# *IN VITRO* EFFECTS OF NEW DERIVATIVES OF CAFFEINE-8-THIOGLYCOLIC ACID ON ISOLATED RAT LIVER MICROSOMES

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**Abstract.** This studyinvestigates the effects of newly synthetized derivatives of caffeine-8 thioglycolic acid on isolated rat liver microsomes. The microsomeswere received by multiple centrifugation. The production of malondialdehyde (MDA) was exam as a marker of lipid peroxidation. The effects were compared to those of caffeine. From all exam compounds, only caffeine, caffeine-8-thioglycolic acid (KTG), JTA-1, JTA-2 and JTA-3 didn't change statistically significant the MDA level. JTA2-Ox increased MDA statistically significant with 17 % compared to the control (non-treated microsomes).All the other compounds revealed higher statistically significant pro-oxidant effects. JTA-4, JTA-5 and JTA-13 increased MDA level with 34 %, JTA-6 – with 37 %, JTA-7 – with 30 %, JTA-8 – with 32 %, JTA-9 – with 31 %, JTA-10 – with 36 %, JTA-11 and JTA-12 – with 33 %, compared to the control. The different effects of the compounds on isolated liver microsomes, might be due to the structures' differences.

**Keywords:** caffeine, isolated rat liver microsomes, pro-oxidant effects

## Introduction

In the resent years, there is information of the ability of caffeine to inhibit the growth of liver tumor cells by a mechanism independent of apoptosis by blocking the cell cycle[1]. Data from recent experimental studies have shown that coffee consumption has beneficial effects in chronic hepatitis C, liver fibrosis and carcinogenicity [2-4].

*In vitro* biotransformation assays are essential for understanding the pharmacokinetic characteristics of certain compounds, optimizing pharmacokinetic parameters, and selecting appropriate molecules in drug developing

process. The production of equivalent data from *in vitro* and *in vivo* studies gives opportunity to pharmaceutical industry to work on the validation of *in vitro* models by means of high technology and to replace animal studies wherever possible.Moreover, *in vitro* test systems are the only humanized models in the early stages of the pharmacological characterization of different compounds [5].

Microsomes are widely used test systems for investigation the metabolic stability and metabolic profile of a large number of during the drug discovery and development phases [6]. Microsomal liver fractions combined with the possibility of automation of incubating process are elevated to high-tech applications. High storage stability of microsomes provides an opportunity to create a human bank for hepatic fractions and to study differences in enzyme activities in the population. Correlation analysis is applied to study metabolic pathways in the context of enzymatic topology [6].

*In vivo* studies in experimental animals show beneficial effects of coffee and caffeine intake on the liver. In a animal study on training rats, after training have been observed elevated levels of hepatic transaminases, increased activity of glutathione peroxidase, superoxide dismutase, catalase, and elevated malondialdehyde (MDA), with caffeine intake decreasing these effects. Results from other studies on rats with induced liver fibrosis, suggest that coffee and caffeine intake has beneficial effect on the development of fibrosis and carcinogenesis [6].

Data from randomized clinical trials show positive effects of coffee consumption in chronic hepatitis C, including reduction of oxidative damage [2]. These data are also supported by meta-analysis that show a reverse relationship between coffee consumption and the risk of developing liver cancer, with a reduction in the risk of consumption vs. non-consumption of 40% [3].

*In vitro* studies on human hepatic cancer cells indicate that caffeine inhibits the growth of these cells by a mechanism independent of apoptosis, but through G0/ G1cell cycle arrest [1].

Regarding the literature data, the aim of the present study was to evaluate the effects of newly synthetized derivatives of caffeine-8-thioglycolic acid on isolated rat liver microsomes.

## Materials and methods

### *Chemicals*

The chemicals used in the biological experiments were: KCl (Merck);TRIS-HCl, $KH_2PO_4$, $K_2HPO_4$(ScharlauChemie SA, Spain), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA), Glycerol (Valerus, Bulgaria).

### *Animals*

For all the experiments, we used 6 male white Wistar rats. The animals were obtained from the National Breeding Center at the Bulgarian Academy of Sciences, Slivnitsa, Bulgaria and grown under standard conditions in plexiglass cells with free access to water and food and 12/12 hours light/dark at $20^0$-$25^0$C.At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinary physician. Twelve hours before each specific study, animal feed was removed.

The vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and made according Ordinance № 15/2006 for humaneness behavior to experimental animals.

### *Isolation of rat liver microsomes[7]*

The liver was removed and weighed, then perfused with 1.15% KCl. A 25% liver homogenate containing 0.1 M TRIS-potassium phosphate buffer with pH = 7.4 was prepared. The homogenate was centrifuged on a "Janetzki" K-24 refrigerated centrifuge for 30 minutes at 10 000 x g. Microsomes were obtained by differential centrifugation of the supernatant for 60 minutes at 106,000 x g in the Beckman-Coultar

refrigerated ultracentrifuge. The supernatant was discarded (cytosol) and the microsomal precipitate was frozen under 0.1 M 20% glycerol potassium phosphate buffer solution with pH 7.5. Before the experiment, the microsomeswere re-suspended with the buffer to a protein content of 1 mg/ml.

All stages of hepatic fraction preparation were performed at temperature 0° C. The re-suspended microsomes were stored at -25° C.

Microsomes were incubated with 100 μ M of the test substances [8].

***Determination of microsomal protein by the method of Lowry et al. [9]***

The content of microsomal protein was determined spectrophotometry at λ = 580 nm, according to the method of Lowry and co-workers, using bovine serum albumin as a standard.

***Determination of malondialdehyde[10]***

The reaction was stopped with 1 ml 25 % trichloroacetic acid and 1 ml 0.67% thiobarbituric acid. The samples were placed at 100 ° C for 20 minutes and then centrifuged at 2000 x g for 10 minutes. The measurement was spectrophotometric at λ = 535 nm. The concentration of MDA was calculated by a molar extinction coefficient of $1.56x10^5M^{-1}sm^{-1}$ of the malondialdehyde thiobarbituric acid complex and was expressed in nmol/mg of protein.

***Statistical analysis***

Statistical analysis was performed using statistical program 'MEDCALC'. Results are expressed as mean ± SEM for 6 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$;$P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant.Three parallel samples were used.

**Results and discussion**

From the exam derivatives, only caffeine, KTG, JTA-1, JTA-2 and JTA-3 did not statistically change the level of MDA. JTA2-Ox increased MDA statistically significantly by 17% relative to the control (non-treated microsomes).

On the isolated rat liver microsomes, more pronounced and statistically significant toxic effect have the compounds: JTA-4, JTA-5 JTA-6, JTA-7, JTA-8, JTA-9, JTA-10, JTA-11, JTA-12 and JTA-13. JTA-4, JTA-5 and JTA-13 increase MDA by 34%. JTA-6 – with 37%, JTA-7 – with 30%, JTA-8 – with 32%, JTA-9 – with 31%, JTA-10 – with 36%, JTA-11 and JTA-12 – with 33% relative to the control.

Based on observed effects on isolated microsomes and the similarities in the chemical structure, the newly synthesized caffeine-8-thioglycolic acid derivatives can be divided into two groups.

From the first group (JTA-1, JTA-2, JTA-2Ox, JTA-3) only JTA-2Ox exhibits a statistically significant pro-oxidant effect and increases the production of MDA by 17% relative to the control (non-treated microsomes). This effect can be explained by the fact that JTA-2Ox is an oxidized form of JTA-2.

The substances of the second group (JTA-4, JTA-5, JTA-6, JTA-7, JTA-8, JTA-9, JTA-10, JTA-11, JTA-12 and JTA-13) exhibit pronounced pro-oxidant effect. Their toxicity is more pronounced than that of caffeine and caffeine-8-thioglycolic acid. In the second group, the replacement of the aromatic system with a cyclohexyl radical or an alcohol group does not lead to a significant change in toxicity. However, the shortening of the ethylene bridge between the amide group and the benzyl radical with a methylene group (JTA-4, JTA-5, JTA-6, JTA-7, JTA-8) results in increased production of MDA.

In the second group compounds, the replacement of the benzyl radical with a cyclohexyl (JTA-9, JTA-10) or alcoholic group (JTA-11, JTA-12, JTA-13) does not reduce the toxicity on the isolated microsomes.

On the isolated rat microsomes JTA-1, JTA-2 and JTA-3 did not exhibit a statistically significant toxic effect, unlike other derivatives of caffeine-8-thioglycollic acid (JTA-5-13), which exhibit pro-oxidant activity.

***Table 1.*** *Effect of 100 μM newly synthesized derivatives of caffeine-8-thioglycolic acid, caffeine and caffeine-8-thioglycollic acid (KTG) on isolated rat microsomes.*

| **Group** | **MDA, nmol/mg protein** | **Effect(%) relative to the control (non-treated microsomes)** |
|---|---|---|
| Control | 1,74 ± 0,05 | 100 |
| Caffeine | 1,66 ± 0,04 | ↓ 4 |
| KTG | 1,78 ± 0,07 | ↓ 2 |
| JTA-1 | 1,67 ± 0,06 | ↓ 4 |
| JTA-2 | 1,82 ± 0,03 | ↑ 5 |
| JTA2-Ox | 2,04 ± 0,05$^{*\,+\,\#}$ | ↑ 17 |
| JTA-3 | 1,75 ± 0,05 | ↑ 1 |
| JTA-4 | 2,34 ± 0,1$^{***\,+++\,\#\#\#}$ | ↑ 34 |
| JTA-5 | 2,34 ± 0,1$^{***\,+++\,\#\#\#}$ | ↑ 34 |
| JTA-6 | 2,38 ± 0,2 $^{***\,+++\,\#\#\#}$ | ↑ 37 |
| JTA-7 | 2,26 ± 0,02 $^{***\,+++\,\#\#\#}$ | ↑ 30 |
| JTA-8 | 2,30 ± 0,05$^{***\,+++\,\#\#\#}$ | ↑ 32 |
| JTA-9 | 2,28 ± 0,04 $^{***\,+++\,\#\#\#}$ | ↑ 31 |
| JTA-10 | 2,36 ± 0,06 $^{***\,+++\,\#\#\#}$ | ↑ 36 |
| JTA-11 | 2,32 ± 0,07 $^{***\,+++\,\#\#\#}$ | ↑ 33 |
| JTA-12 | 2,31 ± 0,06 $^{***\,+++\,\#\#\#}$ | ↑ 33 |
| JTA-13 | 2,33 ± 0,06 $^{***\,+++\,\#\#\#}$ | ↑ 34 |

$^{*}$ $P < 0,05$; $^{***}$ $P < 0,001$ vs control (non-treated microsomes)
$^{+}$ $P < 0,05$; $^{+++}$ $P < 0,001$ vscaffeine
$^{\#}$ $P < 0,05$; $^{\#\#\#}$ $P < 0,001$ vs caffeine-8-thioglycolic acid (KTG)

## Conclusion

The results of our study showed that only JTA-1, JTA-2 and JTA-3 from all 14-th compounds did not revealed statistically significant pro-oxidant effects on isolated rat liver microsomes.

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