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The synthesis and antiviral activity against *yellow fewer* virus of 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides

Aim. To synthesize 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides and study their antiviral activity against yellow fever virus (YFV).

Results and discussion. The target 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides were obtained in three-step format from cyanuric chloride in good to high yields. The carbothioamides synthesized were estimated to possess the antiviral activity against YFV. The results obtained indicate that most of the compounds studied show the inhibitory activity against YFV in concentrations ≤10 μg/mL. For the most active substances, EC_{90} was in the range of 0.06–2.2 μg/mL. Good effective concentration values were accompanied by low levels of cytotoxicity resulting in excellent selectivity index values. The data obtained also indicate that the presence of an alkyl substituent in *ortho*-position of the N-aryl fragment is crucial for an effective inhibition of YFV growth.

Experimental part. 2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides were synthesized starting from cyanuric chloride in three steps by its successive interaction with two equivalents of pyrrolidine, hydrazine and a series of alkyl-/arylisothiocyanates. The antiviral and cytotoxic activities of the target carbothioamides were studied in the Southern Research Institute (SRI, Birmingham, Alabama) by the viral cytopathic effect reduction assay and the virus yield reduction assay.

Conclusions. 2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides synthesized have been proven to be a promising class of compounds for treating such a severe viral disease as yellow fever.

***Key words*:** 1,3,5-triazine; carbothioamides; yellow fever; antiviral activity; cytotoxicity

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Синтез та противірусна активність щодо вірусу жовтої лихоманки 2-(4,6-ди(піролідин-1-іл)-1,3,5-триазин-2-іл)-N-(алкіл, арил)гідразин-1-карботіоамідів

Мета. Синтезувати та вивчити противірусну активність щодо вірусу жовтої лихоманки для 2-(4,6-ди(піролідин-1-іл)-1,3,5-триазин-2-іл)-N-(алкіл, арил)гідразин-1-карботіоамідів.

Результати та їх обговорення. Цільові 2-(4,6-ди(піролідин-1-іл)-1,3,5-триазин-2-іл)-N-(алкіл, арил) гідразин-1-карботіоаміди одержано з ціанурхлориду із середніми та високими виходами, із застосуванням тристадійного підходу. Синтезовані карботіоаміди було досліджено на наявність противірусної активності щодо вірусу жовтої лихоманки. Одержані результати свідчать, що більшість тестованих сполук виявляють інгібувальну активність проти вірусу в концентраціях ≤10 мкг/мл. Для найактивніших субстанцій EC_{90} становила 0,06–2,2 мкг/мл. Гарні значення ефективних концентрацій супроводжувались низьким рівнем цитотоксичності, що зумовило відмінні значення індексу селективності. Одержані дані також є свідченням того, що наявність алкільного замісника в *орто*-положенні N-арильного фрагмента має вирішальне значення для ефективного пригнічення зростання вірусу.

Експериментальна частина. 2-(4,6-Ди(піролідин-1-іл)-1,3,5-триазин-2-іл)-N-(алкіл, арил)гідразин-1-карботіоаміди було синтезовано в три стадії послідовною взаємодією ціанурхлориду з двома еквівалентами піролідину, гідразином та рядом алкіл-/арилізотіоціанатів. Противірусну та цитотоксичну активність цільових карботіоамідів було досліджено в Southern Research Institute (SRI, Birmingham, Alabama) на моделях зменшення вірусного цитопатичного ефекту і зменшення розмноження вірусів.

Висновки. Синтезовані 2-(4,6-ди(піролідин-1-іл)-1,3,5-триазин-2-іл)-N-(алкіл, арил)гідразин-1-карботіоаміди є перспективним класом сполук для лікування такого вірусного захворювання, як жовта лихоманка.

Ключові слова: 1,3,5-триазин; карботіоаміди; жовта лихоманка; противірусна активність; цитотоксичніть

Yellow fever (yellow jack, yellow plague, bronze john) is a severe viral disease resulting from human infection with yellow fever virus (YFV) [1, 2]. It is endemic in tropical regions of Africa, as well as in Central and South America [3–5].

YFV is an RNA virus of the *Flaviviridae* family (genus *Flavivirus*). The virus persists in nature through transmission between non-human primates and mosquitoes fed upon them. The latter usually belong to *Aedes* species in Africa and *Haemagogus* species in the Americas. Humans may become infected with YFV through a sylvatic transmission vector when bitten by an infected mosquito (the so-called *jungle yellow fever*). However, inter-human transmission of the virus may also occur with *Aedes aegypti* as a vector of domestic transmission (the so-called *urban yellow fever*). In Africa, a third transmission vector is possible; it is known as the "intermediate cycle" involving both sylvatic and domestic vector species in inter-human transmission. From the epidemiological point of view, the biggest concern is given to the urban type of transmission as it can cause large-scale epidemics due to the high population density and low or no immunity to the disease [2].

One of the aspects of yellow fever danger lies in the field of its ability to spread rapidly causing the population damage. Due to the developed air and sea communication between the countries, there is a risk of importing infected mosquitoes from the endemic regions and their spread over new territories due to the ongoing climate change.

The clinical course of yellow fever proceeds through three phases, which are infection, remission, and intoxication. The first symptoms appear abruptly 2–9 days after YFV transmission to a living organism through the bite of an infected mosquito (infection phase). They include fever (up to 41°C), headache, myalgia, malaise, nausea and vomiting. A short phase of remission may follow the infection phase, during which many patients (about 88%) recover. Other YFV-infected patients will progress to the intoxication phase, which is accompanied by fever, abdominal pain, nausea and vomiting, oliguria, jaundice, hepatic dysfunction, renal failure, hemorrhagic manifestations, encephalopathy [1].

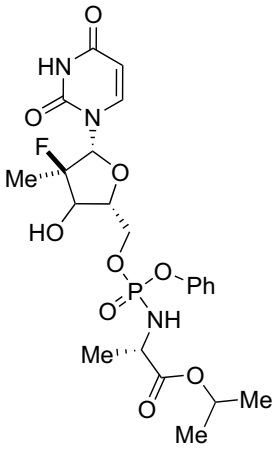
According to the World Health Organization reports, about 200,000 cases of yellow fever are registered worldwide each year. The mortality rate among patients entering the phase of intoxication ranges from 20 to 50% in different outbreaks and case series [1].

Nowadays vaccination is the primary method used to prevent development of the disease [6]. Vaccines against YFV usually produce a strong and long-term immune response in all recipients [7–12]. Nevertheless, one should not consider vaccination as a totally successful and safe way of combating yellow fever. Thus, it can cause severe adverse events, including yellow fever vaccine-associated viscerotropic disease and acute neurotropic disease [13, 14] resulting in the immune system failure [15, 16].

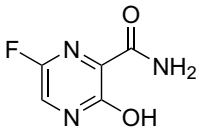
There are no effective antiviral drugs for yellow fever by now. Meanwhile, several antiviral compounds are under investigation for use with this purpose, including sofosbuvir (Fig.) [17]. The study revealed that sofosbuvir may be used as an option to cure yellow fever until other more effective medicines are found and approved for human use.

During the high-throughput screening research aimed at finding novel antiviral substances, a benzodiazepine acetic acid derivative (**BDAA**, Fig.) with the promising antiviral properties was identified [18]. It comprises the 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one core, which is typical for many benzodiazepine drugs, and inhibits YFV potently.

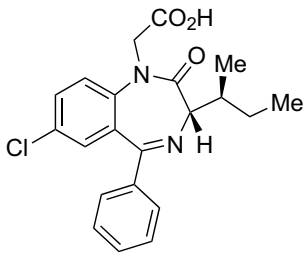
Among other possible cures for yellow fever, one should note Favipiravir (sold under the brand name Avigan®) which is a pyrazinecarboxamide derivative (Fig.). The mechanism of its action is thought to be selective inhibition of viral RNA-dependent RNA polymerase [19].



Sofosbuvir



Favipiravir



BDAA

Fig. The compounds with the confirmed antiviral activity against YFV

Despite the availability of synthetic substances directly affected YFV, the symptomatic treatment is usually applied to people suffering from yellow fever. The treatment may consist of fluid replacement, hemodialysis (in case of kidney failure), blood transfusion (when indicated), vasopressors, antipyretics, antibiotics (if secondary infections appear) [20, 21].

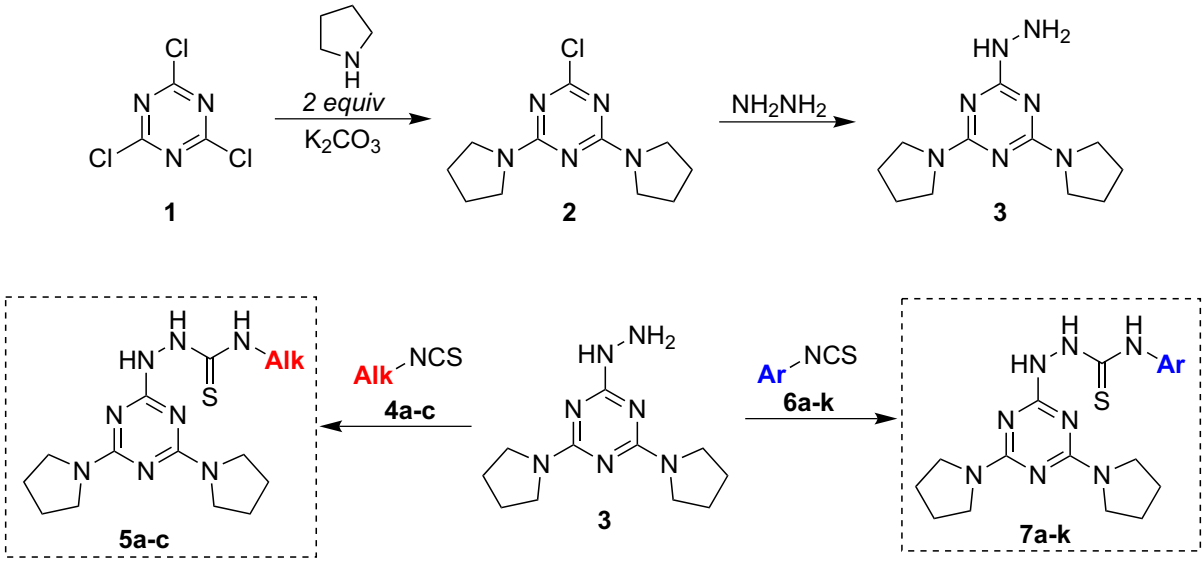
Taking into account the information stated above modern medicine is in urgent need of effective, specific and safe antiviral drugs against yellow fever. Such a state of affairs encouraged us to carry out a research in this field. This paper describes the results of the synthesis and evaluation of the *anti*-YFV activity of some 1,3,5-triazine derivatives. The reason of our attention to the 1,3,5-triazine core is the previously published results revealing valuable pharmacological properties of its derivatives. Thus, 1,3,5-triazines have been found in a number of bioactive molecules, such as herbicides and pharmaceutical products [22]. Compounds comprising this heterocyclic system exhibit the cardiotonic [23], antitumor [24] and anticancer activities [25]. Moreover, antiviral agents of 1,3,5-triazine class have been proven to be effective against the human immunodeficiency viruses (HIV) [26, 27] and herpes simplex virus 1 (HSV-1) [28].

In the current research we set the task to synthesize a series of 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides **5**, **7** (Scheme) and evaluate their usefulness in treating yellow fever. The starting compound in the synthetic strategy towards the target derivatives was cyanuric chloride (**1**). Its interaction with a double amount of pyrrolidine in the acetone solution in the presence of potassium carbonate led to 2-chloro-4,6-di(pyrrolidin-1-yl)-1,3,5-triazine (**2**) [29]. The latter easily gave 2-hydrazinyl-4,6-di(pyrrolidin-1-yl)-1,3,5-triazine (**3**) when refluxed with hydrazine hydrate in propanol-2 [30]. Hydrazine derivative **3** was further used in the reaction with alkyl- **4a–c** and arylisothiocyanates **6a–k** producing the corresponding carbothioamides **5**, **7** [31].

The next step was to study the antiviral activity of compounds **5a–c** and **7a–k** against YFV. These studies were performed in the Southern Research Institute (SRI, Birmingham, Alabama). Testing was done by the viral cytopathic effect (CPE) reduction assay and the virus yield reduction assay (*Virus Yield*). CPE was determined by the microscopic observation of cell culture monolayers (*Visual*), as well as the uptake of neutral red dye (*Neutral Red*). The results obtained are given in the Table below as EC_{50}, EC_{90}, IC_{50} and IS values (see notes under the Table).

The antiviral assay determining CPE is used for the initial screening of potential antiviral compounds. The antiviral activity of the compounds is evaluated based on their ability to prevent the virus from causing viral CPE in a cell culture. Compounds demonstrating the activity in the CPE assay are further evaluated by the virus yield reduction assay. This test evaluates the ability of the compounds to inhibit virus production in a cell culture.

The results obtained indicate that most of the compounds studied showed the inhibitory activity against YFV (EC_{50}≤10 μg/mL) though it was less pronounced as compared to the reference drug Infergen® (Interferon Alphacon) (Table).



5a: Alk = Me; **5b:** Alk = Et; **5c:** Alk = Bn
7a: Ar = 2-Me-C_6H_4; **7b:** Ar = 4-Me-C_6H_4; **7c:** Ar = 2-MeO-C_6H_4; **7d:** Ar = 4-EtO-C_6H_4; **7e:** Ar = 3-Cl-C_6H_4; **7f:** Ar = 4-Cl-C_6H_4; **7g:** Ar = 2,3-diMe-C_6H_4; **7h:** Ar = 2,4-diMe-C_6H_4; **7i:** Ar = 2,5-diMe-C_6H_4; **7j:** Ar = 2,6-diMe-C_6H_4; **7k:** Ar = 3,4-diMe-C_6H_4

Scheme. The synthesis of 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides **5a-c** and **7a-k**

Table

The antiviral activity of 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamide **5** and **7** against YFV

Cmp	R	Assay	Trial*	EC_{50}	EC_{90}	IC_{50}	SI
5a	$NHCH_3$	Neutral Red	1	>100	–	>100	0
5b	NHC_2H_5	Neutral Red	1	31	–	58	1.9
5c	CH_2Ph	Neutral Red	1	>36	–	36	0
7a	2-Me-C_6H_4	Visual	2	1.1	–	23	22
7a	2-Me-C_6H_4	Neutral Red	1	3.2	–	32	10
7a	2-Me-C_6H_4	Neutral Red	2	1.1	–	25	23
7a	2-Me-C_6H_4	Virus Yield	3	–	2.2	–	11
7b	4-Me-C_6H_4	Neutral Red	1	11	–	17	1.5
7c	2-MeO-C_6H_4	Visual	2	5.1	–	270	53
7c	2-MeO-C_6H_4	Neutral Red	1	10	–	>100	>10
7c	2-MeO-C_6H_4	Neutral Red	2	2.8	–	170	61
7c	2-MeO-C_6H_4	Virus Yield	3	–	0.47	–	362
7d	4-EtO-C_6H_4	Neutral Red	1	10	–	29	2.9
7e	3-Cl-C_6H_4	Neutral Red	1	>100	–	>100	0
7f	4-Cl-C_6H_4	Neutral Red	1	10	–	28	2.8
7g	2,3-*di*Me-C_6H_3	Visual	2	2.3	–	18	7.8
7g	2,3-*di*Me-C_6H_3	Neutral Red	1	0.85	–	7.9	9.3
7g	2,3-*di*Me-C_6H_3	Neutral Red	2	1.2	–	18	15
7g	2,3-*di*Me-C_6H_3	Virus Yield	3	–	0.09	–	200
7h	2,4-*di*Me-C_6H_3	Visual	2	1.6	–	18	11
7h	2,4-*di*Me-C_6H_3	Neutral Red	1	3.2	–	32	10
7h	2,4-*di*Me-C_6H_3	Neutral Red	2	1.4	–	18	13
7h	2,4-*di*Me-C_6H_3	Virus Yield	3	–	1.8	–	10
7i	2,5-*di*Me-C_6H_3	Visual	2	0.81	–	32	40
7i	2,5-*di*Me-C_6H_3	Neutral Red	1	1.1	–	10	9.1
7i	2,5-*di*Me-C_6H_3	Neutral Red	2	0.4		18	45
7i	2,5-*di*MeC_6H_3	Virus Yield	3	–	0.06	–	300
7j	2,6-*di*Me-C_6H_3	Neutral Red	1	18	–	89	4.9
7j	2,6-*di*Me-C_6H_3	Visual	1	9	–	16	1.8
7k	3,4-*di*Me-C_6H_3	Neutral Red	1	10	–	18	1.8
Infergen (Interferon Alphacon)		Neutral Red, Visual	–	0.01	–	–	–

Notes: EC_{50} – the half maximal effective concentration causing 50% virus inactivation (μg/mL); EC_{90} – the effective concentration causing 90% virus inactivation (μg/mL); IC_{50} – 50% cytotoxicity value (μg/mL); SI – the selectivity index, it is the ratio that measures the window between cytotoxicity and the antiviral activity by dividing the given IC_{50} value into the EC_{50} or EC_{90} value.
* – Depending on the time the cell cultures are being infected the following cell concentrations were prepared:
100 000 cells/mL – 72 h incubation (3); 200 000 cells/mL – 48 h incubation (2); 400 000 cells/mL – 24 h incubation (1).

Considering the structure of the compounds under research one should note that N-alkyl substituted derivatives **5** demonstrated high values of EC_{50}, hence, were out of interest. Meanwhile, compounds **7** bearing an N-aryl residue turned out to be much more potent against YFV. Their EC_{50} were in the range of 1–10 μg/mL in accordance with the CPE assay, except for 3-chlorophenyl derivative **7e** which was nearly inactive. The more precise virus yield reduction assay provided even less EC_{90} concentration values – 0.06–2.2 μg/mL. It is worth mentioning that good effective concentration values were accompanied by low levels of cytotoxicity resulting in the excellent selectivity index, which was up to 362. It is interesting that the substitution pattern of the N-aryl fragment significantly affected the antiviral potency of compounds **7**. Thus, the most active molecules had a substituent in the *ortho*-position of the aryl residue. The introduction of the second substituent to the aryl residue altered the antiviral activity though it still remained to be high. However,

another *ortho*-position must be free as 2,6-dimethylphenyl derivative **7j** lost in its antiviral efficacy as compared to other N-dimethylphenyl ones.

Thus, 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides **5** and **7** provide a high antiviral activity against YFV. The data obtained confirm the feasibility of further studies of the title compounds as potential antiviral agents.

Experimental part

Chemistry part

^{1}H NMR spectra of compounds **5** and **7** were recorded on a Bruker VXR-400 spectrometer (Germany) operating at a frequency of 400 MHz in DMSO-d_6 using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in ppm using the δ scale. The melting points were measured on a small-sized heating table with an Electrothermal IA 9200 observation device. The elemental analysis was performed on an EuroEA 3000 elemental analyzer.

The general procedure for the synthesis of 2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl) hydrazine-1-carbothioamides 5a–c, 7a–k

The mixture of 2-hydrazinyl-4,6-di(pyrrolidin-1-yl)-1,3,5-triazine (**3**) (0.249 g, 0.01 mol) and the corresponding alkyl- **4a–c** or arylisothiocyanate **6a–k** (0.01 mol) was refluxed in 50 mL of ethanol for 2 h and then cooled. After cooling the solid products formed were filtered off, washed with ethanol, dried in air and recrystallized from propanol-2 or ethanol to afford the pure target carbothioamides **5a–c** and **7a–k**.

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-methylhydrazine-1-carbothioamide (**5a**)

Yield – 2.16 g (67%). M. p. 227–228°C (from ethanol). Anal. Calcd. for $C_{13}H_{22}N_8S$, %: N 34.75; S 9.94. Found, %: N 34.53; S 9.89. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.82–1.85 (8H, m, 2 × CH_2CH_2); 2.85 (3H, d, J = 4.3 Hz, NHC$\underline{H}_3$); 3.38–3.43 (8H, m, 2 × CH_2NCH_2); 7.77 (1H, q, J = 4.3 Hz, N$\underline{H}CH_3$); 8.39 (1H, s, NH); 8.84 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-ethylhydrazine-1-carbothioamide (**5b**)

Yield – 2.15 g (64%). M. p. 191–192°C (from ethanol). Anal. Calcd. for $C_{14}H_{24}N_8S$, %: N 33.30; S 9.53. Found, %: N 33.13; S 9.47. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.03 (3H, t, J = 7.2 Hz, NHCH$_2$C$\underline{H}_3$); 1.82–1.85 (8H, m, 2×CH_2CH_2); 3.38–3.46 (10H, m, NHC$\underline{H}_2$CH$_3$ + 2 × CH_2NCH_2); 7.77 (1H, t, J = 5.6 Hz, N$\underline{H}$CH$_2$CH$_3$); 8.36 (1H, s, NH); 8.79 (1H, s, NH).

N-Benzyl-2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)hydrazine-1-carbothioamide (**5c**)

Yield – 3.03 g (76%). M. p. 226–226°C (from propanol-2). Anal. Calcd. for $C_{19}H_{26}N_8S$, %: N 28.12; S 8.04. Found, %: N 28.31; S 8.13. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.82–1.87 (8H, m, 2 × CH_2CH_2); 3.39–3.45 (8H, m, 2 × CH_2NCH_2); 4.71 (2H, d, J = 5.6 Hz, NHC$\underline{H}_2$), 7.18–7.29 (5H, m, C_6H_5); 7.36 (1H, t, J = 5.6 Hz, N$\underline{H}$CH$_2$); 8.50 (1H, s, NH); 9.07 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(o-tolyl)hydrazine-1-carbothioamide (**7a**)

Yield – 2.79 g (70%). M. p. 173-174°C (from ethanol). Anal. Calcd. for $C_{19}H_{26}N_8S$, %: N 28.12; S 8.04. Found, %: N 28.24; S 8.15. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.80–1.85 (8H, m, 2 × CH_2CH_2); 2.15 (3H, s, CH_3); 3.40–3.45 (8H, m, 2 × CH_2NCH_2); 7.10–7.24 (4H, m, C_6H_4); 7.36 (1H, s, NH); 8.48 (1H, s, NH); 9.17 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(p-tolyl)hydrazine-1-carbothioamide (**7b**)

Yield – 3.07 g (77%). M. p. 167-168°C (from propanol-2). Anal. Calcd. for $C_{19}H_{26}N_8S$, %: N 28.12; S 8.04. Found, %: N 27.91; S 7.96. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.86 (8H, m, 2 × CH_2CH_2); 2.27 (3H, s, CH_3); 3.41–3.45 (8H, m, 2 × CH_2NCH_2); 7.09 and 7.40 (4H, d, J = 8.1 Hz, C_6H_4); 8.53 (1H, s, NH); 9.27 (1H, s, NH); 9.40 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(2-methoxyphenyl)hydrazine-1-carbothioamide (**7c**)

Yield – 2.98 g (72%). M. p. 188–189°C (from propanol-2). Anal. Calcd. for $C_{19}H_{26}N_8OS$, %: N 27.03; S 7.73. Found, %: N 27.33; S 7.87. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.82–1.86 (8H, m, 2 × CH_2CH_2); 3.41–3.45 (8H, m, 2×CH_2NCH_2); 3.75 (3H, s, OCH_3); 6.90–8.37 (4H, m, C_6H_4); 8.76 (1H, s, NH); 8.95 (1H, s, NH); 9.50 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(4-ethoxyphenyl)hydrazine-1-carbothioamide (**7d**)

Yield – 3.47 g (81%). M. p. 161–162°C (from propanol-2). Anal. Calcd. for $C_{20}H_{28}N_8OS$, %: N 26.15; S 7.48. Found, %: N 26.07; S 7.55. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.31 (3H, t, J = 7.2 Hz, OCH$_2$C$\underline{H}_3$); 1.82–1.86 (8H, m, 2 × CH_2CH_2); 3.42–3.46 (8H, m, 2 × CH_2NCH_2); 4.00 (2H, q, J = 7.2 Hz, OC$\underline{H}_2$CH$_3$); 6.84 and 7.31 (4H, d, J = 8.4 Hz, C_6H_4); 8.52 (1H, s, NH); 9.21 (1H, s, NH); 9.36 (1H, s, NH).

N-(3-chlorophenyl)-2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)hydrazine-1-carbothioamide (**7e**)

Yield – 3.69 g (88%). M. p. 221–222°C (from ethanol). Anal. Calcd. for $C_{18}H_{23}ClN_8S$, %: N 26.75; S 7.65. Found, %: N 26.54; S 7.59. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.86 (8H, m, 2 × CH_2CH_2); 3.42–3.46 (8H, m, 2×CH_2NCH_2); 7.14–7.72 (4H, m, C_6H_4); 8.60 (1H, s, NH); 9.52 (1H, s, NH); 9.65 (1H, s, NH).

N-(4-chlorophenyl)-2-(4,6-di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)hydrazine-1-carbothioamide (**7f**)

Yield – 3.81 g (91%). M. p. 206–207°C (from ethanol). Anal. Calcd. for $C_{18}H_{23}ClN_8S$, %: N 26.75; S 7.65. Found, %: N 26.9; S 7.70. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.87 (8H, m, 2 × CH_2CH_2); 3.39–3.43 (8H, m, 2 × CH_2NCH_2); 7.33 and 7.55 (4H, d, J = 8.8 Hz, C_6H_4); 8.54 (1H, s, NH); 9.41 (1H, s, NH); 9.59 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(2,3-dimethylphenyl)hydrazine-1-carbothioamide (**7g**)

Yield – 2.85 g (69%). M. p. 178–179°C (from ethanol). Anal. Calcd. for $C_{20}H_{28}N_8S$, %: N 27.16; S 7.77. Found, %: N 27.23; S 7.83. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.87 (8H, m, 2 × CH_2CH_2); 2.04 (3H, s, CH_3); 2.23 (3H, s, CH_3); 3.42–3.47 (8H, m, 2 × CH_2NCH_2); 6.93–7.05 (3H, m, C_6H_3); 8.56 (1H, s, NH); 9.23 (2H, br. s, 2NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(2,4-dimethylphenyl)hydrazine-1-carbothioamide (**7h**)

Yield – 2.72 g (66%). M. p. 165–166°C (from propanol-2). Anal. Calcd. for $C_{20}H_{28}N_8S$, %: N 27.16; S 7.77. Found, %: N 27.01; S 7.89. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.82–1.85 (8H, m, 2 × CH_2CH_2); 2.13 (3H, s, CH_3); 2.29 (3H, s, CH_3); 3.42–3.47 (8H, m, 2 × CH_2NCH_2); 6.83–7.13 (3H, m, C_6H_3); 8.34 (1H, s, NH), 8.97 (2H, br. s, 2NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(2,5-dimethylphenyl)hydrazine-1-carbothioamide (**7i**)

Yield – 3.30 g (80%). M. p. 175–176°C (from propanol-2). Anal. Calcd. for $C_{20}H_{28}N_8S$, %: N 27.16; S 7.77. Found, %: N 27.11; S 7.85. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.87 (8H, m, 2 × CH_2CH_2); 2.10 (3H, s, CH_3); 2.24 (3H, s, CH_3); 3.42–3.47 (8H, m, 2 × CH_2NCH_2); 6.93–7.06 (3H, m, C_6H_3); 8.57 (1H, s, NH); 9.17 (1H, s, NH); 9.24 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(2,6-dimethylphenyl)hydrazine-1-carbothioamide (**7j**)

Yield – 2.74 g (66%). M. p. 161–162°C (from propanol-2). Anal. Calcd. for $C_{20}H_{28}N_8S$, %: N 27.16; S 7.77. Found, %: N 27.27; S 7.91. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.82–1.87 (8H, m, 2 × CH_2CH_2); 2.14 (6H, s, 2 × CH_3); 3.42–3.47 (8H, m, 2 × CH_2NCH_2); 6.98–7.06 (3H, m, C_6H_3); 8.58 (1H, s, NH); 9.10 (1H, s, NH); 9.21 (1H, s, NH).

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(3,4-dimethylphenyl)hydrazine-1-carbothioamide (**7k**)

Yield – 2.72 g (73%). M. p. 174–175°C (from propanol-2). Anal. Calcd. for $C_{20}H_{28}N_8S$, %: N 27.16; S 7.83. Found, %: N 26.98; S 7.65. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 1.83–1.86 (8H, m, 2 × CH_2CH_2); 2.18 (6H, s, 2 × CH_3); 3.41–3.46 (8H, m, 2 × CH_2NCH_2); 7.03–7.22 (3H, m, C_6H_3); 8.52 (1H, s, NH); 9.23 (1H, s, NH); 9.30 (1H, s, NH).

Pharmacological part

The viral cytopathic effect (CPE) reduction assay and the virus yield reduction assay were performed according to the known procedures [32, 33].

Briefly, the *viral cytopathic effect (CPE)* reduction assay consists in the following. Vero cells were seeded into 96-well clear plates, and all of the plates were incubated for about 18 h prior to use for assays. Compounds were first applied to the cell monolayers, followed within minutes by addition of virus suspensions. Uninfected wells with the compounds tested were used for assessing cytotoxicity (IC_{50}), infected wells – for assessing the antiviral activity, drug-free infected wells – as virus controls, uninfected drug-free wells – as cell controls, background wells contained only water. After incubation the microplates were read visually to estimate the percentage of cell destruction caused by the virus infection or by cytotoxicity. When the maximum CPE was observed, each plate was treated with a neutral red dye followed by incubation of the plates at 37°C. The neutral red solution used was prepared by dilution of its 0.68% solution with the physiological saline solution in the ratio of 1:20. The resulting solution (0.1 mL) was added to each cell monolayer. The plates were incubated for 2 h in order to allow the dye to be adsorbed. After incubation the plates were aspirated dry, and the monolayers were washed twice with a brine/phosphate buffer mixture followed by addition of 0.25 mL of 1:1 Sörensen's citrate buffer (pH 4.2) in ethanol to each well to desorb the dye. To complete desorption of the dye from the cells the plates were placed in a dark place for 30 min at room temperature and after read using the colorimetry method at 540 nm. Absorbance units were converted to percentages of uninfected control cells. Fifty percent virus inhibitory (EC_{50}) or 50% cytotoxicity (IC_{50}) values were determined by linear regression using an Excel spreadsheet.

The *virus yield reduction assay* is a two-step assay where the virus is first produced in Vero cells cultures containing the antiviral substance in varying dilutions, followed later by titration of the samples for the virus titer by the endpoint dilution in 96-well plates. Dilutions of the test compound were assayed, and the effective antiviral concentration was determined by the regression analysis.

Vero cells were infected with the virus at 0.3 pfu/cell and then incubated for 24 h. After incubation the infected cells were exposed to solutions of the test compounds for 72 h. After virus adsorption the culture fluid was diluted to the required concentration by semilogarithmic dilutions of test compounds prepared in the assay medium. Depending on the situation, the supernatants were pooled either 24 h or 72 h after infection, and the extracellular viral infectivity titers were determined in duplicate by analysis of plaques in Vero cell monolayers. The endpoint for the 24-hour assay is the effective concentration (EC_{50}), which reduces the virus yield by 50% compared to control cultures. The effective concentration (EC_{90}) was determined from the data obtained.

Conclusions

2-(4,6-Di(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)-N-(alkyl, aryl)hydrazine-1-carbothioamides synthesized have been proven to be a promising class of compounds for treating such a severe viral disease as yellow fever.

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