single isoforms, modulation of GABA receptor action [27], regulation of intracellular calcium levels and activation of histone deacetylases (HDAC) [20]. In human liver, methylxanthines are metabolised through demethylation by the enzyme cytochrome P450 (CYP) [13]. They do not accumulate in organs or tissues and less than 2% of caffeine administered is excreted unchanged in human urine [3]. Toxicology studies in animals may appear to provide alarming results, concerning

teratology and male reproductive toxicology but these cannot be extrapolated to humans [25].

The *in vitro* systems play an important role in the investigation of the xenobiotic's pharmacological activities and toxicity mechanisms [26]. Different cell models, such as isolated primary cells (isolated rat hepatocytes) or cell cultures are often used for evaluation of cytotoxic/cytoprotective effects of perspective pharmacologically active compounds from synthetic or biological origin. Thus, the pharmacological activities and toxicity of the newly synthesized compounds were deeply characterized in a battery of *in vitro* models.

On the other side, the safety evaluation is an important step in the process of new drugs discovery. Hepatotoxicity implies chemical-driven liver damage and it is the most common reason for a drug to be withdrawn from the market with more than 900 drugs implicated in causing liver injury [23]. Hepatotoxicity and drug-

induced liver injury also account for a substantial number of compound failures, highlighting the need for drug screening assays, such as stem cell-derived hepatocyte-like cells, that are capable of detecting toxicity early in the drug development process [18]. Hepatotoxicity data were used to build a number of QSTR models to identify the structural features of compounds correlated to their hepatotoxicity. QSTR analysis was performed in order to guide a further synthesis of derivatives with an improved hepatotoxicity profile.

Materials and Methods

Chemicals and reagents

The following chemicals and reagents, including pentobarbital sodium (Sanofi, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), NaHCO₃ (Merck), KH₂PO₄ (ScharlauChemie SA, Spain), K₂HPO₄ (ScharlauChemie SA, Spain), glycerol (ScharlauChemie SA, Spain), CaCl₂·2H₂O (Merck), MgSO₄·7H₂O (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fraction V, minimum 98% (Sigma Aldrich), EGTA (Sigma Aldrich), 2-thio-barbituric acid (4,6-dihydropyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK) were used.

Hepatotoxicity evaluation

Animals

The protocol involving the used animals has been conducted in agreement with the requirements of State Regulation number 15/2006 for working with experimental animals of the Ministry of Agriculture and Woods. The experiments were approved by Ethical Agreement for research on animals № 226 from the Bulgarian Agency of Food Safety [8, 24].

Isolation and incubation of microsomes

The isolation of liver rat microsomes and the production of malonaldehyde were performed according to the methodology presented in [9, 14, 15].

Isolation of liver microsomes

Liver is perfused with 1.15% KCl and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH = 7.4. The liver homogenate was centrifuged at 9,000 x g for 30 min at 4°C and the resulting post-mitochondrial fraction (S9) was centrifuged again at 105,000x g for 60 min at 4°C. The microsomal pellets were re-suspended in 0.1 M potassium phosphate buffer, pH = 7.4, containing 20% glycerol. Aliquots of liver microsomes were stored at -70°C until use.

Lipid peroxidation in microsomes

After incubation of microsomes (1 mg/mL) with the compounds, we added to the microsomes 1 mL 25%

(w/v) trichloroacetic acid (TCA) and 1 mL 0.67% 2-thio-barbituric acid (TBA). The mixture is heated at 100°C for 20 min. The absorbance was measured at 535 nm, and the amount of MDA was calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. The calculations were made by using formula: $\text{MDA nmol/mg protein} = E \times 12.8$.

Protein measurement

The protein content was determined using the standard method of Lowry, as described elsewhere [19]. The method used pure Albumin as standard compound for the preparation of the line. The content of the protein, was measured by spectrophotometry, at 550 nm.

The isolation and incubation of the hepatocytes [21] as well as the determination of the followed parameters, such as lactate dehydrogenase (LDH) release [5], glutathione (GSH) depletion and MDA assay [12] was performed using the cited reference methodology as explained below.

Isolation and incubation of hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2 mL/100 g). An optimized *in situ* liver perfusion using less reagents and shorter time of cell isolation was performed. The method provided in higher amount of live and metabolically active hepatocytes.

After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85) and finally HEPES buffer containing collagenase type IV (50 mg/200 mL) and 7 mM CaCl₂ (pH = 7.85). The liver was excised, minced into small pieces, and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin.

Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%). Initial viability averaged 89%.

Cells were diluted with KRB to make a suspension of about 3×10^6 hepatocytes/mL. Incubations were carried out in flasks containing 3 mL of the cell suspension (i.e. 9×10^6 hepatocytes) and were performed in a 5% CO₂ + 95% O₂ atmosphere.

Lactate dehydrogenase (LDH) release

Lactate dehydrogenase release in isolated rat hepatocytes was measured by using LDH kit. The calculations were made by using formula: $\text{LDH } \mu\text{mol/min/mill cells} = E \times 1.34$ (Randox).

Reduced glutathione (GSH) depletion

At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular GSH, which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm.

Malondialdehyde (MDA) assay

Hepatocyte suspension (1 mL) was taken and added to 0.67 mL of 20% (w/v) TCA. After centrifugation, 1 mL

of the supernatant was added to 0.33 mL of 0.67% (w/v) 2-thiobarbituric acid (TBA) and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

The following statistical analysis was conducted according to [21].

Statistical analysis

The MTT-bioassay raw data were normalized as survival fractions (%) relative to the untreated control (set as 100% viable), and the equivalent IC_{50} values (concentrations causing half-maximal decrease of cell survival) were calculated using non-linear regression ('Curve fit'-GraphPad Prizm Software for PC). The statistical processing of MTT data included the paired Student's t-test with $p \leq 0.05$ set as significance level.

Quantitative structure-toxicity relationships (QSTR) study

Data set

QSTR analysis was performed on a set of twelve N^1 -aralkyl xanthine derivatives. The *in vitro* hepatotoxicity was assessed at concentration of 100 μM and evaluated by five parameters (section *In vitro hepatotoxicity evaluation*). Among them, cell viability of hepatocytes, GSH level and LDH activity were considered in the QSTR analysis since only these bioassays provided statistically significant data (p -value < 0.05) for all the compounds.

QSTR protocol

The molecular modelling and geometry optimization of the structures of investigated compounds were performed by HyperChem 7.5 (HyperCubeLtd.). MM+ force field [16] was applied for energy minimization and semiempirical method AM1 [11] for the electron density distribution calculations.

About 50 descriptors were calculated to describe the chemical structures of compounds. A number of 2D descriptors (topological indices and molecular properties like number of H-bond donors and acceptors, molecular weight, number of rings etc.) and 3D general descriptors (ovality, volume, surface area of the molecule etc.) were calculated by MDL QSAR software, version 2.2 (Symyx). LogP that accounts for lipophilicity of compounds was calculated by ACD Labs software (ACD Inc). The values of electronic descriptors - HOMO and LUMO energies and charges at all common heteroatoms were taken from the quantum mechanical calculations in HyperChem 7.5.

A genetic algorithm (GA) [17] as implemented in MDL QSAR software was applied for selection of descriptors relevant to the hepatotoxicity of compounds.

The parameters of GA were set as follows: initial population with size of 32, tournament selection of parents, uniform crossover of chromosomes and one-point mutation. Friedman's lack-of-fit scoring function with parameter of 2 was selected as a fitness function. Finally the descriptor sets were checked for intercorrelation and used for modelling of structure-hepatotoxicity relationships. A number of linear models were generated by ordinary multiple-linear regression (MLR).

The MLR models were assessed by explained variance r^2 , standard error of estimation SEE, F-ratio and leave-one-out cross-validated (LOO-CV) correlation coefficient q^2 . Only models with $r^2 > 0.85$ and $q^2 > 0.45$ were further considered. The validity of the selected descriptor sets was checked by *y scrambling* test. In an iterative procedure the values of the dependent variable were randomized among the compounds, followed by a construction of MLR model. At each step r^2 was computed and after 100 iterations the mean value of r^2 was given as r^2_{scr} . The descriptor set was considered as valid if $r^2_{scr} < r^2$.

Results and Discussion

Chemistry

The evaluated compounds were synthesized by us according to the general synthetic procedure presented in Figure 1, through the methodology described previously [1, 2].

The results of the performed IR, ^1H and ^{13}C NMR and MS spectral analysis were consistent with the assigned structures. The obtained compounds were subjected to a pharmacological evaluation in means of preliminary determination of the performed hepatotoxicity.

In vitro hepatotoxicity evaluation.

In the current research a preliminary hepatotoxicity evaluation for a series of newly obtained in our laboratory theobromine derivatives, comprising aryl/aralkyl moieties at N^1 in theobromine structure with determined antioxidant and antiproliferative effects [1, 2] was performed.

The appearance of signs of hepatotoxicity on sub-cellular and cellular level of the newly synthesized compounds **3a-f** and **4a-f** was evaluated in an attempt to identify the least hepatotoxic agent. The analysis was based on determination of the cell viability, increased LDH leakage and GSH and MDA levels, known as main parameters characterising the functional-metabolic status of hepatocytes.

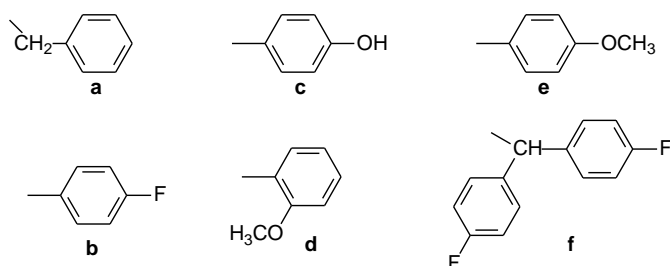
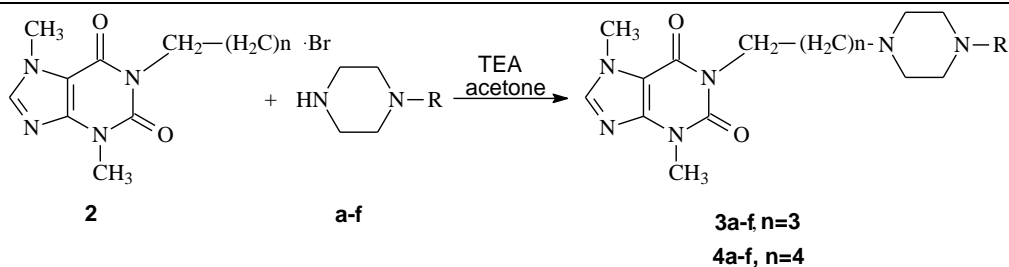


Figure 1.

General synthesis of the target compounds **3a-f** and **4a-f**

Cell viability evaluation.

The cell viability assay determines the ability of the cells to maintain or recover viability. On isolated rat hepatocytes the viability was measured by Trypan blue (0.05%) exclusion. Trypan blue is dye, which

cross the membrane of the death cells and coloured them in blue. The isolated rat hepatocytes were treated with compounds **3a-f** and **4a-f** in concentration of 100 μM [28] and the corresponding data are presented in Figure 2.

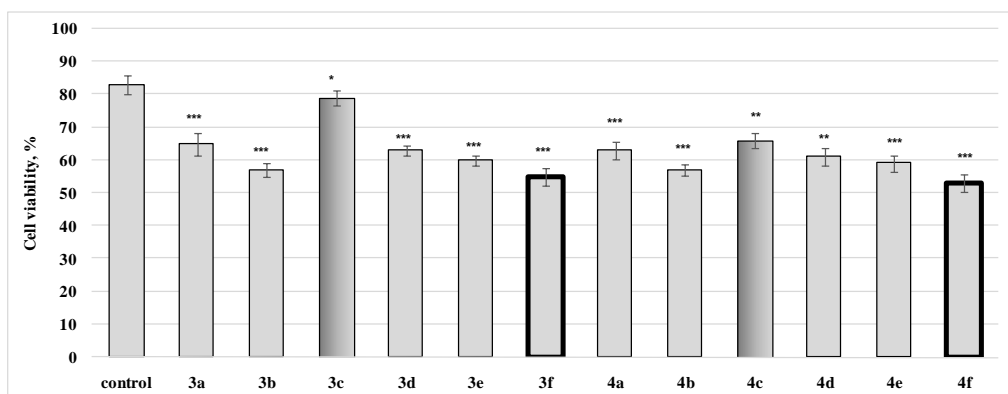


Figure 2.

Effects of **3a-f**, **4a-f** (100 μM) on cell viability of isolated rat hepatocytes

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control (non-treated hepatocytes)

The tested compounds revealed statistically significant cytotoxic effect on cell viability (measured by trypan blue exclusion), when compared to the control non-treated hepatocytes. **3f** and **4f** showed the highest cytotoxicity, decreasing the cell viability by 34% and 36%, respectively. The compounds with lowest toxicity, were **3c** and **4c**, decreasing the cell viability by 5% and by 20%, respectively.

LDH leakage evaluation.

Another biomarker for cytotoxicity is the measurement of lactate dehydrogenase (LDH). Lactate dehydrogenase (LDH) is expressed extensively in the body, such as blood cells, heart muscle, liver cells, etc. The increased LDH release is a sign of

tissue damage, thus it is a valuable biomarker of common injuries and disease.

All tested compounds showed statistically significant toxic effects on LDH enzyme activity, compared to those of control cells (non-treated hepatocytes) (Figure 3). Similarly to the effects obtained by trypan blue exclusion test, compounds **3f** and **4f** were identified as highest toxicity inducing agents, increasing the enzyme activity by 346% and 382%, respectively. Also in LDH viability test assay, compounds **3c** and **4c**, were confirmed to be less toxic, increasing the LDH enzyme activity by 287% and 297%, respectively.

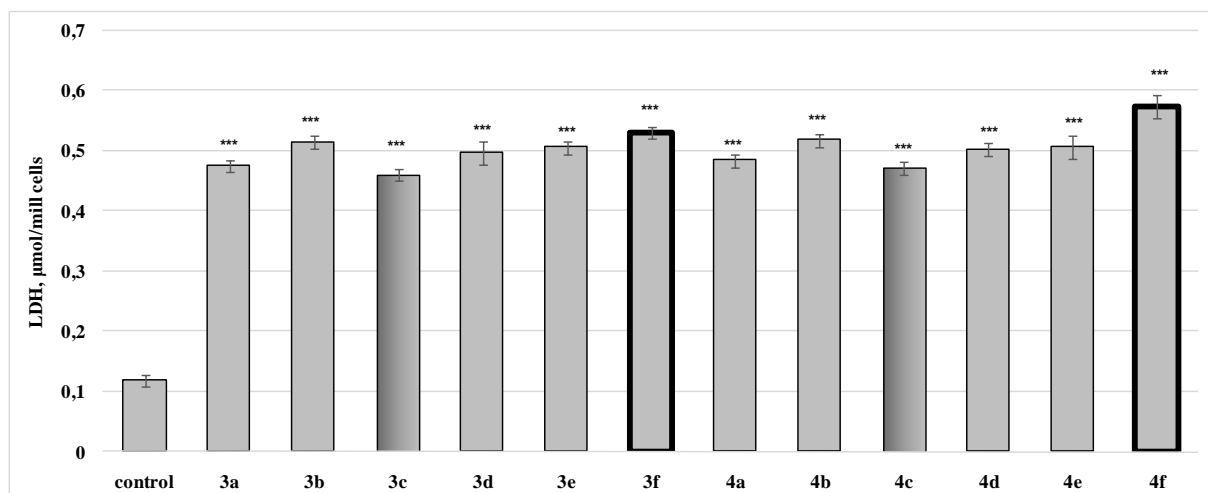


Figure 3.

Effect of **3a-f**, **4a-f** (100 µM) on LDH activity in isolated rat hepatocytes

*** p < 0.001 vs. control (non-treated hepatocytes)

GSH levels evaluation.

The reduced glutathione (GSH) plays a role as main non-enzyme antioxidant cell protector. The GSH depletion serves as a marker for possible toxic

metabolites formation. Both series were tested for their effects on GSH levels in isolated rat hepatocytes (Figure 4).

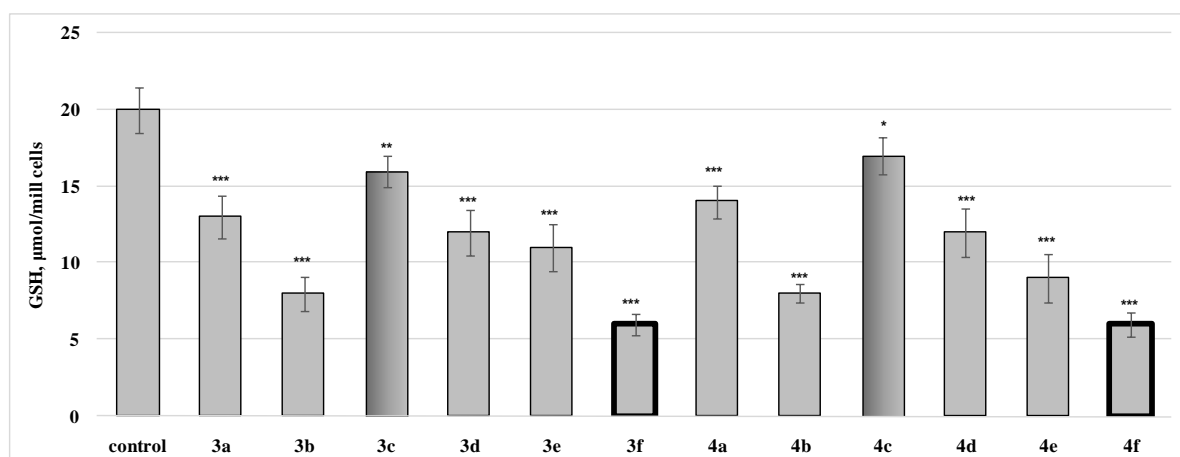


Figure 4.

Effect of **3a-f**, **4a-f** (100 µM) on GSH level in isolated rat hepatocytes

*** p < 0.001 vs. control (non-treated hepatocytes)

On GSH level, the tested compounds revealed statistically significant cytotoxic effect, compared to the control (non-treated hepatocytes). **3f** and **4f** showed highest toxicity with decrease in the reduced glutathione by 67% and 71%, respectively. **3c** and **4c** were found to be with the lowest toxicity, decreasing the GSH levels by 24% and 19%, respectively.

MDA levels evaluation in isolated rat hepatocytes.

Free radicals generate lipid peroxidation processes in the organism. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free

radicals causes overproduction of MDA allowing it to be used as a biomarker for lipid peroxidation.

The tested compounds were subjected to a possibility to affect the MDA levels on two *in vitro* models: isolated rat hepatocytes and rat liver microsomes. The effects are presented in Figures 5 and 6, respectively.

Most of the tested N¹-aralkyl theobromine derivatives didn't reveal statistically significant toxic effect on MDA production in isolated rat hepatocytes. Nevertheless, compounds **3b**, **3f**, **4b** and **4f** increased statistically significant the MDA production, (compared to the untreated control), as follows: **3b**- by 37%, **3f**- by 60%, **4b**- by 43% and **4f**- by 97%.

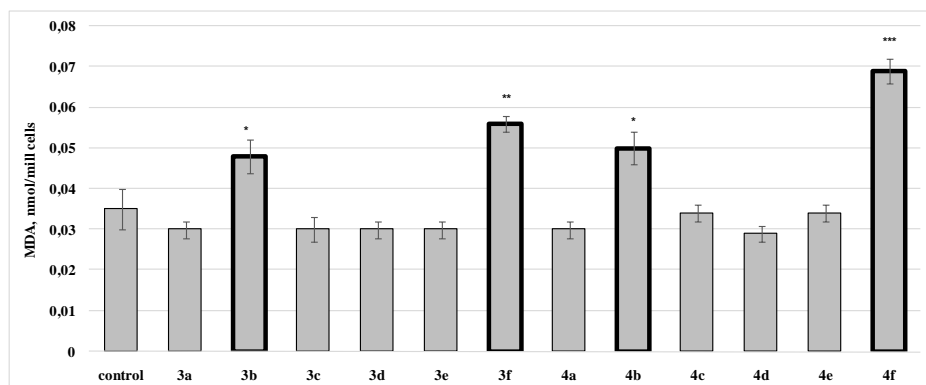


Figure 5.

Effect of **3a-f**, **4a-f** (100 μ M) on MDA production in isolated rat hepatocytes

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control (non-treated hepatocytes)

MDA levels evaluation in isolated rat microsomes. Microsomes are suitable and widely used *in vitro* model in drug metabolism studies, with the advantages of being inexpensive and easy to handle while containing the major drug metabolism enzymes, e.g. cytochromes

P450 (CYPs) and uridine 5'-diphospho-glucuronosyl-transferases (UGTs).

Thus the effects of the tested compounds on MDA levels in isolated rat microsomes were determined (Figure 6).

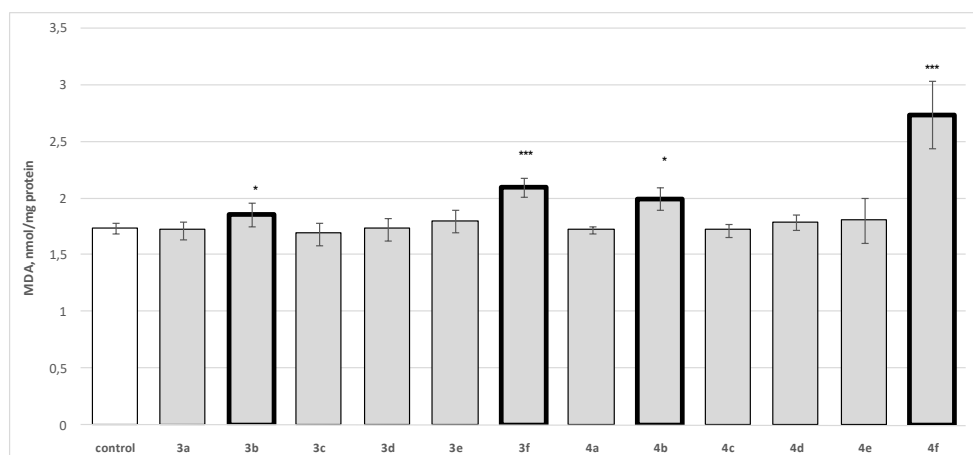


Figure 6.

Effect of **3a-f**, **4a-f** (100 μ M) on MDA production in isolated rat microsomes

* $p < 0.05$; *** $p < 0.001$ vs. control (non-treated microsomes)

No increase in the production of MDA was observed for most of the tested structures, thus showing lack of statistically significant pro-oxidant effects in isolated rat liver microsomes. Nevertheless, some N^1 -aralkyl theobromine derivatives, such as **3b**, **3f**, **4b** and **4f** raised the MDA contents, thus showing to possess statistically significant pro-oxidant effect. The effects of **3f** and **4f**, were most pronounced, with MDA production increase by 21% and 57%, respectively, when compared to the control (non-treated microsomes).

QSTR results

For the purposes of QSTR analysis, hepatotoxicity of compounds was expressed in terms of Cell Viability Decrease - CVD, GSH Depletion - GSHD and LDH Leakage - LDHL (Table I). They were calculated on the basis of *in vitro* data for Cell viability (%), GSH

level (nmol/mill cells) and LDH activity (μ mol/min/mill cells) respectively, using the following expression:

$$Y_{QSTR} = \frac{Y_{in\ vitro} - Y_{negative\ control}}{Y_{positive\ control} - Y_{negative\ control}}$$

Y_{QSTR} – calculated value of CVD, GSHD or LDHL for the tested compounds; $Y_{in\ vitro}$ – value of the respective *in vitro* parameter for the tested compound; $Y_{negative\ control}$ – value of the *in vitro* parameter for the non-treated hepatocytes; $Y_{positive\ control}$ – value of the *in vitro* parameter for the hepatocytes, treated with a positive control (**3a** was selected as such).

Lower values of Y_{QSTR} for CVD, GSHD and LDHL, in respect to the positive control **3a** indicate a decrease in the hepatotoxicity potential of compounds.

Table I

Hepatotoxicity of compounds, expressed in terms of Cell Viability Decrease (CVD), GSH Depletion (GSHD) and LDH Leakage (LDHL)

Compound ID	3a	3b	3c	3d	3e	3f	4a	4b	4c	4d	4e	4f
CVD	1.00	1.44	0.22	1.11	1.28	1.56	1.11	1.44	0.94	1.22	1.33	1.67
GSHD	1.00	1.71	0.57	1.14	1.29	1.86	0.86	1.71	0.43	1.14	1.57	2.00
LDHL	1.00	1.11	0.96	1.06	1.09	1.16	1.03	1.12	0.99	1.08	1.09	1.28

QSTR model based on Cell Viability Decrease (CVD) data

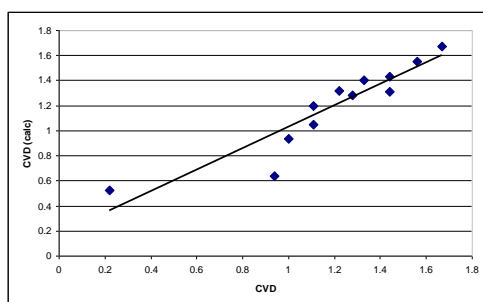
The GA procedure, applied for selection of the best descriptor set, generated the following two parameters MLR equation:

$$CVD = 0.39 \log P / ACD - 6.388 \text{MaxHp} + 1.10939$$

$$n=12 \quad r^2=0.8543 \quad SEE=0.1596$$

$$F=26.38q^2=0.4789 \quad r^2_{scr}=0.1815$$

The descriptor $\log P$ accounts for the lipophilicity of compounds. It increases with the elongation of the polymethylene bridge between xanthine and piperazine moieties and the introduction of bulky and hydrophobic substituents in the molecules (benzhydryl, F-atom). The positive regression coefficient of $\log P$ means that the more lipophilic compounds cause greater decrease of cell viability i.e. possess higher hepatotoxicity. MaxHp is a 3D general descriptor accounting for the largest positive charge on a hydrogen atom in the molecule. MaxHp values for hydroxyphenyl derivatives are higher than for the rest congeners in the series thus discriminating compounds **3c** and **4c**. In their molecules the largest positive charge is associated with the hydrogen atom of the aromatic OH group in the piperazine N¹ substituent. In our study a significant correlation ($r = 0.994$) was detected between MaxHp and the descriptor numHBd (number of hydrogen bond donors). The negative coefficient in the equation indicates that the presence of highly positive H-atoms (hydrogen bond donor groups respectively) in the molecule decreases hepatotoxicity and is favourable for the cell viability of hepatocytes.

**Figure 7.**

Plot of calculated vs. experimentally derived values of Cell Viability Decrease (CVD)

The correlation plot between calculated (MLR model) and the experimentally derived values of CVD is given in Figure 7. None of the compounds can be

considered as an outlier because the highest residual is 0.3.

QSTR model based on GSH Depletion (GSHD) data

The level of reduced glutathione in hepatocytes is a key marker in the toxicological profiling of compounds. Depletion of this cellular protector indicates an increased hepatotoxicity potential. The regression model given below again outlines lipophilic properties of the tested compounds as relevant to their toxicity. Higher values of $\log P$ correspond to increased GSH depletion.

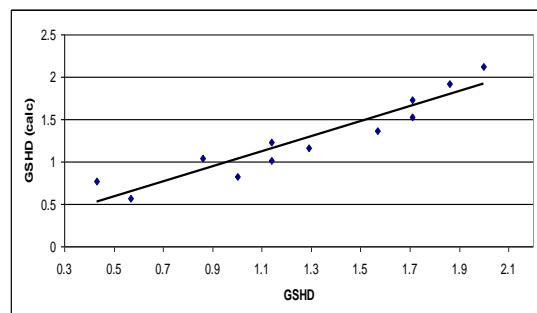
$$GSHD = 0.6836 * \log P / ACD - 0.8193 * ABSQon + 1.70801$$

$$n=12 \quad r^2=0.8888 \quad SEE=0.1869$$

$$F=35.98q^2=0.7986 \quad r^2_{scr}=0.1832$$

The descriptor $ABSQon$ (3D general descriptor) expresses the sum of absolute values of charges on the nitrogen and oxygen atoms in the molecule. The negative regression coefficient means that an increase in $ABSQon$ reduces the GSH depletion i.e. hepatotoxicity. Values of $ABSQon$ range from 1.9486 to 2.3475 and mainly depend upon the substituents at piperazine N¹ atom. The contribution of substituents to $ABSQon$ increases from benzhydryl (**3f** and **4f**) to fluorophenyl (**3b** and **4b**), methoxyphenyl (**3d**, **3e**, **4d**, **4e**), benzyl (**3a**, **4a**) up to hydroxyphenyl (**3c**, **4c**). Hepatotoxicity of derivatives decreases in the same order. High absolute charge of the hydroxyphenyl oxygen (the most influencing $ABSQon$ value) is associated with the ability of aromatic OH group to donate hydrogen that is favourable for preserving the levels of reduced glutathione.

The correlation plot between calculated (MLR model) and the experimentally derived values of GSHD is given in Figure 8.

**Figure 8.**

Vi Plot of calculated vs. experimentally derived values of GSH Depletion (GSHD)

QSTR model based on LDH Leakage (LDHL) data

The best QSTR model for LDH leakage i.e. increased LDH activity is given below:

$$LDHL = 0.136 * \log P/ACD + 0.815866$$

$$n=12 \quad r^2=0.8934 \quad SEE=0.0294$$

$$F=83.83q^2=0.803 \quad r^2_{scr}=0.0740$$

Similarly to the previous two models, increased lipophilicity positively correlates to the hepatotoxicity of compounds, namely higher LogP/ACD values correspond to increased LDH leakage.

The correlation plot between calculated (MLR model) and the experimentally derived values of LDHL is given in Figure 9.

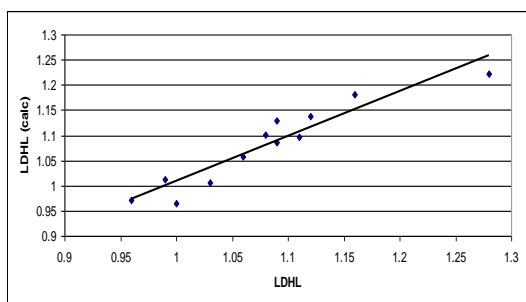


Figure 9.

Plot of calculated vs. experimentally derived values of LDH Leakage (LDHL)

In summary, our study showed a good agreement between the three QSTR models. The lipophilicity of compounds, expressed as LogP, correlates positively to hepatotoxicity. Inversely, the presence of highly positive H-atoms (*MaxHp*) and strongly negative oxygens (*ABSQon*) is advantageous for the cell viability of hepatocytes and preservation of the reduced glutathione levels.

Conclusions

Two series, comprising of N¹-aralkyl theobromine derivatives **3a-f** and **4a-f** were assessed for *in vitro* hepatotoxicity on isolated rat hepatocytes and liver microsomes, determining the following parameters: cell viability, LDH leakage, GSH and MDA levels. On isolated rat hepatocytes, **3c** and **4c**; **3a** and **4a**; **3d** and **4d**; **3e** and **4e**; **3b** and **4b**; **3f** and **4f** showed commensurable toxic effects on the tested parameters. The compound's toxicity increased as follows: **3c** and **4c** (lowest toxicity) < **3a** and **4a** < **3d** and **4d** < **3e** and **4e** < **3b** and **4b** < **3f** and **4f** (highest toxicity).

These compounds showed similar effects on isolated rat liver microsomes – with most evident pro-oxidant effect expressed by compounds **3f** and **4f**, followed by **3b** and **4b**. The rest of the tested structures didn't reveal statistically significant pro-oxidant effects, when compared to the control (non-treated microsomes).

The performed QSTR analysis clearly indicated that the increase in lipophilicity of compounds increases their hepatotoxicity, manifested by a decrease in the cell viability and GSH levels of hepatocytes and an increase in the LDH activity. The presence of an aromatic OH group in the substituent attached to piperazine N¹ is the main structural feature related to an improvement in the hepatotoxicity of compounds. Based on the performed analysis, it seems reasonably the introduction of hydrogen donating and more hydrophilic substituents to be considered in further synthesis.

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Conflict of interest

The authors declare no conflict of interest.

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