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Investigating the therapeutic potential of monothiocarbohydrazones: A comprehensive *in vitro* evaluation of antioxidant, antimicrobial, and cytotoxic activities

GORANA S. MRĐAN[1]*, SANJA S. VLAISAVLJEVIĆ[1], PETAR N. KNEŽEVIĆ[2], ISIDORA N. NIKOLIĆ[2], DINA G. TENJI[2], and BORKO M. MATIJEVIĆ[1]

[1]*University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Novi Sad, Serbia, and* [2]*University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Novi Sad, Serbia.*

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Abstract: Organic compounds, particularly those with nitrogen and sulfur heteroatoms, constitute over 99 % of clinically approved drugs. Among these, thiocarbohydrazones have been extensively studied, with a focus on symmetrical bis-substituted compounds. However, asymmetric and monosubstituted thiocarbohydrazones remain underexplored, despite their demonstrated high biological potential. This study presents an in-depth *in vitro* evaluation of the antioxidant, antimicrobial, and cytotoxic properties of eighteen previously synthesized and characterized monothiocarbohydrazones. The antioxidant potential was assessed using the DPPH assay, while the antimicrobial activity was determined against Gram-positive bacteria using a modified broth microdilution susceptibility method. The cytotoxic effect was evaluated on human hepatocellular carcinoma using the colorimetric MTT assay. The results reveal that the investigated monothiocarbohydrazones exhibit significant antioxidant and antimicrobial activities. Furthermore, their activity and cytotoxicity are influenced by the stereochemistry of the molecule and the nature and position of the substituents. These findings provide valuable insights for future in vivo examinations and underscore the potential of monothiocarbohydrazones in drug development.

Keywords: antimicrobial agents; antioxidant activity; biological activity; cytotoxicity; thiocarbohydrazones.

INTRODUCTION

Thiocarbohydrazones (TCHs) belong to a class of compounds obtained by condensation of thiocarbohydrazide with different types of carbonyl-based compounds. The first synthesis of these derivatives was reported in 1925,[1] but most

* Corresponding author. E-mail: gorana.mrdjan@dh.uns.ac.rs
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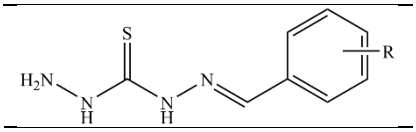
studies were conducted from the late 1970s when thiocarbohydrazide became commercially available.[2] At the very beginning TCHs have mostly been used in analytical chemistry as spectrophotometric reagents for the determination of elements such as palladium, ruthenium, iridium, gallium, zinc, etc.[3-6] Today some of them are also used for analytical research,[7-9] but most studies on thiocarbohydrazones are concerning their potential biological activity. The majority of the compounds tested so far belong to bisubstituted TCHs mainly derived from isatin, pyridyl, salicylaldehyde, and related carbonyl compounds. They have proven to be excellent antioxidant,[10-16] antimicrobial,[10,17-20] antiviral,[21] anticancer,[22-24] and antitumor[25] agents. On the other hand, monosubstituted TCHs are much less investigated, although some studies confirm their exceptional biological activity too, somewhere even more significant regarding analogous bisubstituted derivatives.[20-22] Continuing our work on monothiocarbohydrazones (mTCHs) where we have published synthesis and physicochemical characterization of eighteen compounds,[26] in this paper we present the results of the investigation on their potential biological activity. The antioxidant and antimicrobial activities of eighteen mTCHs were evaluated, along with their cytotoxic effects, using the human hepatocellular carcinoma HepG2 cell line, a standard model in toxicity assays, representing the liver, the primary organ responsible for biotransformation and detoxification of numerous xenobiotics.[27,28]

EXPERIMENTAL

Synthesis and structures of mTCHs

All thiocarbohydrazone derivatives tested within this paper were previously synthesized and characterized by Mrđan *et al.* 2021.[26] Structures and numbering of these compounds are presented in Table I.

Table I. Structures and numbering of thiocarbohydrazones



Compound	R
1	H
2/3/4	2/3/4 -OH
5/6/7	2/3/4 -CH_3
8/9/10	2/3/4 -NO_2
11/12/13	2/3/4 -OCH_3
14/15	3/4 -Cl
16/17	3/4 -Br
18	4-F

Evaluation of antioxidant activity

The antioxidant activity of selected thiocarbohydrazones was evaluated using DPPH (2,2-diphenyl-1-picryl hydrazyl) assay. Measurements were performed according to the early described spectrophotometric method with some modifications.[29] Stock solutions of TCHs were prepared in DMSO at a concentration of 5 mg mL-1. After 30 minutes incubation, the absorbance was measured at 515 nm. All measurements were performed in triplicate. Results obtained were expressed as Trolox equivalent capacity per gram of dry weight of tested solutions (mg TE/g d.w.).

Antimicrobial agents

Eighteen different compounds of mTCH were dissolved in 100 % DMSO to prepare stock solutions with a concentration of 20 mg mL-1. Stock solutions were stored at room temperature. The final concentration of DMSO was not more than 1 % in broth.

Bacterial strains and culture conditions

Four reference Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19111), *Enterococcus faecalis* (ATCC 2912) and *Bacillus subtilis* (ATCC 6633) were used in antimicrobial tests. The overnight cultures were grown at 37°C on Muller Hinton agar (MHA), except *L. monocytogenes* and *E. faecalis* which were grown on Brain Heart Infusion agar (BHIA).

Antibacterial activity of monothiocarbohydrazones

The modified broth microdilution susceptibility method was used to determine the antibacterial activity of monothiocarbohydrazones (CLSI 2015). Serial dilutions of tested agents were prepared in a 96-well microtiter plate ranging from 128 to 2 μg mL^{-1} (DMSO concentration in the final volume was ≤ 0.8 %). The overnight cultures were used for preparing bacterial suspensions by adjusting an optical density of 0.5 using McFarland Densitometer (Biosan, Latvia). Muller Hinton broth (MHB) and Brain Heart Infusion (BHIB) were used in all experiments. The bacterial suspensions were diluted with MHB or BHIB (1:100, v/v) to achieve an inoculum density of approximately $2.0x10^6$ CFU mL^{-1}. After the addition of equal volumes of the previously prepared inocula (1:1, v/v) to each well, the final bacterial count was approximately $1x10^6$ CFU mL^{-1}. The microtitre plates were incubated for 24h at 37 °C. Each experiment was performed in triplicate, in at least two independent repeats and results were presented as geometric mean of the obtained values.

Cell culture

Human hepatocellular carcinoma HepG2 (European Collection of Authenticated Cell Cultures, ECACC 85011430, Salisbury, UK), cells were grown in Minimum Essential Medium Eagle (MEM; Sigma) growth medium, containing 10 % FBS (Gibco), sodium bicarbonate, penicillin/streptomycin, sodium pyruvate (0,11 g/L) and 10 mM HEPES buffer. The cell culture process followed conditions and protocol previously described by Vulin *et al.* 2022.[30]

Cytotoxicity assay

The cytotoxic effect of tested substances was assessed using a colorimetric MTT assay. For the experiments, HepG2 cells were seeded at a density of 20.000 cells/well in 96-well plates and incubated overnight before the treatment with test substances. After the 24h incubation period, the test medium was replaced with MTT (final concentration 0.5 mg mL-1 in growth medium) and incubated for an additional 3h, followed by medium removal. The formazan crystals were dissolved in 0.04M HCl in isopropanol. The optical density was measured at 540

and 690 nm, using a Multiskan™ GO microplate spectrophotometer (Thermo Scientific). The activity of mitochondrial dehydrogenase (%) was calculated from the absorbances. The cell viability (CV) was expressed in percentages and calculated from the absorbances according to the formula:

$$CV\ (\%) = 100 - \left(\frac{1 - A_{tested\ substance}}{control}\right) \text{x } 100 \quad (1)$$

where $A_{tested\ substance}$ and $A_{control}$ represent the absorbances measured in the experimental and control wells, respectively. Hence, the values of the control are presented as 100 %.

Each concentration of all tested substances was examined in hexaplicates, in three independent experiments including treatment-free control (growth medium), solvent controls, and blank wells (cell free wells). The final concentration of DMSO in treatments did not exceed 1 % and did not result in any background response.

EC50 value was calculated based on dose-response data using concentrations of the tested substances and the corresponding effects on cell growth in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, California). The dose-response curve was determined using log inhibitor vs. normalized response-variable slope procedure.[31]

RESULTS AND DISCUSSION

Antioxidant activity of monothiocarbohydrazones

Many diseases occur as a result of oxidative stress, which represents an imbalance between free radicals and antioxidants present in the body. Antioxidants are substances that counteract and stop the action of free radicals by creating products that are non-toxic and do not cause damage to cellular structures or cells. One of the most used *in vitro* assays for the determination of the free-radical scavenging activity is the DPPH assay, used in this paper. Eighteen compounds of monothiocarbohydrazones were tested and the results obtained are presented as milligrams of Trolox equivalents (TE) per gram of dry weight (mg TE/g d.w.). Obtained values are shown in Table II.

Table II. Results of DPPH assay for monothiocarbohydrazones

	Compound / mg TE/g dw				
1	152.85 ± 0.08	7	106.63 ± 0.06	13	155.30 ± 0.04
2	145.50 ± 0.04	8	151.86 ± 0.10	14	156.06 ± 0.06
3	156.51 ± 0.11	9	103.89 ± 0.09	15	159.50 ± 0.09
4	153.57 ± 0.05	10	152.88 ± 0.08	16	114.03 ± 0.02
5	152.01 ± 0.05	11	152.72 ± 0.05	17	95.50 ± 0.02
6	153.39 ± 0.02	12	113.54 ± 0.03	18	152.03 ± 0.06

In general, all examined compounds have shown great antioxidant activity in the range of 95 to 160 mg TE/g d.w.

It is well known that the effectiveness of a compound as an antioxidant can depend on its structure, i.e. the position and type of substituent present. As can be seen from Table II the highest activity have shown compounds **14** and **15** with chlorine as a substituent. Previous studies of structurally similar compounds have

shown that derivatives with halogen substituents, especially chlorine, stand out with the highest activity.[10,14,32] Compound **18** with fluorine as a substituent has also exhibited good activity, while the compounds with bromine (**16** and **17**) have less antioxidant power. Compounds **13** (4-OCH_3) and **3** (3-OH) also have great scavenging capacity which is in agreement with studies where the compounds with these two functional groups also stand out as great antioxidants.[15,16,33]

Antimicrobial activity of monothiocarbohydrazones

Our previous studies showed that monothiocarbohydrazones are active only against Gram-positive bacteria, so in the present study, we focused on this group of microorganisms. It is known that gram-negative bacteria easily bypass the action of most drug molecules because of their unique outer membrane, that acts as a potent barrier restricting the entry of compounds into the cell. Low molecular weight and low lipophilicity are identified as two important parameters to potentiate drug entry into Gram-negative bacteria.[34] Thus, high lipophilic nature of examined monothiocarbohydrazones could render them inactive in Gram-negative bacteria. As can be seen from Table III, selected mTCHs showed activity against *S. aureus* and *B. subtilis*, but not against *L. monocytogenes* and *E. faecalis* at the highest applied concentration (128 μg mL^{-1}). This difference in susceptibility can be a consequence of different medium application since both resistant bacteria were grown using BHIA. As BHIA contains calf brain and beef heart extracts, it is possible that the extracts contain components that react with mTCHs and neutralize them. In order to confirm this assumption some of the compounds were examined again against *S. aureus* and *B. subtilis* but now bacterial suspension was prepared in BHIA, and obtained results are presented in Table IV. MIC values were higher, compared to the previous results. It can be concluded that the use of BHIA leads to a decrease in mTCHs activity, probably due to the inhibition by broth components and this can be the reason why there is no activity against *L. monocytogenes* and *E. faecalis*. Considering that these two bacterial species grow poorly in MHA and that BHIA affects the activity of the given compounds, it is necessary to find an alternative broth that would support growth and not affect the activity of the compounds in order to precisely determine the MIC values.

Table III. Susceptibility of selected Gram positive bacteria to monothiocarbohydrazones

	MIC (μg mL^{-1})			
mTCHs	*S. aureus* (ATCC 25923)	*L. monocytogenes* (ATCC 19111)	*E. faecalis* (ATCC 2912)	*B. subtilis* (ATCC 6633)
1	>128	>128	>128	45
2	64	>128	>128	45
3	64	>128	>128	45
4	128	>128	>128	23
5	32	>128	>128	32
6	32	>128	>128	16
7	32	>128	>128	32
8	>128	>128	>128	>128
9	>128	>128	>128	>128
10	128	>128	>128	45
11	64	>128	>128	23
12	>128	>128	>128	64
13	>128	>128	>128	76
14	32	>128	>128	23
15	16	>128	>128	27
16	>128	>128	>128	>128
17	>128	>128	>128	54
18	32	>128	>128	64

Table IV. Susceptibility of S. aureus and B. subtilis (BHIA) to selected mTCHs

	MIC (μg mL^{-1})	
mTCHs	*S.aureus* (ATCC 25923)	*B. subtilis* (ATCC 6633)
3	128	>128
4	>128	>128
5	>128	>128
6	128	>128
7	>128	>128
11	>128	>128
14	128	128
18	>128	>128

The antibacterial activity of mTCHs against *S. aureus* and *B. subtilis* (Table III) can be ascribed to their lipophilic nature and easier penetration into the cells[17,35] and/or oxidation of thiono group by hydrogen peroxide and formation of toxic reaction compounds.[36,37] In general mTCHs have shown good activity against *S. aureus* and *B. subtilis.* The most active compounds were compounds **14** and **15** which have chlorine as a substituent. In previous studies[36,37] it has been proven that compounds with halogen elements as substituents show high activity against

bacteria. This trend also follows compound **18** with fluorine as a substituent (MIC 32 and 64), but not compounds **16** and **17** that contain bromine. Significant activity have also shown compounds **2**, **3**, and **4** with OH-group as a substituent (2-OH, 3-OH, and 4-OH respectively) and compounds **5**, **6**, and **7** with CH_3-group as a substituent (2-CH_3, 3-CH_3, and 4-CH_3 respectively).

Cytotoxicity of monothiocarbohydrazones

The cytotoxicity of monothiocarbohydrazones (mTCHs) was evaluated using the HepG2 cell line, with effective concentrations (EC50) ranging from 20.12 to 138.04 μg mL-1 (Table V).

Table V. Effective concentrations (EC50) of tested mTCHs for HepG2 cell line

Comp.	EC_{50} (μg mL^{-1})	r^2	Comp.	EC_{50} (μg mL^{-1})	r^2
1	36.22	0.960	**10**	88.94	0.901
2	34.67	0.980	**11**	50.12	0.926
3	109.65	0.950	**12**	44.17	0.980
4	138.04	0.962	**13**	70.78	0.880
5	28.84	0.994	**14**	133.80	0.939
6	43.71	0.936	**15**	27.54	0.970
7	29.51	0.986	**16**	32.20	0.980
8	20.12	0.990	**17**	29.76	0.980
9	36.16	0.840	**18**	120.23	0.963

The results showed that the cytotoxicity of thiocarbohydrazones varied depending on the nature of the substituents and other factors. The most cytotoxic compound was compound **8**, which had a -NO_2 group, with an EC50 of 20.12 μg mL-1. This substituent is a strong electron-withdrawing group that can increase the intramolecular charge transfer and the lipophilicity of the thiocarbohydrazone molecule, which may enhance its cellular uptake and interaction with DNA. Moreover, this group can act as a redox-active center that can induce oxidative stress in cells, leading to apoptosis.[40,41] However, compounds **9** and **10** with the same -NO_2 group showed weaker cytotoxicity, suggesting other influencing factors.

Compounds **5**, **6** and **7**, with -CH_3 group as a substituent, also showed significant cytotoxicity in the lowest tested concentration (8 μg mL-1). This group is a weak electron-donating group that can also increase the intramolecular charge transfer and the lipophilicity of the thiocarbohydrazone molecule, which may facilitate its cellular uptake and interaction with DNA.[42] In addition, this group can act as a methylating agent that can transfer a methyl group to nucleophilic sites on DNA, RNA, or proteins, causing DNA damage and apoptosis.[39]

The least cytotoxic compound was **4**, which had a -OH group as a substituent. This group is a weak electron-donating group that can decrease the intramolecular charge transfer and the lipophilicity of the thiocarbohydrazone molecule, which may hinder its cellular uptake and interaction with DNA.[40] Furthermore, this group can act as a hydrogen bond donor that can form intermolecular interactions with water molecules and increase the solubility of the thiocarbohydrazone compound, which may reduce its bioavailability and efficacy.[41]

These results suggest that the cytotoxicity of thiocarbohydrazones is influenced by several factors. Previous studies have reported that some of these factors including the nature and position of the substituents, the stereochemistry and conformation of the molecules, the cell line used for testing, and the metal complexation of the ligands may modulate the physicochemical and biological properties of thiocarbohydrazones and their metal complexes, affecting their molecular interactions and biological activities.[15,40-42] Thiocarbohydrazones have been tested for cytotoxicity on various, both cancer and non-cancer cell lines in different studies such as mouse fibroblast 3T3 cell line, human erythroleukemia HEL cell line, human normal keratinocyte NCTC-2544, MDA-MB-231 breast cancer and PC-3 human prostate adenocarcinoma cell lines, showing higher cytotoxic potential in cancer call lines.43,44 Therefore, the difference between compounds with the same substituent on the same places suggest the need for further elucidation of the exact molecular targets and pathways involved in the cytotoxic effect.

CONCLUSION

In this paper, the results of the investigation on the potential biological activity of eighteen monothiocarbohydrazones were presented. The antioxidant and antimicrobial activity of mTCHs was evaluated, as well as their cytotoxic effect. Using the DPPH assay, it was concluded that all examined compounds have great antioxidant activity. The highest activity has shown compounds with chlorine as a substituent, in agreement with previous studies. Compounds with fluorine, methoxy, and hydroxyl groups also show excellent activity. Antimicrobial examinations showed that mTCHs have activity against *S. aureus* and *B. subtilis*, but not against *L. monocytogenes* and *E. faecalis*. Results obtained showed that the use of BHIA leads to a decrease in mTCHs activity, probably due to the inhibition by broth components and that is probably the reason why there is no activity against *L. monocytogenes* and *E. faecalis*. The antibacterial activity of mTCHs can be ascribed to their lipophilic nature and easier penetration into the cells and/or oxidation of thiono group by hydrogen peroxide and formation of toxic reaction compounds. Again, the most active compounds were compounds with chlorine, as well compounds with fluorine, hydroxyl, and methyl group. In the end, the cytotoxic effect of tested substances was assessed using a colorimetric MTT assay.

Results obtained showed that the most cytotoxic compound was a compound with nitro group (para position). Compounds with methyl group as a substituent (all positions), also showed significant cytotoxicity, while the least cytotoxic compound was a compound that had hydroxyl group as a substituent (also para position). This group is a weak electron-donating group that can decrease the intramolecular charge transfer and the lipophilicity of the thiocarbohydrazone molecule, which may hinder its cellular uptake and interaction with DNA. These results suggest that the cytotoxicity of thiocarbohydrazones is influenced by several factors: the nature and position of the substituents, the stereochemistry, and conformation of the molecules, the cell line used for testing, etc.

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И З В О Д

ИСПИТИВАЊЕ ТЕРАПЕУТСКОГ ПОТЕНЦИЈАЛА МОНОКАРБОХИДРАЗОНА: *IN VITRO* ПРОЦЕНА АНТИОКСИДАНТНЕ, АНТИМИКРОБНЕ И ЦИТОТОКСИЧНЕ АКТИВНОСТИ

ГОРАНА С. МРЂАН[1*], САЊА С. ВЛАИСАВЉЕВИЋ[1], ПЕТАР Н. КНЕЖЕВИЋ[2], ИСИДОРА Н. НИКОЛИЋ[2], ДИНА Г. ТЕЊИ[2], БОРКО М. МАТИЈЕВИЋ[1]

[1]Универзитет у Новом Саду, Природно-математички факултет, Департман за хемију, биохемију и заштиту животне средине, Нови Сад, Србија, и [2]Универзитет у Новом Саду, Природно-математички факултет, Департман за биологију и екологију, Нови Сад, Србија.

Органска једињења, посебно она која у свом саставу садрже атоме азота и сумпора, чине преко 99 % клинички одобрених лекова. Као једни од припадника поменутих једињења, симетрични бисупституисани деривати тиокарбохидразона су детаљно изучавани. Са друге стране, асиметрични и моносупституисани тиокарбохидразони нису довољно испитани, упркос њиховом израженом биолошком потенцијалу. У овом раду су приказани резултати *in vitro* процене антиоксидативних, антимикробних и цитотоксичних својстава осамнаест претходно синтетисаних и окарактерисаних монотиокарбохидразона. Антиоксидативни потенцијал је одређен применом DPPH теста, док је антимикробна активност испитана на Грам-позитивним бактеријама применом модификоване микродилуционе методе. Цитотоксични ефекат је испитан на ћелијама хуманог хепатоцелуларног карцинома применом колориметријског MTT теста. Добијени резултати показују да испитивани монотиокарбохидразони имају значајно антиоксидативно и антимикробно дејство. Такође је установљено да на њихову активност и цитотоксичност велики утицај имају структура испитиваног молекула, као и природа и положај присутног супституената. Ови резултати пружају добар увид за будућа *in vivo* испитивања и истичу потенцијалну примену монотиокарбохидразона у развоју лекова.

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