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Research Article

Synthesis, *in silico* prediction of sites of metabolism and *in-vitro* hepatotoxicity evaluationofnew seriesN'-substituted 3-(1,3,7-trimethyl-xanthin-8-ylthio) propanehydrazides

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Abstract

New series of 3-(1,3,7-trimethyl-xanthin-8-ylthio)propanehydrazide derivatives were designed and synthesized. The targed compounds were obtained in yields of 54 to 100% and their structures were elucidated by FTIR, ^{1}H NMR, ^{13}C NMR, MS and microanalyses.

The tested compounds were subjected to *in silico* prediction of sites of metabolism (SOMs). The predictions show thatthe main metabolic changes will be primarily related to oxidation of the sulfur atom in the side chain, carried out under the action of CYP2C19, as well as O-demethylation of compounds containing methoxy groups.The N-demethylation of the xanthine fragment was determined to be regulated by CYP1A2, CYP2C9, CYP2D6 and CYP3A4. Theperformed *in vitro* studies confirmed for two of the tested compounds to be low hepatotoxic, due to the presented prooxidant effects at subcellular level (isolated rat liver microsomes). These results highlight these molecules as promising hydrazide-hydrazone structures for the design of compounds with low hepatotoxicity.

Keywords

hepatotoxicity, caffeine containing acyl hydrazones, isolated rat liver microsomes, SOMs prediction

Introduction

Hydrazones are a class of organic compounds that can be considered as derivatives of carbonyl compounds (ketones and aldehydes) in which the oxygen atom in the functional group is replaced by the NNHR function. The hydrazone structural fragment plays an important role in heterocyclic chemistry. The C=N bond of the hydrazone is conjugated to the unshared electron pair of the terminal nitrogen atom and is responsible for the physical and chemical properties of this class of compounds. The C-atom in the hydrazone is both electrophilic and nucleophilic in nature, and both N-atoms are nucleophilic, although the amine type of nitrogen atom is more reactive (Ali et al. 2012). The alpha-hydrogen atom of hydrazones is more reactive than that of ketones, as the alpha-hydrogen atom of hydrazones is 10 times more acidic

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than that of ketones (Corey and Enders 1976a, b; Belskaya et al. 2010).The compounds of general formula ArCONH-N=C(R)Ar are known as N-acyl hydrazones and can be considered as obtained by condensation of acylhydrazine and carbonyl reagent. They represent another molecular skeleton from which new biologically active compounds can be derived. Acyl hydrazones have been extensively studied in recent years, as they have been found to be associated with a variety of biological activities, have promising analytical properties, and can be used as catalysts. Also, the double bond between C and N in the structure of hydrazones contributes to the formation of geometric isomers (sin and anti). Geometric isomerism can play an important role in the bioactivity of acylhydrazones, which is why their research is essential in the development of synthetic methods for the selective synthesis of a specific isomer (Singh et al. 2013). In recent years, extensive research on hydrazides and their derivatives has demonstrated a variety of biological activities. Hydrazones with azomethine –NHN = CH– proton are an important class of compounds for the development of new drugs. Recently, hydrazide hydrazones have become important due to their diverse biological properties, including antibacterial, antifungal, anticonvulsant, anti-inflammatory, antimalarial and antituberculous activities(Rollas and Küçükgüzel 2007; Sarıgöl et al. 2015), as well as anti-cancer and cytoprotective potency (Hristova-Avakumova et al. 2017), antidepressant, antiparkinsonian and antioxidant (Gohil et al. 2010). The latter fact is very important because it is known that oxidative stress is associated with the pathogenesis of many diseases such as neurodegenerative disorders (Parkinson‘s and Alzheimer‘s disease), atherosclerosis, diabetes, cancer. Oxidative stress leads to damage, as free radicals damage a number of structures such as lipids, proteins and DNA. Oxidative stress also plays an important role in the physiological adaptation and regulation of signaling cell transduction (Gohil et al. 2010).

Caffeine (1,3,7-trimethylxanthine) is a methylxanthine alkaloid found in a number of natural sources. At the same time, it is the most widely used psychoactive drug in the world (Nehlig et al. 1992). The most common biological targets for caffeine are adenosine receptors, phosphodiesterases, calcium channels and GABA receptors. Due to its effects on various systems and organs in the human body, caffeine is used in many clinical conditions: modulation of pain (Sawynok 2011), headache (Camann et al. 1990), migraine (Baratloo et al. 2015), as well as to improve fatigue and cognitive function and Parkinson‘s disease (Al Deeb et al. 2002; Kolayli et al. 2004; Lorist and Tops 2003). Although there is a great number of preclinical evidence to support the neuroprotective effects of caffeine against neurodegenerative diseases such as AD and PD, clinical trials in this area are scarce. Blockade of adenosine A_{2A}receptors and antioxidant activity can be mentioned as possible mechanisms leading to the manifestation of a neuroprotective effect (Negida et al. 2017).It was well established that oxidative stress is involved in Alzheimer’s disease (Resende et al. 2008) and Parkinson’s disease (Lang 2007). Caffeine has been recognized as an efficient biological antioxidant with inhibitory activity on lipid peroxidation in rat liver microsomes (Onwuka and Erhabor 2012). 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 (Barcelos et al. 2014), where malondialdehyde (MDA) is known to be the most frequently used biomarker of lipid peroxidation and oxidative stress evaluation.

In this study, based on the biological activity profiles of caffeine and the active pharmacophore (-CONH-N=CH-) of acyl hydrazones, we sought to develop hybrid molecules by combining these pharmacophore fragments with spacer chain in one structure. The various functional substituents in the new molecules may allow structure optimization, which could further modify the pharmacological effect, as well as favorably affect solubility and bioavailability. In addition, we aimed to predict the site of metabolism (SOM) and to evaluate the effects of the newly synthesized series of hydrazide-hydrazone derivatives of caffeine-8-(3-thio)-propanoic acid on isolated rat liver microsomes as a model of hepatotoxicity at the subcellular level, applying the level of generated malondialdehyde (MDA) in rat liver microsomes as a measure of hepatotoxicity.

Experimental

General information

Synthetic gradechemicals and solvents used in the study were purchased from Acros (Spain). Thin layer chromatography (TLC) applications performed on Kieselgel 60, F254 (Germany) in the mobile phase: 25% NH_4OH:acetone:$CHCl_3$:n-butanol (1:3:3:4 v/v parts)were used for monitoring reactions and chemical purities of the compounds. The melting points of the studied compounds were determined with a Büchi 535 electrothermal apparatus(Switzerland) and were uncorrected. The UV spectra reported in the present work wererecordedon a Jenway 6715 UV/VIS Spectrophotometer (UK). Infrared spectra were recorded on a Nicolet iS10 FT-IR spectrometer with with Smart iTR adapter. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 using Bruker-250 WM spectrometer at 250MHz and 75 MHz, respectively. Tetramethylsilane (TMS) was used as an internal standard. Elemental analyses were performed using a EuroEA3000-Single analyzer (EuroVector S.p.A, Italy). Mass spectrometry was carried out on a Dionex Ultimate 3000 RS LC/ Q Exactive Plus orbitrap MS spectrometer system. The chemical nameswere generated by using structure-to-name algorithm of the software ChemBioDraw Ultra 11.0 (CambridgeSoft).

Chemistry

Synthesis of 3-((1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)thio)propanoic acid (2)

The reaction was carried out analogously to the procedure described in our previous publication(Mitkov et al. 2012). Yield84%. M.p.: 219–220 °C. FTIR (ATR, cm^{-1}): 1728 (νCO - carcboxyl), 1710(νCO - xanthine),1694 (νCO - xanthine), 1558 with shoulder at 1539 (νC=N, νC=C – vanthine).^{1}H NMR (DMSO-d_6) δ, ppm: 2.80 (t, 2H, –CH_2–, *J* = 6.9 Hz),3.41 (s, 3H, N^1–CH_3), 3.47 (s, 3H, N^3–CH_3), 3.81 (s, 3H, N^7–CH_3), 4.31 (s, 2H, S–CH_2, *J* = 6.9 Hz).

Synthesis of methyl 3-((1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)thio)propanoate (3)

The reaction was carried out analogously to the procedure described in our previous publication(Mitkov et al. 2012). Yield96%. M.p.: 123 - 125 °C. FTIR (ATR, cm^{-1}): 1735 (νCO - ester), 1694(νCO - xanthine),1652 (νCO - xanthine), 1558 with shoulder at 1539 (νC=N, νC=C – xanthine).^{1}H NMR (DMSO-d_6) δ, ppm: 2.68 (t, 2H, –CH_2–, *J* = 6.9 Hz), 3.41 (s, 3H, N^1–CH_3), 3.47 (s, 3H, N^3–CH_3), 3.65 (s, 3H, O–CH_3),3.81 (s, 3H, N^7–CH_3), 4.31 (t, 2H, S–CH_2, *J* = 6.9 Hz).

Synthesis of 3-((1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)thio)propano hydrazide (4)

To methyl caffeine-8-thiopropanoate (4.32 g, 0.014 mol) dissolved in 50 ml hot ethanol, hydrazine hydrate (0.04 mol, 2 ml) was added. Reaction time 2 hours - determined by TLC on starting acid depletion. After cooling, the crystallized product was filtered and recrystallized from 70% ethanol.Yield88%. M.p.: 210 – 212 °C. IR (ATR, cm^{-1}): 3270 (νNH, NH_2),1710(νCO – xanthine),1687(νCO – xanthine),1670with shoulders at1622 and 1637 (νCO – amide I), 1590with shoulders at 1567 and 1551 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 2.59 (t, 2H, –CH_2–, *J* = 6.9 Hz), 3.41 (s, 3H, N^1–CH_3), 3.47 (s, 3H, N^3–CH_3), 3.81 (s, 3H, N^7–CH_3), 4.31 (t, 2H, S–CH_2, *J* = 6.9 Hz).

General procedure for syntheses of hydrazide-hydrazones of 3-((1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)thio)propanoic acid (6a-p)

To a solution of **4** (0.0032 mol) in 25 ml of ethanol the corresponding aldehyde (0.0036 mol) was added. The reaction mixture was stirred under reflux on an electromagnetic stirrer until the starting hydrazide was exhausted (TLC control).Then the solvent was removed under reduced pressure and the dry residue was recrystallized from ethanol / water (1: 1).

Synthesis of N′-benzylidene-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6a)

Reaction time 3 hours.Yield 61%. M.p.: 189 - 192 °C. UV λ_{max}: 220, 298 368 nm, FTIR (ATR, cm^{-1}): 3171 (νN-H),1670(νCO – xanthine),1660(νCO – xanthine),1609 (νCO – amide I), 1539 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 8.09 (s, H,N=CH), 7.64–7.50 (m, 5H, Ar), 4.08 (t, 2H, S-CH_2), 3.38 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.7 (t, 2H, CH_2, *J* = 7.04 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO), 154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 149.2 (C^4), 140.9 (–CH=), 139.5 (C^1_{arom}), 128.6 (C^2, C^6_{arom}), 128.4 (C^3, C^5_{arom}), 127.8 (C^4_{arom}),107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{18}H_{21}N_6O_3S$(Mm = 401.46) calculated: C 53.85% H 5.27% N 20.93% S 7.99%; found: C 53.81%, H 5.24%, N 20.90%, S 7.92%.LC-MS (70 eV) *m/z*:$[M+2H]^{+2}$/$[M+H]^{+}$: 402/401.

Synthesis of N′-(4-nitrobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6b)

Reaction time 1 hour.Yield 100%. M.p.: 239 - 240 °C (decomposition). UV λ_{max}: 218, 292 338 nm, FTIR (ATR, cm^{-1}): 3174 (νNH),1698(νCO – xanthine),1657(νCO – xanthine),1610 (νCO – amide I), 1537 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 8.37 (s, H,N=CH), 8.20 (d, 2H, Ar, *J* = 8.32 Hz), 7.67 (d, 2H, Ar, *J* = 8.32 Hz), 3.38 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.71 (t, 3H, CH_2,*J* = 7.06 Hz).^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO), 164.3 (C^3_{arom}), 154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 148.8 (C^4), 140.9 (–CH=), 134.1 (C^1_{arom}), 128.6 (C^6_{arom}), 129.1 (C^5_{arom}), 120.9 (C^4_{arom}), 119.3 (C^2_{arom}),107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{18}H_{20}N_7O_5S$(Mm = 446.46) calculated: C 48.42% H 4.52% N 21.96% S 7.18%; found: C 48.40%, H 4.49%, N 21.93%, S 7.15%. LC-MS (70 eV) *m/z*:$[M+2H]^{+2}$/$[M+H]^{+}$: 446/447.

Synthesis of N′-(2,6-dichlorobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6c)

Reaction time 2 hours.Yield 93%. M.p.: 110 - 114 °C. UV λ_{max}: 226, 292 nm, FTIR (ATR, cm^{-1}): 3171 (νNH),1695(νCO – xanthine),1658(νCO – xanthine),1610 (νCO – amide I), 1539 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.21 (s, H,N=CH), 7.43 (m, 3H, Ar), 3.38 (t, 2H, S-CH_2, *J* = 7.05 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.05 Hz).^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO),154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 148.8 (C^4), 140.9 (–CH=), 132.7 (C^1_{arom}), 132.0 (C^2, C^6_{arom}), 128.7 (C^3, C^5_{arom}), 129.4 (C^4_{arom}),107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{18}H_{19}Cl_2N_6O_3S$(Mm = 470.35) calculated: C 45.97%

H 4.07% Cl 15.08% N 17.87% S 6.82%; found: C 45.95%, H 4.05%, Cl 15.02%,N 17.88%, S 6.80%. LC-MS (70 eV) *m/z*:$[M+2H]^{+2}$/$[M+H]^{+}$: 471 /470

Synthesis of N'-(3-chlorobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6d)

Reaction time 3 hours.Yield 60%. M.p.: 106 - 110 °C. UV λ_{max}: 226, 292 nm, FTIR (ATR, cm^{-1}): 3175 (νNH),1697(νCO – xanthine),1670(νCO – xanthine),1660 (νCO – amide I), 1536 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.19 (s, H,N=CH), 7.52 - 7.27 (m, 4H, Ar), 3.38 (t, 2H, S-CH_2, *J* = 7.05 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.05 Hz).^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO),154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 148.8 (C^4), 140.9 (–CH=), 134.1 (C^1_{arom}), 130.4 (C^3_{arom}), 128.8 (C^2_{arom}),138.6 (C^6_{arom}), 128.7 (C^5_{arom}), 127.0 (C^4_{arom}),107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{18}H_{20}ClN_6O_3S$(Mm = 435.10) calculated: C 49.60% H 4.62% Cl 8.13% N 19.28% S 7.35%; found: C 49.58%, H 4.65%, Cl 8.10%,N 19.24%, S 7.25%. LC-MS (70 eV) *m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 436 /437.

Synthesis of N'-(4-bromobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6e)

Reaction time 2 hours.Yield 72%. M.p.: 116 - 120 °C. UV λ_{max}: 220, 296 nm, FTIR (ATR, cm^{-1}): 3172 (νNH),1700(νCO – xanthine),1657(νCO – xanthine),1605 (νCO – amide I), 1542 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.19 (s, H,N=CH), 7.43 (t, 2H, CAr, *J* = 8.57 Hz),7.15 (t, 2H, Ar, *J* = 8.57 Hz), 3.36 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.73 (t, 3H, CH_2,*J* = 7.06 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO), 154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 148.8 (C^4), 140.9 (–CH=), 139.5 (C^1_{arom}), 131.7 (C^3, C^5_{arom}), 127.5 (C^2, C^6_{arom}), 122.3 (C^4_{arom}),107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{18}H_{20}BrN_6O_3S$(Mm = 480.36) calculated: C 45.01% H 4.20% Br 16.63% N 17.50% S 6.67%; found: C 44.98%, H 4.18%, Br 16.33%, N 17.42%, S 6.57%.LC-MS (70 eV) *m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 480 /481.

Synthesis of N'-(4-(trifluoromethyl)benzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6f)

Reaction time 2 hours.Yield 70%. M.p.: 234 - 236 °C. UV λ_{max}: 214, 298 nm, FTIR (ATR, cm^{-1}): 3172 (νNH),1699(νCO – xanthine),1668(νCO – xanthine),1607 (νCO – amide I), 1545 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.23 (s, H,N=CH), 7.65 (t, 2H, Ar, *J* = 8.31 Hz),7.40 (t, 2H, Ar, *J* = 8.31 Hz), 3.69 (t, 2H, S-CH_2, *J* = 7.04 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.73 (t, 3H, CH_2,*J* = 7.04 Hz).^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 167.1 (-CO), 154.5 (C^6=O), 152.2 (C^2=O), 150.5 (C^8), 148.8 (C^4), 140.9 (–CH=), 139.5 (C^1_{arom}), 130.3 (C^4_{arom}),128.5 (C^2, C^6_{arom}), 125.7 (C^3, C^5_{arom}), 123.8 (CF_3), 107.1 (C^5),30.7 (N7-CH_3),30.5 (–CH_2–), 30.0 (–CH_2–S),29.7 (N3-CH_3), 28.1 (N1-CH_3). For $C_{19}H_{20}F_3N_6O_3S$(Mm = 469.46) calculated: C 48.61% H 4.29% F 12.14% N 17.90% S 6.83%; found: C 48.58%, H 4.22%, F12.10%, N 17.85%, S 6.77%.MS (70 eV) *m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 469 /470.

Synthesis of N'-(3,4-dimethoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6g)

Reaction time 3 hours.Yield 82%. M.p.: 182 - 186 °C. UV λ_{max}: 210, 308 nm, FTIR (ATR, cm^{-1}): 3164 (νNH),1698(νCO – xanthine),1654(νCO – xanthine),1600 (νCO – amide I), 1545 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.23 (s, H,N=CH), 7.13 - 7.09 (m, 3H, Ar), 6.68 (d, 2H, Ar, *J* = 8.45 Hz),3.89 (s,3H, Ar-OCH_3-*p*), 3.80 (s,3H, Ar-OCH_3-*m*),3.39 (t, 2H, S-CH_2, *J* = 7.04 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.73 (t, 3H, CH_2,*J* = 7.04 Hz). MS (70 eV) *m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 461 M/462 M.

Synthesis of N'-(3,4,5-trimethoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6h)

Reaction time 2 hours. Yield 54%. In methanol the reaction time is 15 minutes and the yield is 70%.M.p.: 186 - 189 °C. UV λ_{max}: 216, 316 nm, FTIR (ATR, cm^{-1}): 3170 (νNH),1698(νCO – xanthine),1654(νCO – xanthine),1610 (νCO – amide I), 1539 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.18 (s, H,N=CH), 6.90 (s, 2H, Ar),3.82 (s,3H, Ar-OCH_3-*p*), 3.81 (s,6H, Ar-OCH_3-*m*),3.39 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.73 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV) *m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 492 M/493.

Synthesis of N'-(4-hydroxy-3,5-dimethoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6i)

Reaction time 2 hours. Yield 78%. M.p.: 187 - 190 °C. UV λ_{max}: 218, 302, 352 nm. FTIR (ATR, cm^{-1}): 3300 (νOH),3194 (νNH),1690(νCO – xanthine),1643(νCO – xanthine),1607 (νCO – amide I), 1540 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.18 (s, H,N=CH), 6.92 (s, 2H, Ar),3.81 (s,6H, Ar-OCH_3-*m*),3.38 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV)*m/z*:$[M+H]^{+}$/ $[M+2H]^{+2}$: 477 /478.

Synthesis of N'-(4-hydroxy-3-methoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6j)

Reaction time 4 hours. Yield 61%. In methanol the reaction time is 1 hour and the yield is 94%.M.p.: 228 - 232 °C. UV λ_{max}: 220, 302, 354 nm. FTIR (ATR, cm^{-1}): 3305

(νOH),3201 (νNH),1698(νCO – xanthine),1643(νCO – xanthine),1602 (νCO – amide I), 1538 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.11 (s, H,N=CH), 7.11 (s, 2H, Ar), 6.69 (d, 1H, Ar, *J* = 8.45 Hz),3.79 (s,3H, Ar-OCH_3-*m*),3.36 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 447 /448.

Synthesis of N'-(2,3-dimethoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6k)

Reaction time 3 hours.Yield 50%. M.p.: 206 - 209 °C. UV λ_{max}: 228, 312 nm. FTIR (ATR, cm^{-1}): 3201 (νNH),1696(νCO – xanthine),1643(νCO – xanthine),1607 (νCO – amide I), 1552 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.06 (s, H,N=CH), 7.11 (m, 2H, Ar), 6.62 (d, 1H, Ar, *J* = 8.62 Hz),3.91 (s,3H, Ar-OCH_3-*o*), 3.79 (s,3H, Ar-OCH_3-*m*),3.36 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 461 /462.

Synthesis of N'-(2-hydroxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6l)

Reaction time 10 minutes.Yield 68%. M.p.: 232 - 234 °C. UV λ_{max}: 222, 298, 354 nm. FTIR (ATR, cm^{-1}): 3301 (νOH),3170 (νNH),1699(νCO – xanthine),1650(νCO – xanthine),1610(νCO – amide I), 1538 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.01 (s, H,N=CH), 7.51 (d, 1H, Ar, *J* = 7.86 Hz), 7.18 (m, 2H, Ar), 6.93 (d, 1H, Ar, *J* = 7.86 Hz), 3.36 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 417 /418.

Synthesis of 3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)-N'-(2,4,6-trimethylbenzylidene)propanehydrazide (6m)

Reaction time 3 hours.Yield 70%. M.p.: 115 - 120 °C. UV λ_{max}: 220, 300 nm. FTIR (ATR, cm^{-1}): 3170 (νNH),1693(νCO – xanthine),1659(νCO – xanthine),1618 (νCO – amide I), 1558 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 7.97 (s, H,N=CH), 6.91 (d, 1H, Ar, *J* = 7.86 Hz), 3.36 (t, 2H, S-CH_2, *J* = 7.05 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.71 (t, 2H, CH_2,*J* = 7.05 Hz)q 2.21 (s, 3H, CH_3), 2.13 (s, 6H, CH_3). MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$:443 / 444.

Synthesis of N'-(2-hydroxy-3-methoxybenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6n)

Reaction time 2 hours.Yield 77%. In methanol the reaction time is 1 hour and the yield is 94%.M.p.: 215 - 218 °C. UV λ_{max}: 220, 301 nm. FTIR (ATR, cm^{-1}): 3300 (νOH),3170 (νNH),1693(νCO – xanthine),1659(νCO – xanthine),1618 (νCO – amide I), 1558 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm:8.11 (s, H,N=CH), 7.10 (m, 2H, Ar), 6.63 (d, 1H, Ar, *J* = 8.61 Hz),3.79 (s,3H, Ar-OCH_3-*m*),3.36 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.72 (t, 3H, CH_2,*J* = 7.06 Hz). MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 447 /448.

Synthesis of N'-(3-nitrobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6o)

Reaction time 3 hours. Yield 100%. M.p.: 249 - 250 °C (decomposition). UV λ_{max}: 218, 292 302 nm, FTIR (ATR, cm^{-1}): 3168 (νNH),1690(νCO – xanthine),1652(νCO – xanthine),1620 (νCO – amide I), 1557 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 8.54 (s, H,Ar), 8.37 (s, H,N=CH), 7.53 (m, 3H, Ar), 3.38 (t, 2H, S-CH_2, *J* = 7.06 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.70 (t, 3H, CH_2,*J* = 7.06 Hz).MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 446 /447.

Synthesis of N'-(4-hydroxy-3-methoxy-5-nitrobenzylidene)-3-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)propanehydrazide (6p)

Reaction time 3 hours.Yield 96%. M.p.: 250 °C (decomposition). UV λ_{max}: 219, 298 nm, FTIR (ATR, cm^{-1}): 3301 (νOH),3168 (νNH),1690(νCO – xanthine),1652(νCO – xanthine),1620 (νCO – amide I), 1557 (νC=N, νC=C – xanthine, δNH – amide II).^{1}H NMR (DMSO-d_6) δ, ppm: 8.16 (s, H,N=CH), 8.05 (s, H,Ar),7.15 (s, H, Ar), 3.79 (s, 3H, Ar-OCH_3), 3.38 (t, 2H, S-CH_2, *J* = 7.05 Hz), 3.75 (s, N^7-CH_3), 3.41 (s, 3H, N^3-CH_3), 3.21 (s,3H, N^1-CH_3), 2.70 (t, 3H, CH_2,*J* = 7.05 Hz).MS (70 eV) *m/z*:$[M+H]^+$/ $[M+2H]^{+2}$: 492 /493.

Biological evaluation

Animals

Male Wistar rats (body weight 200–250 g) were used. The rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 °C ± 2 °C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according to ISO 9001:2008.

Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinary physician. 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 to Ordinance № 15/2006 for humaneness behavior to experimental animals.

Isolation and incubation of microsomes

The liver microsomes were isolated by ultracentrifugation, using Beckman L8-M centrifuge with a 70Ti rotor, for 1 hour, following Guengerich's methodology (Guengerich 1989). The protein measurement was carried out using method of Lowry et al (Lowry et al. 1951).

Measurement of malondialdehyde (MDA) in isolated rat microsomes

The concentration of MDA was determined spectrophotometrically at 535 nm by the Debby&Gutier method(Deby and Goutier 1990).

Statistical analysis

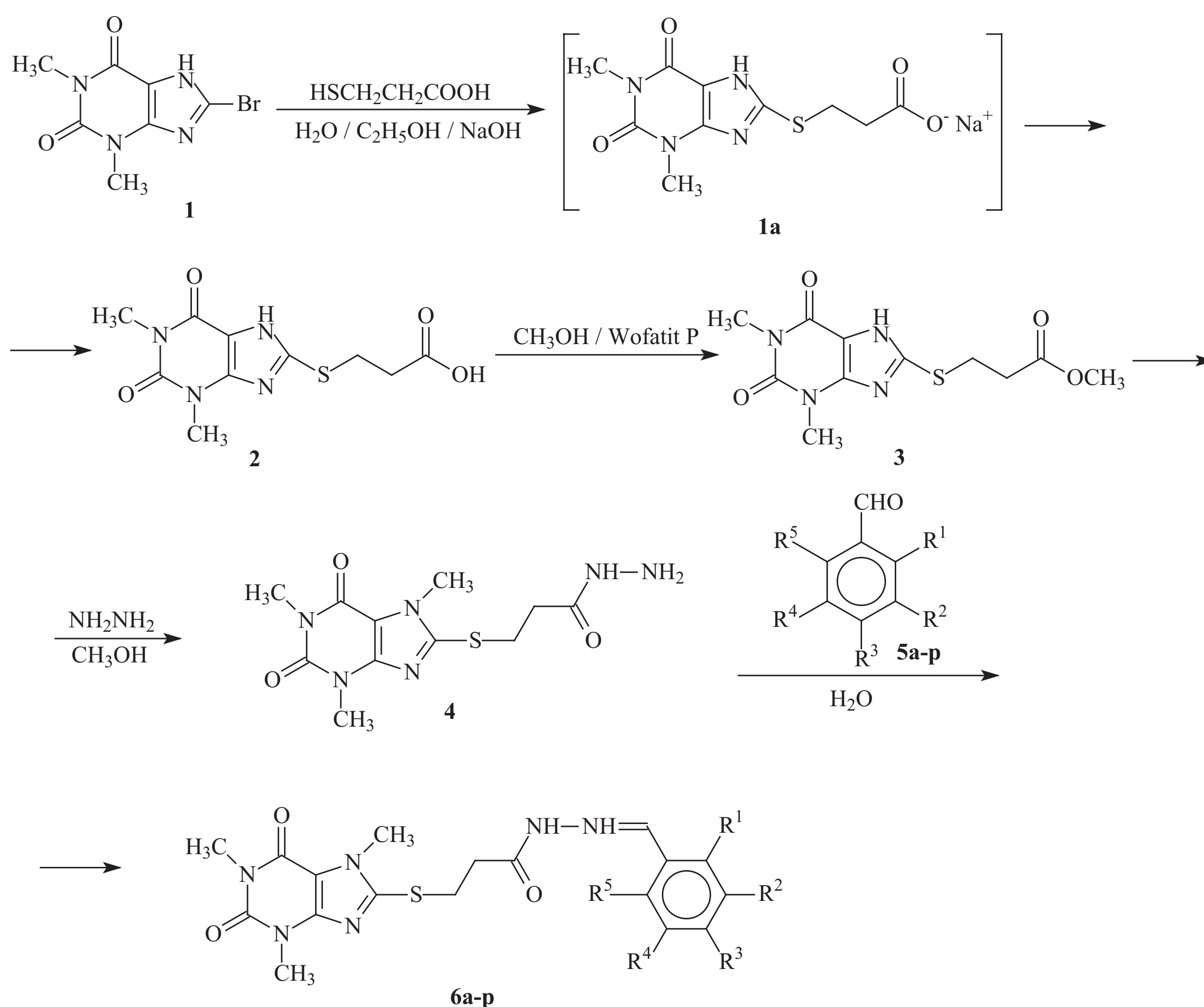
Statistical analysis was performed using statistical program "MEDCALC". Results are expressed as mean ± SEM for 7 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant.Three parallel samples were used.

Results and Discussion

Chemistry

The preparation of the target compounds was carried out synthetically as illustrated in Scheme 1. For the preparation of 8-bromocaffeine (**1**), 3-(1,3,7-xanthinyl-8-thio) propanoic acid (**2**) and its methyl ester (**3**) are applied methods described in detail in our previous publication(Mitkov et al. 2012).The next two steps of the synthesis involve the hydrazinolysis of 3-(1,3,7-xanthinyl-8-thio) propanoic acid methyl ester (**3**) to intermediate hydrazide **4**, which is subsequently reacted with the selected carbonyl partners.The choice of methyl ester as a starting compound is due to its high yield and its easy isolation and purification. A second major advantage is the ease with which the methanol fragment is replaced by nucleophilic groups.

The reaction proceeds smoothly when the reactants are boiled in an alcoholic medium. The reaction time determined by TLC in systems 1 and 2 is 2 hours, with yields of the target hydrazide in the range of 88%.



Scheme 1. The synthetic pathwayof hydrazide-hydrazone compounds **6a-p**.

Table 1. Type and location of substituents, reaction medium, reaction times and yields of newly obtained compounds.

Compound	R^1	R^2	R^3	R^4	R^5	R^6	media	Reaction time [h]	Yield [%]
6a	H	H	H	H	H	H	EtOH	3	61
6b	H	H	NO_2	H	H	H	EtOH	1	100
6c	Cl	H	H	H	Cl	H	EtOH	2	93
6d	H	H	H	Cl	H	H	EtOH	3	60
6e	H	H	Br	H	H	H	EtOH	2	72
6f	H	H	CF_3	H	H	H	EtOH	2	70
6g	H	OCH_3	OCH_3	H	H	H	EtOH	3	82
6h	H	OCH_3	OCH_3	OCH_3	H	H	EtOHMeOH	2	54
								15 min.	70
6i	H	OCH_3	OH	OCH_3	H	H	EtOH	2	78
6j	H	H	OH	OCH_3	H	H	EtOHMeOH	4	61
								1	94
6k	OCH_3	OCH_3	H	H	H	H	EtOH	3	50
6l	OH	H	H	H	H	H	EtOH	10 min.	68
6m	CH_3	H	CH_3	H	CH_3	H	EtOH	3	70
6n	OH	OCH_3	H	H	H	H	EtOH	2	77
6o	H	NO_2	H	H	H	H	EtOH	3	100
6p	H	NO_2	OH	OCH_3	H	H	EtOH	3	96

Attempts were made to obtain hydrazide **2** by direct reaction of the acid with hydrazine hydrate, which did not give a satisfactory result. We found that at a reaction temperature below 70 °C no reaction took place. As the temperature increased, the reaction mixture began to darken rapidly, resinating above 100 °C. In the reaction products we found the presence of chromatographic traces (system 2) of the target product, as well as a large number of compounds with unclear structure.

The second stage of the synthesis of hydrazide-hydrazone derivatives - condensation with selected carbonyl partners is carried out at a molar ratio of reactants 1: 1.25 in a medium of methyl or ethyl alcohol at boiling. Reaction times (Table 1) were determined by TLC - control of starting hydrazide depletion.

From the results presented in Table 1 it can be seen that when the reaction was carried out in ethanol the yields vary between 54 and 100%. Some compounds showed a significant reduction in the amount of isolated product due to their better solubility in this solvent. Replacing it with methanol, which is significantly more polar than ethanol, we found that this strongly shortened the reaction times and increased the yield of isolated product. A disadvantage is the use of larger volumes of methanol as a reaction medium due to the poor solubility of most of the reactants used in this solvent.

The structures of the newly obtained compounds were characterized by melting point and data from IR, ^{1}H-, ^{13}C-NMR, UV and LS-MS spectroscopy.

Evaluation of newly synthesized compounds

The liver performs various important functions, mainly related to the regulation of glucose metabolism, plasma protein synthesis, detoxification of endogenous and exogenous substances. At the same time, drug-induced liver damage (DILI) is one of the leading causes of a number of side effects and toxicity. On the other hand, the safety assessment of a molecule is an important step in the early stages of the development of biologically active substances and drug candidates.Hepatotoxicity involves damage to the liver caused by chemical agents, leading to the withdrawal from the market of more than 900 drugs with proven effects of liver damage(Pegg 1989). An important element of this assessment is the study of the relationship between the pharmacodynamics and pharmacokinetics of the drug candidate. The kinetic profile of the compound is highly dependent on phase I metabolism, in which cytochrome P450 (CYPs) plays a major role.

Human cytochromes P450 (CYP) are oxidoreductases with a heme cofactor that are responsible for the Phase I metabolism of 75% of drugs in the human body(Guengerich 2008).There are 57 mammalian isoforms known and their inhibition, induction, or allosteric effects by various small molecules often lead to a number of drug–drug interactions. Cytochromes P450 catalyze a wide variety of reactions, that are in general improving the water solubility either of their endogenous substrates or xenobiotics. These reactions take place at the functional groups of the molecules, also known as sites of metabolism (SOM)(Jandova et al. 2019).

Taking in mind the presented specifics, in the present work we conducted studies on the newly synthesized compounds, including *in silico* prediction of the site of metabolism (SOM) and assessment of the probabble hepatotoxicity at the subcellular level.

Prediction of the site of metabolism (SOM)

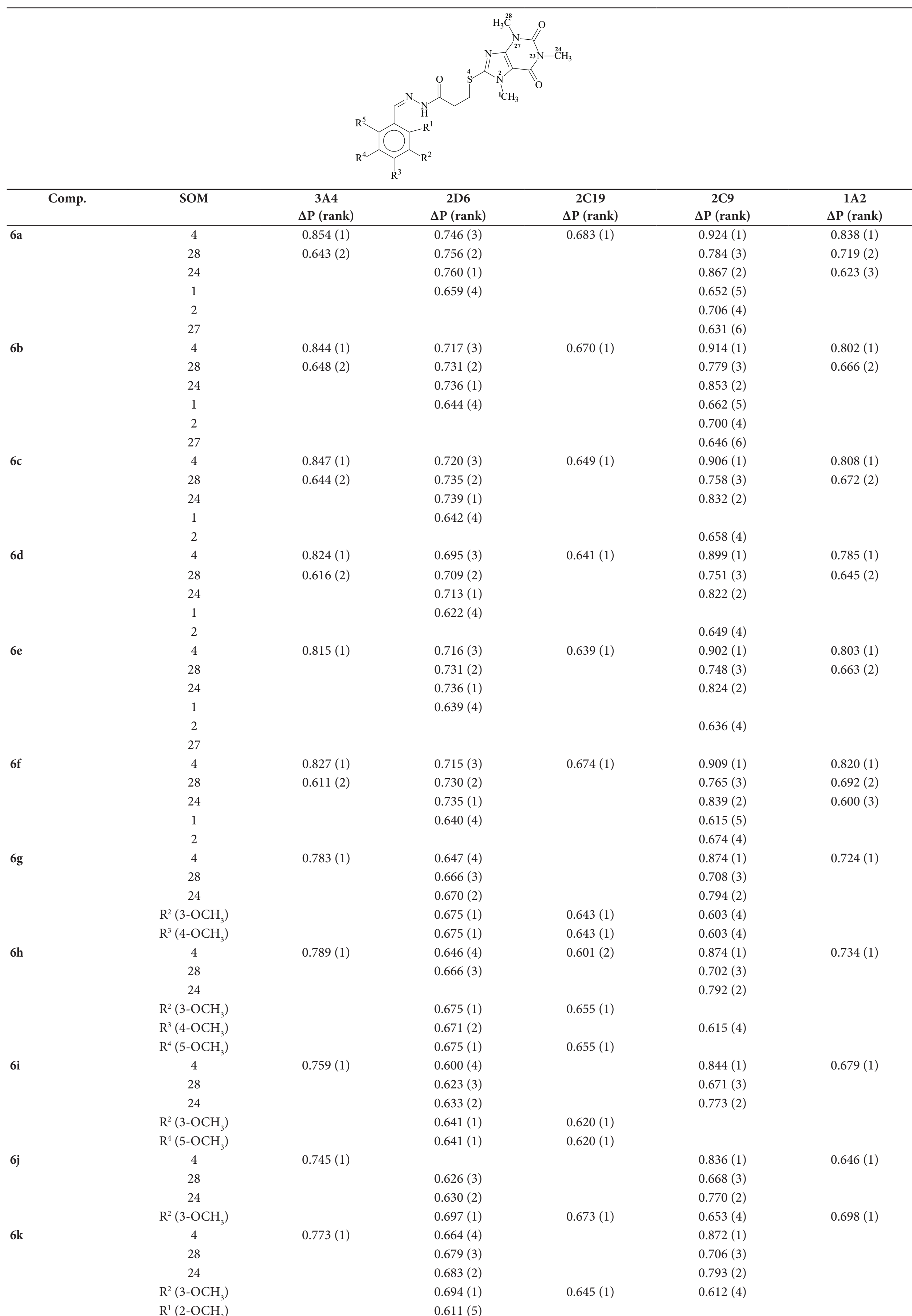
Prediction of sites of metabolism (SOM) for newly synthesized compounds is based onPASS (Prediction of Activity Spectra for Substances) technology, LMNA descriptors and applied for prediction of the SOMs of the five major human cytochrome P450s: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 using SOMP web-service (http://www.way2drug.com/SOMP/) (Rudik et al. 2015). The detailed description of the algorithm was described in literature (Rudik et al. 2014). The SOM prediction results (Table 2) include a calculated values for each non-hydrogen atom in the molecular structure of the probability to be attacked by each enzyme (ΔP) and their ranks in descending order according to this. Only values above 0.600 for ΔP are shown.

Caffeine is known to be almost completely metabolized, when less than 3% was eliminated unchanged with the urine. The main pathway in the first stage of metabolism in humans (70–80%) is by N-demethylation to 1,7-dimethylxanthine (paraxanthine), 1,3-dimethylxanthine (theophylline) and 3,7-dimethylxanthine (theobromine) (Thorn et al. 2012). The latter undergo additional metabolic degradation. The CYP1A2 enzyme is responsible for 90% of caffeine metabolism, and for 84%, 12%, and 4% of the metabolism of caffeine's metabolites: paraxanthine, theophylline, and theobromine, respectively(Nehlig 2018).

The compounds we studied are caffeine derivatives that carry a substituent at 8th place.

The results of the prediction indicate that possible metabolic changes in the test compounds would be primarily related to the oxidation of the S-atom in the

Table 2. Prediction of the site of metabolism (SOM) of compounds 6a-p.



Comp.	SOM	3A4 ΔP (rank)	2D6 ΔP (rank)	2C19 ΔP (rank)	2C9 ΔP (rank)	1A2 ΔP (rank)
6a	4	0.854 (1)	0.746 (3)	0.683 (1)	0.924 (1)	0.838 (1)
	28	0.643 (2)	0.756 (2)		0.784 (3)	0.719 (2)
	24		0.760 (1)		0.867 (2)	0.623 (3)
	1		0.659 (4)		0.652 (5)	
	2				0.706 (4)	
	27				0.631 (6)	
6b	4	0.844 (1)	0.717 (3)	0.670 (1)	0.914 (1)	0.802 (1)
	28	0.648 (2)	0.731 (2)		0.779 (3)	0.666 (2)
	24		0.736 (1)		0.853 (2)	
	1		0.644 (4)		0.662 (5)	
	2				0.700 (4)	
	27				0.646 (6)	
6c	4	0.847 (1)	0.720 (3)	0.649 (1)	0.906 (1)	0.808 (1)
	28	0.644 (2)	0.735 (2)		0.758 (3)	0.672 (2)
	24		0.739 (1)		0.832 (2)	
	1		0.642 (4)			
	2				0.658 (4)	
6d	4	0.824 (1)	0.695 (3)	0.641 (1)	0.899 (1)	0.785 (1)
	28	0.616 (2)	0.709 (2)		0.751 (3)	0.645 (2)
	24		0.713 (1)		0.822 (2)	
	1		0.622 (4)			
	2				0.649 (4)	
6e	4	0.815 (1)	0.716 (3)	0.639 (1)	0.902 (1)	0.803 (1)
	28		0.731 (2)		0.748 (3)	0.663 (2)
	24		0.736 (1)		0.824 (2)	
	1		0.639 (4)			
	2				0.636 (4)	
	27					
6f	4	0.827 (1)	0.715 (3)	0.674 (1)	0.909 (1)	0.820 (1)
	28	0.611 (2)	0.730 (2)		0.765 (3)	0.692 (2)
	24		0.735 (1)		0.839 (2)	0.600 (3)
	1		0.640 (4)		0.615 (5)	
	2				0.674 (4)	
6g	4	0.783 (1)	0.647 (4)		0.874 (1)	0.724 (1)
	28		0.666 (3)		0.708 (3)	
	24		0.670 (2)		0.794 (2)	
	R^2 (3-OCH_3)		0.675 (1)	0.643 (1)	0.603 (4)	
	R^3 (4-OCH_3)		0.675 (1)	0.643 (1)	0.603 (4)	
6h	4	0.789 (1)	0.646 (4)	0.601 (2)	0.874 (1)	0.734 (1)
	28		0.666 (3)		0.702 (3)	
	24				0.792 (2)	
	R^2 (3-OCH_3)		0.675 (1)	0.655 (1)		
	R^3 (4-OCH_3)		0.671 (2)		0.615 (4)	
	R^4 (5-OCH_3)		0.675 (1)	0.655 (1)		
6i	4	0.759 (1)	0.600 (4)		0.844 (1)	0.679 (1)
	28		0.623 (3)		0.671 (3)	
	24		0.633 (2)		0.773 (2)	
	R^2 (3-OCH_3)		0.641 (1)	0.620 (1)		
	R^4 (5-OCH_3)		0.641 (1)	0.620 (1)		
6j	4	0.745 (1)			0.836 (1)	0.646 (1)
	28		0.626 (3)		0.668 (3)	
	24		0.630 (2)		0.770 (2)	
	R^2 (3-OCH_3)		0.697 (1)	0.673 (1)	0.653 (4)	0.698 (1)
6k	4	0.773 (1)	0.664 (4)		0.872 (1)	
	28		0.679 (3)		0.706 (3)	
	24		0.683 (2)		0.793 (2)	
	R^2 (3-OCH_3)		0.694 (1)	0.645 (1)	0.612 (4)	
	R^1 (2-OCH_3)		0.611 (5)			

Comp.	SOM	3A4 ΔP (rank)	2D6 ΔP (rank)	2C19 ΔP (rank)	2C9 ΔP (rank)	1A2 ΔP (rank)
6l	4	0.816 (1)	0.682 (3)	0.611 (1)	0.889 (1)	0.753 (1)
	28	0.606 (2)	0.696 (2)		0.732 (3)	0.613 (2)
	24		0.700 (1)		0.809 (2)	
	1		0.604 (4)			
	2				0.619 (4)	
6m	4	0.819 (1)	0.662 (3)	0.611 (1)	0.899 (1)	0.753 (1)
	28		0.679 (2)		0.739 (3)	0.603 (2)
	24		0.683 (1)		0.818 (2)	
6n	4	0.762 (1)	0.615 (4)		0.841 (1)	0.672 (1)
	28		0.640 (3)		0.672 (3)	
	24		0.644 (2)		0.773 (2)	
	R^2 (3-OCH_3)		0.712 (1)	0.670 (1)	0.658 (4)	0.772 (1)
6o	4	0.833 (1)	0.694 (3)	0.647 (1)	0.905 (1)	0.634 (2)
	28		0.708 (2)		0.767 (3)	
	24		0.712 (1)		0.836 (2)	
	1		0.623 (4)		0.636 (5)	
	2				0.679 (4)	
6p	4	0.759 (1)			0.837 (1)	0.633 (1)
	28		0.623 (3)		0.680 (3)	
	24		0.627 (2)		0.773 (2)	
	R^2 (3-OCH_3)		0.692 (1)	0.669 (1)	0.668 (4)	

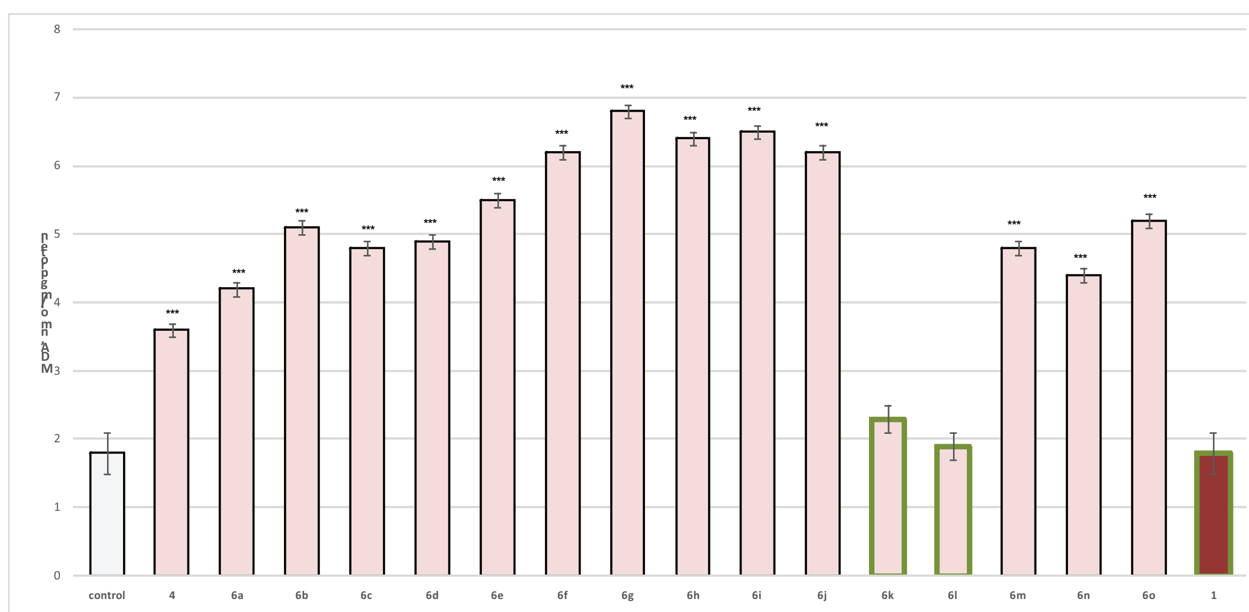


Figure 1. Influence of hydrazide-hydrazones of 3 - [(caffeine-8-yl) thio] propanoic acid (**6a**-**o**), applied separately, on the production of MDA. *** P <0.001 relative to control (untreated microsomes).

side chain, and this is most likely to occur under the action of CYP2C19. Then there occurs the probability of demethylation at the nitrogen atoms.The most likely SOMs for the test compounds were 4S, $1CH_3$, $24CH_3$, $28CH_3$ (see numbering in Table 2). For derivatives having methoxy groups in their structure, they are SOM, and according to the obtained data they are attacked mainly by CYP2C19.N–demethylation of the xanthine fragment is carried out under the action ofCYP1A2, CYP2C9, CYP2D6 and CYP3A4.

Hepatotoxicity evaluation– effects on isolated rat liver microsomes

The probable hepatotoxicity of compounds **6a-p** at the subcellular levelwas performed on liver microsomes. Microsomes are widely used as subcellular *in vitro* systems to study the metabolic profile of a large number of compounds as well as their pro- or antioxidant properties (Brandon et al. 2003; Zaidi et al. 1993) as they contain the major drug-metabolizing enzymes of the Cytochrome P450 (CYPs) and Uridine 5 families and 1-diphospho-glucuronosyl transferase (UGTs). Their storage stability (Meier et al. 1983; Pearce et al. 1996; Yamazaki et al. 1997) provides an opportunity to create a human bank of liver fractions (von Bahr et al. 1980), which allows to study the differences in enzyme activities in different populations (Beaune et al. 1986). Microsomes are also used to assess the substrate specificity of different isoforms of CYP, using various proven inhibitors with affinity for the studied isoform (Birkett et al. 1993; Donato and Castell 2003; Newton et al. 1995; Rettie et al. 1995).

The described model was used to evaluate the effect of newly synthesized hydrazide-hydrazone derivatives on

isolated rat liver microsomes by measuring the level of MDA produced. MDA is a three carbon, low molecular weight aldehyde, which is the end-product of membrane lipid peroxidation, which is documented as a primary biomarker of free radical mediated lipid damage and oxidative stress. It is characterized by a high toxicity due to its ability to react with other molecules like DNA and protein (Metro et al. 2017). Due to this MDA can be used as an indicator of membrane lipid peroxidation, and it's concentration can indicate the degree of cell peroxidation (Chi et al. 2014).

The results obtained are summarized on Fig. 1. As can be seen, only substances **6k** and**6l** applied to liver microsomes did not show a statistically significant pro-oxidant effect compared to controls (untreated microsomes). Caffeine (as positive control) also didn't induce lipid peroxidation in statistically significant manner on isolated microsomes.All other substances increase statistically significantly the production of MDA, which is a marker of lipid peroxidation and damage.

The obtined results indicate that the replacement of the mehtoxy group in the carbonyl fragment from ortho (6k) to para position (6g) of the phenyl core leads to increase in the oxidative potential of the structure, thus underlining compound 6g as most pro-oxidant structure. This observation should be taken into account in future design of analogous derivatives.

Conclusion

The present study describes the synthesis of a series of new derivatives of 3-(1,3,7-trimethyl-xanthin-8-ylthio)-propanehydrazide derivatives that have undergone *in silico* prediction ofsites of metabolism (SOMs). Based on the data obtained, we believe that the main metabolic changes will be primarily related to the oxidation of the sulfur atom in the side chain, carried out under the action of CYP2C19. Most likely, the same enzyme will be involved in the O-demethylation of compounds containing methoxy groups. N-demethylation of the xanthine fragment is performed under the action of CYP1A2, CYP2C9, CYP2D6 and CYP3A4. *In vitro* studies confirmed that two of the tested compounds (**6k** and **6l**) did not show statistically significant hepatotoxicity due to prooxidant effects at the subcellular level (isolated microsomes of rat liver). These results highlight these molecules as promising hydrazide-hydrazone structures for the design of compounds with low hepatotoxicity.

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