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Synthesis, modification and cytotoxic properties of new 7-amino-5-arylazolo[1,5-*a*]pyrimidine-6-carbonitriles

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Abstract

Azolo[1,5-*a*]pyrimidine is one of the most promising scaffold in the development of potent anticancer agents. However, the structural motifs in such development are often limited to 5,7-substituted azolo[1,5-*a*]pyrimidines due to the well-known synthesis route and existing drugs (e.g., Dinaciclib) on their basis. To expand the understanding of rare substituent combinations in this research field, a new series of 7-amino-5-aryl-6-cyanoazolo[1,5-*a*]pyrimidines with various azole moieties were synthesized and studied on A172, Rd, Hos and HEK293 cell lines. The obtained derivatives were further modified by acylation to introduce an additional pharmacophore moiety. During the study of anticancer activity, compounds with a pronounced cytotoxic effect were found. The analysis of activity against the embryonal rhabdomyosarcoma (Rd) cell line suggested a mechanism of cytotoxic action causing a significant decrease in mitochondrial potential in neoplastic cells.

Key findings

- The synthetic methodology for obtaining 7-amino-5-arylazolo[1,5-*a*]pyrimidines with post-synthetic modifications was significantly expanded.
- The cytotoxic activity of compounds on Rd, A172, HOS and HEK293 cell lines was studied.
- The mechanism of cytotoxic activity of the synthesized compounds associated with a significant decrease in mitochondrial potential in neoplastic cells was proposed.

Accompanying information

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Supplementary information
Supplementary materials: READ
Transparent peer review: READ

Sustainable Development Goals

1. Introduction

The development of target-based bioactive compounds for individual therapy of socially significant diseases is a key area of medicinal chemistry. A separate niche in the context of this research area is occupied by synthetic analogues of natural purines - azolo[1,5-*a*]pyrimidines, which have proven themselves as bioactive substances and therapeutic agents with a diverse spectrum of activity (Figure 1), including anticancer[1, 2], antidiabetic [3], hypnotic [4], antiplatelet/vasodilatory [5, 6], antiviral [7, 8], and some others [9–13].

From the given examples (Figure 1), it is obvious that the biological activity of pyrazolo- and 1,2,4-triazolo[1,5-*a*]pyrimidines depends on combinations of pharmacophoric substituents in the azole and pyrimidine fragments of the azoloazine scaffold. Therefore, the search for methods for the synthesis of azolo[1,5-*a*]pyrimidines with the widely varying profile of their chemical environment may be of interest in the development of new targeted therapeutic agents.

Of interest in this direction are 7-amino-5-aryl-6-cyanoazolo[1,5-*a*]pyrimidines, which can be obtained on the basis of synthetically available aminoazoles and arylidene malononitriles, but with a limited observed range of substituents, reaction conditions [14–18] and antitumor [17, 18], antimalarial [19] activity.

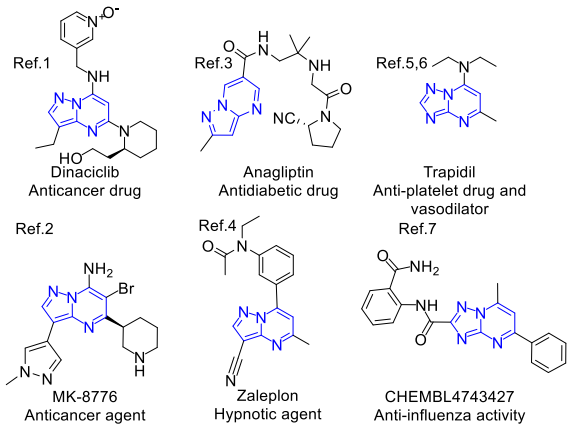

Figure 1 Structures of some bioactive azolo[1,5-a]pyrimidines.

This series of compounds deserves special attention due to the possibilities of their structural modification and pharmacophore similarity to known biologically active compounds with action against the molecular targets of tumour pathologies (Figure 2): casein kinase type 2 (CK2), an enzyme associated with the processes of cell proliferation [20–24] and A2A adenosine receptor (A2A AR), a G protein associated with activation of the PIK3/AKT signalling pathway, the development of anti-apoptotic effects in tumour cells [25], as well as the development of some neurodegenerative pathologies [26–28].

2. Experimental

2.1. Chemistry

All solvents and commercially available reagents were used as received unless otherwise stated. Non-commercial starting materials were prepared as described below or according to the literature procedures. One-dimensional ^{1}H and ^{13}C NMR spectra were obtained on a «Bruker AVANCE II – 400» (400 and 101 MHz, respectively) or «Bruker Avance NEO 600» (600 and 151 MHz, respectively) equipped with a Prodigy broadband gradient cryoprobe using DMSO-d_6 as a solvent and TMS as an internal standard.

IR spectra were recorded on a Bruker Alpha FTIR spectrometer equipped with an ATR attachment. The progress of the reaction was monitored by TLC on 0.25 mm plates with silica gel (Merck 60F 254), eluent – ethyl acetate. Melting points were determined on a Stuart ST.PL.3 instrument at a heating rate of 1 °C/min.

Arilidenemalononitriles **2a**, **2b** were synthesized as described in [29].

2.2. Preparation of 7-amino-6-cyano-4,7-dihydroazolo[1,5-a]pyrimidines (3a–u)

The corresponding aminoazole **1a-k** (1 mmol) and arylidenemalononitriles **2a-b** (1 mmol) were suspended in 5 ml of the required solvent (*i*-PrOH for **3a-k**, acetonitrile for **3l-t**); then 0.14 ml (1 mmol) of Et_3N were added. The mixture was refluxed until a new precipitate formed, and the progress of the reaction was monitored by TLC. Then the formed suspension was cooled; the precipitate was filtered, washed with 5 ml of cold solvent and dried in air.

2.2.1. 7-amino-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-3,6-dicarbonitrile (3a)

Yield: 60%, Brown powder. mp 236–238 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.13 (*d*, *J* = 2.9 Hz, 1H), 7.82 (*s*, 1H), 7.37 (*dt*, *J* = 26.9, 7.5 Hz, 5H), 7.08 (*s*, 2H), 5.29 (*d*, *J* = 2.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 147.29, 144.96, 142.37, 141.23, 127.14, 126.55, 124.59, 117.02, 111.88, 69.73, 54.91, 52.17. IR (KBr, cm^{-1}): 2188.60 (C≡N), 2225.96 (C≡N). Found %: C, 64.10; H, 3.82; N, 32.01, $C_{14}H_{10}N_6$. Calculated, %: C, 64.11; H, 3.84; N, 32.04.

2.2.2. ethyl 7-amino-6-cyano-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-3-carboxylate (3b)

Yield: 65%, Beige powder. mp 220 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 7.98 (*d*, *J* = 3.3 Hz, 1H), 7.71 (*s*, 1H), 7.46–7.23 (*m*, 5H), 6.98 (*s*, 2H), 5.29 (*d*, *J* = 3.2 Hz, 1H), 4.22 (*q*, *J* = 7.1 Hz, 2H), 1.30 (*t*, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 162.62, 147.98, 147.41, 144.21, 143.16, 129.22, 128.37, 126.15, 119.59, 94.18, 59.74, 56.54, 54.01, 14.91. IR, cm^{-1}: 2191.94 (C≡N). Found %:62.05; H, 4.78; N, 22.71, $C_{16}H_{15}N_5O_2$. Calculated, %: C, 62.13; H, 4.89; N, 22.64.

2.2.3. 7-amino-2-(methylthio)-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3c)

Yield: 31%, Beige powder. mp 287 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.92 (*d*, *J* = 2.8 Hz, 1H), 7.47–7.23 (*m*, 5H), 7.12 (s, 2H), 5.31 (*d*, *J* = 2.7 Hz, 1H), 2.50 (*s*, 3H, CH_3) ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 162.67, 154.98, 147.20, 143.51, 129.20, 128.51, 126.52, 119.64, 55.32, 54.21, 13.76. IR, cm^{-1}: 2190.26 (C≡N). Found %: C, 54.89; H, 4.24; N, 29.59; S, 11.15, $C_{13}H_{12}N_6S$. Calculated, %: C, 54.91; H, 4.25; N, 29.56; S, 11.28.

2.2.4. 7-amino-2-(ethylthio)-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3d)

Yield: 47%, Light yellow powder. mp 265–267 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.85 (*d*, *J* = 2.8 Hz, 1H), 7.55–7.21 (*m*, 5H), 6.91 (*s*, 2H), 5.28 (*d*, *J* = 2.7 Hz, 1H), 3.07 (*q*, *J* = 7.3 Hz, 2H), 1.37 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 161.34, 154.34, 146.67, 143.01, 128.70, 128.01, 126.04, 119.12, 54.88, 53.73, 24.87, 15.17. IR, cm^{-1}: 2177.28 (C≡N). Found %: C, 56.35; H, 4.77; N, 28.15; S, 10.79, $C_{14}H_{14}N_6S$. Calculated, %: C, 56.36; H, 4.73; N, 28.17; S, 10.75.

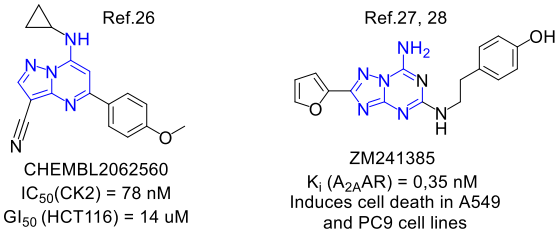

Figure 2 Highly active azoloazine-based effectors of CK2 and A2A AR with observed cytotoxicity on cancer cell lines.

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2.2.5. 7-amino-5-phenyl-2-(propylthio)-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3e)

Yield: 21%, White powder. mp 220 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.92 (*d*, *J* = 2.8 Hz, 1H), 7.44–7.28 (*m*, 5H), 7.12 (*s*, 2H), 5.31 (*d*, *J* = 2.7 Hz, 1H), 3.04 (*t*, *J* = 7.3 Hz, 2H), 1.68 (*h*, *J* = 7.3 Hz, 2H), 0.97 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 161.97, 154.82, 147.17, 143.51, 129.19, 128.51, 126.54, 119.63, 55.33, 54.23, 32.88, 23.23, 13.56. IR, cm^{-1}: 2184.88 (C≡N). Found %: C, 57.69; H, 5.20; N, 26.85; S, 10.20, $C_{15}H_{16}N_6S$. Calculated, %: C, 57.67; H, 5.16; N, 26.90; S, 10.26.

2.2.6. 7-amino-5-phenyl-2-(prop-2-yn-1-ylthio)-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3f)

Yield: 54%, Yellow powder. mp 255 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.03 (*d*, *J* = 2.9 Hz, 1H), 7.37 (*dt*, *J* = 30.7, 7.6 Hz, 5H), 7.19 (*s*, 2H), 5.36 (*d*, *J* = 2.7 Hz, 1H), 3.96 (*s*, 0H), 3.21 (*d*, *J* = 2.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 160.39, 154.98, 147.11, 143.41, 129.22, 128.56, 126.57, 119.50, 80.48, 74.53, 55.57, 54.27, 19.45. IR, cm^{-1}: 2184.28 (C≡N). Found %: C, 58.49; H, 3.95; N, 27.31; S, 10.47, $C_{15}H_{12}N_6S$. Calculated, %: C, 58.43; H, 3.92; N, 27.25; S, 10.40.

2.2.7. 7-amino-2-(benzylthio)-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3g)

Yield: 54%, White powder. mp 330 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.96 (*d*, *J* = 2.7 Hz, 1H), 7.46–7.24 (m, 10H), 7.18 (*s*, 2H), 5.33 (*d*, *J* = 2.6 Hz, 1H), 4.35 (*s*, 2H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 160.97, 154.40, 146.62, 143.03, 137.76, 128.92, 128.71, 128.39, 128.02, 127.22, 126.01, 119.10, 54.99, 53.75, 34.46. IR, cm^{-1}: 2192.61 (C≡N). Found %: C, 63.27; H, 4.55; N, 23.31; S, 8.82, $C_{19}H_{16}N_6S$. Calculated, %: C, 63.31; H, 4.47; N, 23.32; S, 8.90.

2.2.8. 7-amino-5-phenyl-2-(thiophen-2-yl)-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3h)

Yield: 50%, Yellow-green powder. mp 255–257 °C (from *i*-PrOH). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 8.96 (*d*, *J* = 2.6 Hz, 1H), 7.67 (*dd*, *J* = 5.0, 1.2 Hz, 1H), 7.61 (*dd*, *J* = 3.6, 1.2 Hz, 1H), 7.40 (*dd*, *J* = 8.1, 6.8 Hz, 2H), 7.33–7.35 (*m*, 3H), 7.21 (*s*, 2H), 7.17 (*dd*, *J* = 5.0, 3.6 Hz, 1H), 5.38 (*d*, *J* = 2.5 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6), δ, ppm: 157.42, 154.85, 147.25, 143.62, 133.45, 129.24, 128.76, 128.55, 128.50, 127.60, 126.57, 119.52, 56.64, 54.30. IR, cm^{-1}: 2191.08 (C≡N). Found %: C, 60.05; H, 3.75; N, 26.18; S, 9.91, $C_{16}H_{12}N_6S$. Calculated, %: C, 59.98; H, 3.78; N, 26.23; S, 10.01.

2.2.9. 7-amino-2-(furan-2-yl)-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3i)

Yield: 72%, Beige powder. mp 243 °C (from *i*-PrOH). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 8.94 (*d*, *J* = 2.6 Hz, 1H), 7.84 (*dd*, *J* = 1.9, 0.8 Hz, 1H), 7.40 (*dd*, *J* = 8.2, 7.0 Hz, 2H), 7.34 (*d*, *J* = 7.3 Hz, 3H), 7.24 (*s*, 2H), 6.95 (*dd*, *J* = 3.4, 0.8 Hz, 1H), 6.65 (*dd*, *J* = 3.4, 1.8 Hz, 1H), 5.38 (*d*, *J* = 2.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6), δ, ppm: 154.78, 154.30, 147.28, 145.96, 144.97, 143.65, 129.23, 128.54, 126.56, 119.48, 112.33, 111.63, 56.73, 54.35. IR, cm^{-1}: 2191.67 (C≡N). Found %: C, 63.12; H, 4.09; N, 27.55, $C_{16}H_{12}N_6O$. Calculated, %: C, 63.15; H, 3.97; N, 27.62.

2.2.10. 7-amino-5-phenyl-2-(pyridin-3-yl)-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3j)

Yield: 80%, Yellow powder. mp 295–297 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.15 (*d*, *J* = 2.2 Hz, 1H), 9.02 (*d*, *J* = 2.6 Hz, 1H), 8.65 (*dd*, *J* = 4.8, 1.7 Hz, 1H), 8.27 (*dt*, *J* = 8.0, 2.0 Hz, 1H), 7.52 (*dd*, *J* = 8.0, 4.8 Hz, 1H), 7.44–7.33 (*m*, 5H), 7.30 (*s*, 2H), 5.41 (*d*, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 158.50, 154.62, 150.55, 147.18, 146.74, 143.05, 133.44, 128.73, 128.07, 126.11, 126.03, 123.82, 118.92, 56.37, 53.91. IR, cm^{-1}: 2188.94 (C≡N). Found %: C, 64.77; H, 4.13; N, 31.11, $C_{17}H_{13}N_7$. Calculated, %: C, 64.75; H, 4.16; N, 31.09.

2.2.11. 7-amino-2-(5-nitrofuran-2-yl)-5-phenyl-4,5-dihydropyrazolo[1,5-a]pyrimidine-6-carbonitrile (3k)

Yield: 44%, Brown powder. mp 270–272 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.10 (*d*, *J* = 2.7 Hz, 1H), 7.82 (*d*, *J* = 3.6 Hz, 1H), 7.45–7.31 (*m*, 7H, –C_6H_5, –NH_2 (7.36, *s*)), 7.25 (*d*, *J* = 3.5 Hz, 1H), 5.43 (*d*, *J* = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 154.45, 151.86, 151.69, 147.42, 146.33, 142.93, 128.76, 128.15, 126.15, 118.56, 114.51, 113.68, 57.43, 54.00. IR, cm^{-1}: 2195.03(C≡N), 1498.38 (-NO2). Found %: C, 49.98; H, 3.24; N, 28.07, $C_{16}H_{11}N_7O_3$. Calculated, %: C, 55.02; H, 3.17; N, 28.07.

2.2.12. ethyl 7-amino-6-cyano-5-(4-methoxyphenyl)-4,5-dihydropyrazolo[1,5-a]pyrimidine-3-carboxylate (3l)

Yield: 25%, Yellow powder. mp 175 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 7.82 (*d*, *J* = 3.1 Hz, 1H), 7.69 (*s*, 1H), 7.25 (*d*, *J* = 8.3 Hz, 2H), 6.93 (*s*, 2H), 6.89 (*d*, *J* = 8.3 Hz, 2H), 5.24 (*d*, *J* = 3.0 Hz, 1H), 4.21 (*q*, *J* = 7.1 Hz, 2H), 1.30 (*t*, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 162.63, 159.46, 147.85, 147.36, 143.07, 136.20, 127.60, 119.59, 114.54, 94.17, 59.71, 56.78, 55.60, 53.52, 14.91. IR, cm^{-1}: 2187.47(C≡N). Found %: C, 60.12; H, 5.10; N, 20.59, $C_{17}H_{17}N_5O_3$. Calculated, %: C, 60.17; H, 5.05; N, 20.64.

2.2.13. 7-amino-5-(4-methoxyphenyl)-2-(methylthio)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3m)

Yield: 37%, Beige powder. mp 270 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.75 (*d*, *J* = 2.6 Hz, 1H), 8.33 (*s*, 1H), 8.00 (*d*, *J* = 8.5 Hz, 2H), 7.24 (*d*, *J* = 8.2 Hz, 2H), 7.13 (*d*, *J* = 8.5 Hz, 2H), 6.90 (*d*, *J* = 8.3 Hz, 2H), 6.83 (*s*, 2H), 5.21 (*d*, *J* = 2.7 Hz, 1H), 3.92 (*s*, 3H), 3.78 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 162.57, 159.53, 154.90, 147.11, 135.51, 127.93, 119.65, 114.50, 55.63, 53.77, 13.76. IR, cm^{-1}: 2174.46 (C≡N). Found %: C, 53.42; H, 4.58; N, 26.69; S, 10.13, $C_{14}H_{14}N_6OS$. Calculated, %: C, 53.49; H, 4.49; N, 26.73; S, 10.20.

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2.2.14. 7-amino-2-(ethylthio)-5-(4-methoxyphenyl)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3n)

Yield: 25%, Beige powder. mp 275 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.76 (*d*, *J* = 2.7 Hz, 1H), 7.24 (*d*, *J* = 8.3 Hz, 2H), 6.91 (*d*, *J* = 8.3 Hz, 2H), 6.87 (*s*, 2H), 5.22 (*d*, *J* = 2.6 Hz, 1H), 3.78 (*s*, 3H), 3.06 (*q*, *J* = 7.3 Hz, 2H), 1.37 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 161.75, 159.53, 154.77, 147.09, 135.51, 127.95, 119.64, 114.50, 55.69, 55.62, 53.78, 25.36, 15.68. IR, cm^{-1}: 2190.05 (C≡N). Calculated, %: C, 54.86; H, 4.91; N, 25.59; S, 9.76; $C_{15}H_{16}N_6OS$. Found %: C, 54.87; H, 4.89; N, 25.55; S, 9.87.

2.2.15. 7-amino-5-(4-methoxyphenyl)-2-(propylthio)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3o)

Yield: 23%, Light yellow powder. mp 235 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.76 (*d*, *J* = 2.8 Hz, 1H), 7.24 (*d*, *J* = 8.3 Hz, 2H), 6.91 (*d*, *J* = 8.5 Hz, 2H), 6.88 (*s*, 2H), 5.22 (*d*, *J* = 2.7 Hz, 1H), 3.04 (*dt*, *J* = 7.6, 3.7 Hz, 2H), 1.73 (*q*, *J* = 7.3 Hz, 2H), 1.02 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 161.88, 159.53, 154.74, 147.09, 135.51, 127.95, 119.65, 114.49, 55.64, 55.62, 53.78, 32.88, 23.24, 13.55. IR, cm^{-1}: 2187.95 (C≡N). Found %: C, 56.08; H, 5.39; N, 24.51; S, 9.28, $C_{16}H_{18}N_6OS$. Calculated, %: C, 56.12; H, 5.30; N, 24.54; S, 9.36.

2.2.16. 7-amino-5-(4-methoxyphenyl)-2-(prop-2-yn-1-ylthio)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3p)

Yield: 43%, White powder. mp 245 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.85 (*d*, *J* = 2.7 Hz, 1H), 7.31–7.19 (*m*, 2H), 6.95–6.84 (m, 4H), 5.23 (*d*, *J* = 2.6 Hz, 1H), 3.88 (*dd*, *J* = 2.7, 1.2 Hz, 2H), 3.78 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 160.30, 159.56, 154.89, 147.02, 135.41, 127.98, 119.51, 114.52, 80.50, 74.52, 55.88, 55.63, 53.82, 19.44. IR, cm^{-1}: 2184.76 (C≡N). Found %: C, 56.80; H, 4.17; N, 24.85; S, 9.50, $C_{16}H_{14}N_6OS$. Calculated, %: C, 56.79; H, 4.17; N, 24.84; S, 9.48.

2.2.17. 7-amino-2-(benzylthio)-5-(4-methoxyphenyl)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3q)

Yield: 52%, Beige powder. mp 250 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 8.80 (*d*, *J* = 2.7 Hz, 1H), 7.42–7.39 (*m*, 2H), 7.30 (*t*, *J* = 7.4 Hz, 2H), 7.25 (*dd*, *J* = 7.6, 5.3 Hz, 3H), 6.93 (*s*, 2H), 6.91 (*d*, *J* = 8.7 Hz, 2H), 5.23 (*d*, *J* = 2.6 Hz, 1H), 4.32 (*d*, *J* = 2.2 Hz, 2H), 3.78 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 161.41, 159.55, 154.82, 147.04, 138.28, 135.52, 129.42, 128.89, 127.94, 127.71, 119.61, 114.51, 55.80, 55.63, 53.82, 34.95. IR, cm^{-1}: 2192.81 (C≡N). Found %: C, 61.48; H, 4.70; N, 21.41; S, 8.10, $C_{20}H_{18}N_6OS$. Calculated, %: C, 61.52; H, 4.65; N, 21.52; S, 8.21.

2.2.18. 7-amino-5-(4-methoxyphenyl)-2-(thiophen-2-yl)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3r)

Yield: 35%, Brown powder. mp 290–293 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.79 (*d*, *J* = 2.6 Hz, 1H), 7.57 (*dd*, *J* = 3.6, 1.3 Hz, 1H), 7.52 (*dd*, *J* = 5.0, 1.2 Hz, 1H), 7.30–7.24 (*m*, 2H), 7.11 (*dd*, *J* = 5.1, 3.7 Hz, 1H), 6.94–6.87 (*m*, 4H), 5.27 (*d*, *J* = 2.5 Hz, 1H), 3.78 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 159.55, 157.37, 154.77, 147.15, 135.61, 133.49, 128.69, 128.47, 127.97, 127.53, 119.49, 114.53, 57.01, 55.62, 53.87. IR, cm^{-1}: 2187.48 (C≡N). Found %: C, 58.25; H, 4.01; N, 23.91; S, 9.04. $C_{17}H_{14}N_6OS$. Calculated, %: C, 58.27; H, 4.03; N, 23.98; S, 9.15.

2.2.19. 7-amino-2-(furan-2-yl)-5-(4-methoxyphenyl)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3s)

Yield: 41%, Light yellow powder. mp 270 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 8.83 (*d*, *J* = 2.5 Hz, 1H), 7.84 (*d*, *J* = 1.8 Hz, 1H), 7.26 (*d*, *J* = 8.7 Hz, 2H), 7.17 (*s*, 2H), 7.04–6.92 (*m*, 3H), 6.65 (*dd*, *J* = 3.5, 1.8 Hz, 1H), 5.32 (*d*, *J* = 2.4 Hz, 1H), 3.76 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 159.55, 154.69, 154.26, 147.18, 145.99, 144.92, 135.64, 127.96, 119.46, 114.52, 112.31, 111.55, 57.11, 55.62, 53.92. IR, cm^{-1}: 2187.67 (C≡N). Found %: C, 61.11; H, 4.17; N, 25.04, $C_{17}H_{14}N_6O_2$. Calculated, %: C, 61.07; H, 4.22; N, 25.14.

2.2.20. 7-amino-5-(4-methoxyphenyl)-2-(pyridin-3-yl)-4,5-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (3t)

Yield: 60%, Light yellow powder. mp 290 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.13 (*s*, 1H), 8.89 (*d*, J = 3.7 Hz, 1H), 8.62 (*d*, *J* = 4.9 Hz, 1H), 8.26 (*d*, *J* = 7.4 Hz, 1H), 7.49 (*dd*, *J* = 8.0, 4.6 Hz, 1H), 7.27 (*d*, *J* = 8.2 Hz, 2H), 7.13 (*d*, *J* = 9.2 Hz, 2H), 6.93 (*d*, *J* = 8.1 Hz, 2H), 5.31 (*s*, 1H), 3.77 (*d*, *J* = 2.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 159.57, 158.95, 155.05, 151.06, 147.70, 147.15, 147.04, 135.57, 133.89, 132.96, 128.02, 126.56, 124.31, 124.13, 119.44, 114.53, 57.19, 55.62, 53.95. IR, cm^{-1}: 2189.86 (C≡N). Found %: C, 62.59; H, 4.39; N, 28.35, $C_{18}H_{15}N_7O$. Calculated, %: C, 62.60; H, 4.38; N, 28.39; O, 4.63.

2.3. Preparation of 7-amino-5aryl-6-cyano-azolo[1,5-a]pyrimidines 4a-t

Method A. To the suspension of the corresponding dihydro derivative **3a–u** (1 mmol) in 15 ml of MeCN, the dry DDQ (1 mmol) were added slowly. The suspension was stirred at 20 C for 0.5 h, then filtered, washed with acetonitrile and dried in air.

2.3.1. 7-amino-5-phenyl-pyrazolo[1,5-a]pyrimidine-3,6-dicarbonitrile (4a)

Yield: 77%, Yellow powder. mp 274–276 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.32 (*s*, 2H), 8.67 (*s*, 1H), 7.90–7.88 (*m*, 2H), 7.58 (*d*, *J* = 6.6 Hz, 3H). ^{13}C NMR

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(151 MHz, DMSO-d_6), δ, ppm: 162.98, 151.84, 150.93, 148.46, 137.05, 131.21, 129.24, 128.91, 115.99, 113.91, 82.33, 76.42. IR, cm^{-1}: 2236.47 (C≡N), 2213.68 (C≡N). Found %: C, 64.58; H, 3.15; N, 32.27, $C_{14}H_8N_6$. Calculated, %: C, 64.61; H, 3.10; N, 32.29.

2.3.2. ethyl 7-amino-6-cyano-5-phenyl-pyrazolo[1,5-a]pyrimidine-3-carboxylate (4b)

Method A: Yield: 65%. **Method B:** The solution of 0.5 mmol of **3b** in 5 ml of TFAAA was stirred well at room temperature with additional air bubbling for 2 hours. Then the solvent was evaporated, and the residue was treated with KOH solution (2M) until pH = 9. The resulting suspension was filtered and washed with water. The crude product was recrystallized from acetonitrile and dried in air. Yield: 80% (method B), White powder. mp 248–250 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.08 (*s*, 2H), 8.52 (*s*, 1H), 7.91–7.89 (*m*, 2H), 7.58–7.56 (*m*, 3H), 4.30 (*q*, *J* = 7.1 Hz, 2H), 1.37 (*t*, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 162.18, 161.98, 151.64, 148.14, 147.86, 137.62, 130.92, 129.24, 128.78, 116.25, 103.21, 75.48, 60.01, 14.82. IR, cm^{-1}: 2219.80 (C≡N). Found %: C, 62.54; H, 4.25; N, 22.81, $C_{16}H_{13}N_5O_2$. Calculated, %: C, 62.53; H, 4.26; N, 22.79.

2.3.3. 7-amino-2-(methylthio)-5-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4c)

Yield: 94%, white powder. mp 246–247 °C (from MeCN).^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.11 (*s*, 2H), 7.85–7.83 (*m*, 2H), 7.59–7.56 (*m*, 3H), 2.68 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 167.60, 164.22, 155.44, 150.13, 136.85, 130.58, 128.69, 128.32, 115.59, 75.64, 13.39. IR, cm^{-1}: 2207.67 (C≡N). Found %: C, 55.28; H, 3.55; N, 29.73; S, 11.45, $C_{13}H_{10}N_6S$. Calculated, %: C, 55.30; H, 3.57; N, 29.77; S, 11.36.

2.3.4. 7-amino-2-(ethylthio)-5-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4d)

Yield: 93%, white powder. mp 208–212 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.12 (*s*, 2H), 7.85–7.83 (*m*, 2H), 7.58–7.56 (*m*, 3H), 3.26 (*q*, *J* = 7.3 Hz, 2H), 1.41 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 166.91, 164.21, 155.36, 150.14, 136.86, 130.57, 128.69, 128.32, 115.59, 75.66, 25.10, 15.17. IR, cm^{-1}: 2216.10 (C≡N). Found %: C, 56.77; H, 4.09; N, 28.29; S, 10.81, $C_{14}H_{12}N_6S$. Calculated, %: C, 56.74; H, 4.08; N, 28.36; S, 10.82.

2.3.5. 7-amino-5-phenyl-2-(propylthio)-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4e)

Yield: 73%, white powder. mp 205 °C (from MeCN) ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.10 (*s*, 2H), 7.85–7.83 (*m*, 2H), 7.59–7.54 (*m*, 3H), 3.24 (*t*, *J* = 7.2 Hz, 2H), 1.77 (*h*, *J* = 7.3 Hz, 2H), 1.02 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 167.04, 164.22, 155.33, 150.12, 136.86, 130.57, 128.68, 128.32, 115.60, 75.65, 32.51, 22.76, 13.06. IR, cm^{-1}:2221.02 (C≡N). Found %: C, 58.01; H, 4.57; N, 27.11; S, 10.27, $C_{15}H_{14}N_6S$. Calculated, %: C, 58.05; H, 4.55; N, 27.08; S, 10.33.

2.3.6. 7-amino-5-phenyl-2-(prop-2-yn-1-ylthio)-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4f)

Yield: 89%, white powder. mp 227–230 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.19 (*s*, 2H), 7.86–7.83 (*m*, 2H), 7.58–7.56 (*m*, 3H), 4.16 (*d*, *J* = 2.6 Hz, 2H), 3.24 (*d*, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 165.89, 164.96, 155.89, 150.80, 137.31, 131.13, 129.24, 129.14, 128.84, 116.01, 76.48, 19.64. IR, cm^{-1}:2216.59 (C≡N). Found %: C, 58.77; H, 3.25; N, 27.48; S, 10.45, $C_{15}H_{10}N_6S$. Calculated, %: C, 58.81; H, 3.29; N, 27.43; S, 10.47.

2.3.7. 7-amino-2-(benzylthio)-5-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4g)

Yield: 65%, Beige powder. mp 224–230 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.07 (*s*, 2H), 7.86 (*d*, *J* = 4.9 Hz, 2H), 7.56–7.49 (*m*, 5H), 7.32–7.22 (*m*, 3H), 4.53 (*s*, 2H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 166.54, 164.35, 155.38, 150.19, 137.75, 136.85, 130.59, 128.98, 128.70, 128.41, 128.33, 127.27, 115.56, 75.82, 34.42. IR, cm^{-1}: 2221.00 (C≡N). Found %: C, 63.65; H, 3.91; N, 23.49; S, 8.87, $C_{19}H_{14}N_6S$. Calculated, %: C, 63.67; H, 3.94; N, 23.45; S, 8.95.

2.3.8. 7-amino-5-phenyl-2-(thiophen-2-yl)-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4h)

Yield: 80%, Light yellow powder. mp 326–328 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.20 (*s*, 2H), 7.92–7.84 (*m*, 3H), 7.81 (*d*, *J* = 4.9 Hz, 1H), 7.58 (*dd*, *J* = 5.2, 2.0 Hz, 3H), 7.26 (*dd*, *J* = 5.0, 3.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 164.49, 160.80, 155.56, 151.00, 136.92, 132.72, 130.60, 129.78, 128.70, 128.62, 128.35, 115.61, 75.93. IR, cm^{-1}: 2212.20 (C≡N). Found %: C, 60.35; H, 3.21; N, 26.37; S, 10.14, $C_{16}H_{10}N_6S$. Calculated, %: C, 60.36; H, 3.17; N, 26.40; S, 10.07.

2.3.9. 7-amino-2-(furan-2-yl)-5-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4i)

Yield: 71%, Beige powder. mp 335–338 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.26 (*s,* 2H), 7.97 (*d*, *J* = 1.8 Hz, 1H), 7.87–7.85 (*m*, 2H), 7.59–7.57 (*m*, 3H), 7.25 (*d*, *J* = 3.4 Hz, 1H), 6.75 (*dd*, *J* = 3.4, 1.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 164.64, 157.50, 155.50, 151.18, 145.45, 145.31, 136.93, 130.59, 128.70, 128.35, 115.58, 112.89, 112.23, 75.94. IR, cm^{-1}:2212.40 (C≡N). Found %: C, 63.55; H, 3.33; N, 27.70, $C_{16}H_{10}N_6O$. Calculated, %: C, 63.57; H, 3.33; N, 27.80.

2.3.10. 7-amino-5-phenyl-2-(pyridin-3-yl)-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4j)

Yield: 59%, White powder. mp 365 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 9.40 (*d*, *J* = 2.1 Hz, 1H), 9.32 (*s*, 2H), 8.76 (*dd*, *J* = 4.8, 1.7 Hz, 1H), 8.53 (*dt*, *J* = 7.9, 2.0 Hz, 1H), 7.90–7.85 (*m*, 2H), 7.64 (*ddd*, *J* = 7.9, 4.8, 0.9 Hz, 1H), 7.63–7.57 (*m*, 3H). ^{13}C NMR (151 MHz, DMSO-d_6), δ, ppm: 165.11, 162.91, 156.29, 151.98, 151.80, 148.32, 137.40, 134.83, 131.17, 129.23, 128.90, 126.50, 124.65, 116.13, 76.53. IR, cm^{-1}: 2215.82 (C≡N). Found %: C, 65.19;

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H, 3.58; N, 31.25, $C_{17}H_{11}N_7$. Calculated, %: C, 65.17; H, 3.54; N, 31.29.

2.3.11. 7-amino-2-(5-nitrofuran-2-yl)-5-phenyl-pyrazolo[1,5-a]pyrimidine-6-carbonitrile (4k)

Yield: 87%, Brown powder. mp 300–305 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.45 (*s*, 2H), 7.89–7.87 (*m*, 3H), 7.61–7.55 (*m*, 4H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 165.26, 155.65, 155.46, 152.27, 151.51, 147.08, 136.76, 130.77, 128.77, 128.66, 128.41, 115.36, 114.46, 114.30, 76.64. IR, cm^{-1}: 2217.73 (-C≡N), 1505.59 (-NO2). Found %: C, 55.31; H, 2.69; N, 28.20, $C_{16}H_9N_7O_3$. Calculated, %: C, 55.33; H, 2.61; N, 28.23.

2.3.12. ethyl 7-amino-6-cyano-5-(4-methoxyphenyl)-pyrazolo[1,5-a]pyrimidine-3-carboxylate (4l)

Yield: 77%, White powder. mp 208–210 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.08 (*s*, 2H), 8.58 (*s*, 1H), 7.89 (*d*, *J* = 8.5 Hz, 2H), 7.13 (*d*, *J* = 8.5 Hz, 2H), 4.27 (*q*, *J* = 7.1 Hz, 2H), 3.87 (*s*, 3H), 1.31 (*t*, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 160.44, 160.09, 159.80, 150.14, 146.52, 146.28, 129.37, 128.15, 114.92, 112.61, 101.32, 73.22, 58.35, 54.30, 13.22. IR, cm^{-1}: 2214.52 (C≡N). Found %: C, 60.58; H, 4.45; N, 20.69; $C_{17}H_{15}N_5O_3$. Calculated, %: C, 60.53; H, 4.48; N, 20.76.

2.3.13. 7-amino-5-(4-methoxyphenyl)-2-(methylthio)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4m)

Yield: 88%, Gray powder. mp 307–310 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.04 (*s*, 2H), 7.85 (*d*, *J* = 8.3 Hz, 2H), 7.10 (*d*, *J* = 8.4 Hz, 2H), 3.85 (*s*, 3H), 2.68 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 166.37, 162.38, 160.11, 154.31, 149.06, 129.33, 127.85, 114.74, 112.59, 73.83, 54.25, 12.24. IR, cm^{-1}: 2204.64 (C≡N). Found %: C, 53.81; H, 3.84; N, 26.87; S, 10.35, $C_{14}H_{12}N_6OS$.Calculated, %: C, 53.83; H, 3.87; N, 26.91; S, 10.27.

2.3.14. 7-amino-2-(ethylthio)-5-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4n)

Yield: 91%, White powder. mp 212–214 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 8.96 (*s*, 2H), 7.87 (*d*, *J* = 8.9 Hz, 2H), 7.07 (*d*, *J* = 8.9 Hz, 2H), 3.89 (*s*, 3H), 3.26 (*q*, J = 7.3 Hz, 2H), 1.46 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 165.20, 161.87, 159.62, 153.73, 148.58, 128.83, 127.37, 114.25, 112.09, 73.34, 53.75, 23.45, 13.55. IR, cm^{-1}: 2217.15 (C≡N). Found %: C, 55.18; H, 4.33; N, 25.70; S, 9.88, $C_{15}H_{14}N_6OS$. Calculated, %: C, 55.20; H, 4.32; N, 25.75; S, 9.82.

2.3.15. 7-amino-5-(4-methoxyphenyl)-2-(propylthio)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4o)

Yield: 98%, White powder. mp 211–212 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.04 (*s*, 2H), 7.85 (*d*, *J* = 8.9 Hz, 2H), 7.10 (*d*, *J* = 8.8 Hz, 2H), 3.23 (*t*, *J* = 7.2 Hz, 2H), 1.77 (*h*, *J* = 7.3 Hz, 2H), 1.01 (*t*, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 167.45, 164.01, 161.74, 155.84, 150.69, 130.96, 129.50, 116.40, 114.23, 75.47, 55.88, 32.99, 23.27, 13.56. IR, cm^{-1}: 2212.16 (C≡N). Found %: C, 56.41; H, 4.79; N, 24.61; S, 9.50, $C_{16}H_{16}N_6OS$.Calculated, %: C, 56.45; H, 4.74; N, 24.69; S, 9.42.

2.3.16. 7-amino-5-(4-methoxyphenyl)-2-(prop-2-yn-1-ylthio)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4p)

Yield: 96%, White powder. mp 195–199 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.11 (*s*, 2H), 7.86 (*d*, *J* = 8.8 Hz, 2H), 7.11 (*d*, *J* = 8.8 Hz, 2H), 4.15 (*d*, *J* = 2.6 Hz, 2H), 3.86 (*s*, 3H), 3.23 (*t*, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 165.31, 163.73, 161.29, 155.40, 150.36, 130.49, 128.93, 115.80, 113.74, 79.93, 75.27, 74.06, 55.39, 19.12. IR, cm^{-1}: 2213.75 (C≡N). Found %: C, 57.11; H, 3.62; N, 25.03; S, 9.48, $C_{16}H_{12}N_6OS$. Calculated, %: C, 57.13; H, 3.60; N, 24.98; S, 9.53.

2.3.17. 7-amino-2-(benzylthio)-5-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4q)

Yield: 90%, White powder. mp 265–270 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 9.02 (*s*, 2H), 7.87 (*d*, *J* = 8.8 Hz, 2H), 7.51–7.49 (*m*, 2H), 7.32–7.29 (*m*, 2H), 7.26–7.23 (*m*, 1H), 7.07 (*d*, *J* = 8.9 Hz, 2H), 4.53 (*s*, 2H), 3.89 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 166.92, 164.15, 161.77, 155.89, 150.75, 138.26, 130.99, 129.48, 128.92, 127.77, 116.37, 114.25, 75.63, 55.89, 34.88. IR, cm^{-1}: 2218.76 (C≡N). Found %: C, 61.84; H, 4.14; N, 21.65; S, 8.27, $C_{20}H_{16}N_6OS$. Calculated, %: C, 61.84; H, 4.15; N, 21.63; S, 8.25.

2.3.18. 7-amino-5-(4-methoxyphenyl)-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4r)

Yield: 82%, Beige powder. mp 320 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.12 (*s*, 2H), 7.90–7.87 (*m*, 3H), 7.80 (*d*, *J* = 1.3 Hz, 1H), 7.26 (*dd*, *J* = 5.0, 3.6 Hz, 1H), 7.12 (*d*, *J* = 8.7 Hz, 2H), 3.86 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 163.77, 161.27, 160.75, 155.57, 151.06, 132.79, 130.47, 129.70, 129.06, 128.54, 128.32, 115.90, 113.76, 75.27, 55.39. IR, cm^{-1}: 2210.98 (C≡N). Found %: C, 58.63; H, 3.46; N, 24.15; S, 9.18, $C_{17}H_{12}N_6OS$.Calculated, %: C, 58.61; H, 3.47; N, 24.12; S, 9.20.

2.3.19. 7-amino-2-(furan-2-yl)-5-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4s)

Yield: 77%, Beige powder. mp 305–308 °C (from MeCN). ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 9.07 (*s*, 2H), 7.91–7.89 (*m*, 2H), 7.85 (*d*, *J* = 1.8 Hz, 1H), 7.21 (*d*, *J* = 3.4 Hz, 1H), 7.08 (*d*, *J* = 8.8 Hz, 2H), 6.67 (*dd*, *J* = 3.4, 1.8 Hz, 1H), 3.90 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6), δ, ppm: 163.90, 161.26, 157.46, 155.50, 151.24, 145.39, 145.37, 130.47, 129.05, 115.86, 113.74, 112.81, 112.20, 75.27, 55.38. IR, cm^{-1}: 2218.45 (C≡N). Found %: C, 61.40; H, 3.68; N, 25.21, $C_{17}H_{12}N_6O_2$.Calculated, %: C, 61.44; H, 3.64; N, 25.29.

2.3.20. 7-amino-5-(4-methoxyphenyl)-2-(pyridin-3-yl)-[1,2,4]triazolo[1,5-a]pyrimidine-6-carbonitrile (4t)

Yield: 61%, White powder. mp 384–385 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.39 (*d*, *J* = 2.2 Hz, 1H), 9.16 (*s*, 2H), 8.71 (*dd*, *J* = 4.9, 1.7 Hz, 1H), 8.53 (*dt*, *J* = 8.0, 2.0 Hz, 1H), 7.95–7.89

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(*m*, 2H), 7.58 (*dd*, *J* = 8.0, 4.8 Hz, 1H), 7.10 (*d*, *J* = 8.7 Hz, 2H), 3.90 (*s*, 3H). ^{13}C NMR (151 MHz, DMSO-d_6), δ, ppm: 164.41, 162.85, 161.81, 156.32, 151.93, 151.87, 148.30, 134.82, 131.02, 129.55, 126.56, 124.66, 116.43, 114.30, 75.88, 55.91. IR, cm^{-1}: 2217.42 (C≡N). Found %: C, 62.94; H, 3.86; N, 28.53, $C_{18}H_{13}N_7O$. Calculated, %: C, 62.97; H, 3.82; N, 28.56.

2.3.21. 7-amino-5-(4-methoxyphenyl)-2-(5-nitrofuran-2-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carbonitrile (4u)

The corresponding aminoazole **1a–k** (1 mmol) and arylidenemalononitriles **2a–b** (1 mmol) were suspended in 5 ml of MeCN, then 0.14 ml (1 mmol) of Et_3N were added. The mixture was refluxing until a new precipitate forms, monitoring the progress of the reaction by TLC. Then the formed suspension is cooled, the precipitate was filtered and washed with 5 ml of cold solvent. Then the solid residue was suspended in 10 ml of MeCN and 1 mmol of DDQ was added slowly. The suspension was stirred at 20 °C for 0.5 h, then filtered, washed with MeCN and dried in air. Yield: 50%, Brown-green powder. mp 285–290 °C (from MeCN). ^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm: 9.39 (*s*, 2H), 7.89–7.87 (*m*, 3H), 7.53 (*d*, *J* = 3.9 Hz, 1H), 7.13 (*d*, *J* = 8.9 Hz, 2H), 3.87 (*s*, 3H). ^{13}C NMR (151 MHz, DMSO-d_6), δ, ppm: 164.95, 161.90, 156.13, 155.89, 152.72, 152.05, 147.64, 131.04, 129.34, 116.18, 115.78, 114.90, 114.30, 76.41, 55.91. IR, cm^{-1}: 2226.59 (-C≡N), 1514.29 (-NO2). Found %: C, 54.10; H, 2.91; N, 26.07, $C_{17}H_{11}N_7O_4$. Calculated, %: C, 54.11; H, 2.94; N, 25.99.

2.3.22. Preparation of benzoylated 7-amino-5-aryl-6-cyanoazolo[1,5-*a*]pyrimidine derivatives (6a-c)

Acylating agent **5a** (3 mmol) was added to a solution of the corresponding amine **4** (1 mmol) in 5 ml of pyridine, and the resulting mixture was refluxed for 3 h. Then the mixture was cooled and poured into 10 ml of Na_2CO_3 (1 M) and extracted with chloroform (2x20 ml). The combined organic layers were dried with sodium sulfate and evaporated off. The product was recrystallized from i-PrOH and dried in air.

2.3.23. N-(6-cyano-2-(furan-2-yl)-5-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)benzamide (6a)

Yield 71% Brown powder. mp 255–257 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12–8.01 (*m*, 2H), 7.91–7.82 (*m*, 3H), 7.47 (*ddd*, *J* = 14.5, 7.8, 6.2 Hz, 3H), 7.15–7.05 (*m*, 3H), 6.67 (*dd*, *J* = 3.4, 1.8 Hz, 1H), 3.86 (*s*, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) 171.36, 163.99, 160.69, 156.73, 155.83, 151.32, 146.36, 144.49, 137.84, 130.71, 130.30, 130.27, 128.88, 127.77, 118.36, 113.49, 111.88, 111.14, 81.30, 55.30. Found %: C 66.03, H 3.73, N 19.24, $C_{24}H_{16}N_6O_3$. Calculated, %: C, 66.05; H, 3.70; N, 19.26.

2.3.24. N-(6-cyano-2-(furan-2-yl)-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)benzamide (6b)

Yield 20% Yellow powder. mp 290–292 °C (from *i*-PrOH).^{1}H NMR (600 MHz, DMSO-d_6) δ 8.15 (*d*, *J* = 7.0 Hz, 1H), 8.02–7.98 (*m*, 2H), 7.90 (*d*, *J* = 1.7 Hz, 1H), 7.72 (*t*, *J* = 7.4 Hz, 1H), 7.67–7.60 (*m*, 4H), 7.32 (*d*, *J* = 3.4 Hz, 1H), 6.71 (*dd*, *J* = 3.4, 1.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.02, 165.17, 157.22, 156.34, 151.28, 146.81, 145.03, 138.62, 138.23, 131.27, 130.33, 129.38, 129.14, 128.59, 128.30, 118.58, 112.39, 111.69, 82.15. Found %: C 67.98; H 3.55; N 20.61, $C_{23}H_{14}N_6O_2$. Calculated, %:C 67.97; H 3.47; N 20.68.

2.3.25. N-(6-cyano-5-(4-methoxyphenyl)-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)benzamide (6c)

Yield 87% Light beige powder. mp 275–276 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05–8.03 (*m*, 2H), 7.86 (*d*, *J* = 8.4 Hz, 2H), 7.71–7.70 (*m*, 2H), 7.49–7.47 (*m*, 3H), 7.20 (*t*, *J* = 4.3 Hz, 1H), 7.10 (*d*, *J* = 8.7 Hz, 2H), 3.87 (*s*, 3H). ^{13}C NMR (151 MHz, CDCl3) δ 177.14, 169.18, 165.92, 164.09, 162.07, 156.00, 143.17, 139.27, 135.94, 135.54, 135.52, 134.04, 133.62, 133.29, 133.04, 132.47, 123.67, 118.73, 86.21, 60.54. Found %: C 63.65; H 3.69; N 18.41; S 7.22, $C_{24}H_{16}N_6O_2S$. Calculated, %: C 63.70; H 3.56; N18.57; S 7.09.

2.4. Preparation of furoylated 7-amino-5-aryl-6-cyanoazolo[1,5-*a*]pyrimidine derivatives (6d–i)

Acylating agent **5b** (1.1 mmol) was added to a solution of the corresponding amine **4** (1 mmol) in 5 ml of pyridine, and the mixture was refluxed for 3 h. The formed dark solution was cooled and poured into 10 ml of Na_2CO_3 (1 M) and 20 ml of chloroform. Then the mixture was stirred vigorously for 10 min. The formed precipitate was filtered, washed with 30 ml of diethyl ether and dried in air.

2.4.1. N-(6-cyano-2-(furan-2-yl)-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)furan-2-carboxamide (6d)

Yield 44% Brown powder. mp 280–282 °C (from *i*-PrOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.88 (*d*, *J* = 1.7 Hz, 1H), 7.85–7.81 (*m*, 2H), 7.77–7.75 (*m*, 1H), 7.57–7.52 (m, 3H), 7.10–7.06 (*m*, 1H), 7.01 (*dd*, *J* = 2.3, 1.2 Hz, 1H), 6.68 (*dd*, *J* = 3.4, 1.8 Hz, 1H), 6.58 (*dd*, *J* = 3.4, 1.6 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.16, 163.61, 157.14, 156.40, 152.29, 146.70, 145.15, 145.11, 138.50, 130.40, 129.14, 128.62, 118.53, 114.66, 112.42, 112.15, 111.80, 82.20. Found %: C 67.98; H 3.55; N 20.61. $C_{23}H_{14}N_6O_2$. Calculated, %:C 67.97; H 3.47; N 20.68.

2.4.2. N-(6-cyano-2-(5-nitrofuran-2-yl)-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)furan-2-carboxamide (6e)

Yield 82% Dark brown powder. mp 330 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88–7.85 (*m*, 2H), 7.71–7.70 (*m*, 1H), 7.65 (*s*, 1H), 7.52–7.51 (*m*, 3H), 7.36 (*d*, *J* = 3.7 Hz, 1H), 7.02 (*d*, *J* = 3.3 Hz, 1H), 6.53 (*dd*, *J* = 3.3, 1.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.89, 163.78, 157.35, 154.39, 152.39, 152.00, 150.48, 148.77, 145.41, 138.36, 130.53, 129.16, 128.64, 118.24, 115.06, 115.02, 114.37, 112.22, 82.38. Found %: C 57.09; H 2.55; N 22.17, $C_{21}H_{11}N_7O_5$. Calculated, %: C 57.15; H 2.51; N 22.21.

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2.4.3. N-(6-cyano-5-phenyl-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)furan-2-carboxamide (6f)

Yield 45% White powder. mp 290–292 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.55–8.54 (*d*, *J* = 3.9 Hz, 1H), 7.88–7.85 (*m*, 2H), 7.74 (*tt*, *J* = 7.7, 1.9 Hz, 1H), 7.64 (*dd*, *J* = 5.0, 1.3 Hz, 1H), 7.54–7.53 (*m*, 2H), 7.34 (*ddd*, *J* = 7.6, 4.2, 1.5 Hz, 1H), 7.20 (*dd*, *J* = 5.0, 3.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.12, 164.00, 159.46, 157.26, 152.31, 150.54, 145.12, 138.54, 134.37, 130.38, 129.14, 129.00, 128.61, 128.56, 127.83, 118.56, 114.54, 112.15, 81.90. Found %: C 61.21; H 2.89; N 20.27; S 7.74, $C_{21}H_{12}N_6O_2S$. Calculated, %: C 61.16; H 2.93; N 20.38; S 7.77.

2.4.4. N-(2-(benzylthio)-6-cyano-5-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)furan-2-carboxamide (6g)

Yield 49% Beige powder. mp 220 °C (from *i*-PrOH). ^{1}H NMR (400 MHz, DMSO-d_6) (400 MHz, DMSO-d_6) δ 7.83 (*s*, 2H), 7.62 (*s*, 1H), 7.49 (*d*, *J* = 4.4 Hz, 3H), 7.42–7.30 (*m*, 3H), 7.23 (*dt*, *J* = 12.8, 7.0 Hz, 3H), 6.94 (*s,* 1H), 6.50 (*s*, 1H), 4.30 (*s*, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.81, 164.25, 164.02, 157.17, 152.20, 148.78, 145.27, 138.60, 138.47, 130.35, 129.44, 129.09, 128.81, 128.59, 127.56, 118.68, 114.62, 112.18, 81.73, 34.76. Found %: C 63.77; H 3.54; N 18.41; S 6.97, $C_{24}H_{16}N_6O_2S$. Calculated, %: C 63.70; H 3.56; N 18.57; S 7.09.

2.4.5. N-(6-cyano-2-(furan-2-yl)-5-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)furan-2-carboxamide (6h)

Yield 77% Yellow powder. mp 318–320 °C (from *i*-PrOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.87 (*s*, 1H), 7.84–7.83 (*m*, 2H), 7.75 (*d*, *J* = 1.7 Hz, 1H), 7.10–7.06 (*m*, 3H), 6.99 (*d*, *J* = 3.4 Hz, 1H), 6.67 (*dd*, *J* = 3.4, 1.8 Hz, 1H), 6.57 (*dd*, *J* = 3.5, 1.7 Hz, 1H), 3.85 (*s*, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.48, 163.47, 161.21, 157.15, 156.37, 152.40, 151.47, 146.76, 145.06, 130.81, 130.65, 118.81, 114.53, 114.00, 113.87, 112.40, 112.11, 111.72, 81.83, 62.49. Found %: C 61.93; H 3.35; N 19.65, $C_{22}H_{14}N_6O_4$. Calculated, %: C 61.97; H 3.31; N 19.71.

2.4.6. N-(6-cyano-5-(4-methoxyphenyl)-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)furan-2-carboxamide (6i)

Yield 57%. White powder. mp 284–286 °C (from *i*-PrOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.85 (*d*, *J* = 8.2 Hz, 2H), 7.75 (s, 1H), 7.70 (*d*, *J* = 5.7 Hz, 2H), 7.19 (*s,* 1H), 7.10 (*d*, *J* = 8.3 Hz, 2H), 7.01 (*d*, *J* = 3.4 Hz, 1H), 6.57 (*d*, *J* = 4.1 Hz, 1H), 3.86 (*s*, 4H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.44, 163.84, 161.21, 159.45, 157.26, 152.41, 151.09, 150.09, 145.04, 136.61, 134.43, 130.81, 130.68, 128.95, 128.55, 127.79, 124.38, 118.84, 114.45, 114.00, 112.12, 81.56, 62.50. Found %: C 59.75; H 3.14; N 18.90; S 7.21, $C_{22}H_{14}N_6O_3S$. Calculated, %: C 59.72; H 3.19; N 18.99; S 7.25.

2.5. Crystal data

X-ray diffraction analysis was carried out using the equipment of the Center for Collective Use “Spectroscopy and Analysis of Organic Compounds” of the Institute of Organic Synthesis, Ural Branch of the Russian Academy of Sciences. The experiment was carried out on an automatic X-ray diffractometer “Xcalibur 3” with a CCD detector according to the standard procedure (Mo Kα radiation, graphite monochromator, ω-scanning with a step of 1° at *T* = 295(2) K). An empirical correction for absorption was introduced. The solution and refinement of the structure were carried out in the Olex2 software [30]. The structure was solved using the ShelXT program [31] by the internal phase method and refined in the ShelXL program [32] by full-matrix least squares on F2 in the anisotropic approximation for non-hydrogen atoms. The hydrogen atoms of C-H bonds were placed in the calculated positions; the protons of NH groups are localized along the peaks of the spatial electron density and were refined independently in the isotropic approximation.

$C_{20}H_{16}N_6OC$, *M* = 388.45, orthorhombic, *a* = 13,8476(8) Å, *b* = 9,0055(9) Å, *c* = 30,460(2) Å, *V* = 3798,5(5) Å^3, *T* = 295(2), *Pbca*, *Z* = 8, μ(Mo Kα)= 0.194 mm^{-1}, 5.34<2θ<54.2° 10831 reflections: 4181 unique r_e (R_{int} = 0.0649). w*R*2 = 0.2071 (all data), *R*1 = 0.0760 (*I*>2σ(*I*)), Δρē = 0.41/–0.36 ēÅ^{-3}. CCDC 2363180 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk.

2.6. Bioassays

2.6.1. Cell lines and culture of cells

The studies were carried out on cultured human glioblastoma cells (A172, ATCC CRL 1620), human osteosarcoma cells (HOS, ATCC CRL 1543), human rhabdomyosarcoma cells (Rd, ATCC CRL 136) and human embryonic kidney cells (HEK293, ATCC CRL 1573), which were obtained from the Shared research facility “Vertebrate cell culture collection” (Institute of Cytology RAS, Russia). All the cell lines were grown in Dulbecco’s Modified Eagle’s Medium / F12 (DMEM/ F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained in an incubator at 37 °C and 5% CO_2 humidified atmosphere. The stock cells were subcultured with 0.25% trypsin when cell confluence reaches 90%.

2.6.2. MTT assay

The substances were dissolved in DMSO-d_6 and diluted in culture medium DMEM/F-12 (10% FBS) to obtain solutions of the tested concentration in the range from 8 to 1024 μM. For the less soluble substances the concentration range was 4–512 μM. The final concentration of DMSO-d_6 in the cells is 0.1%.

The cells were seeded in 96-well plates, at a density of $4{\cdot}10^3$ per well, and incubated overnight at 37 °C prior to experimentation. After 24 h, the test compounds were added to the wells in a given concentration range. Then the cells were incubated for 24 h, after which 20 μL (5 mg/mL) of a solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added to the cultures in

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the wells. After 2.5 h, the medium was removed from the wells. The formazan crystals were solubilized with 200 μL of a mixture of DMSO-d_6 and isopropanol, 1:1, and the plates were shaken for 10 min. The absorbance was measured at 570 nm using a plate reader.

2.6.3. Acridine orange (AO)-ethidium bromide (EB) double staining

Cell viability was determined using vital dye acridine orange (5 μg/ml) and propidium iodide (15 μg/ml). The cells were incubated in 35 mm Petri dishes and then cultured for 24 h to allow complete attachment to the surface of the plates. Subsequently, solutions of the test compounds and cisplatin were added at a concentration of 64 μM. After being cultured for 72 h, the cells were removed and centrifuged at 200 g for 5 min. The sediment was resuspended in 25 μl phosphate buffer at room temperature. Dual fluorescent staining solution containing AO and EB was added to each suspension, and then the suspensions were transferred to glass slides and covered with a coverslip. The morphology of apoptotic cells was examined, and 300 cells were counted within 20 min using a fluorescent microscope XDS-3FL4 (Optika, Italy). Cisplatin served as the positive control.

2.6.4. Proliferative activity

A Click-iT EdU-555 assay (G1602, Wuhan Servicebio Technology Co., Ltd., China) was used to assess the impact of cell proliferative activity. 5-Ethynyl-2′-deoxyuridine (EdU) is a thymidine analogue which is incorporated into the DNA of dividing cells.

Cells were pre-dispersed into wells of a 96-well plate at a seeding concentration of $4{\cdot}10^3$ cells per well the day before addition of the test substances and incubated in a CO_2 incubator for 24 h. Subsequently, the suspensions of the test compounds were added at a concentration of 64 μM. After 24 h, EdU reagent was added, and the cultures were incubated for 2 h. Then the cells were fixed with 10% formaldehyde and permeabilized with 0.5% Triton X-100. After that the cells were stained with fluorescent mixture with iF555 and microscopically examined using a fluorescent microscope XDS-3FL4 (Optika, Italy). Cisplatin served as the positive control.

2.6.5. Morphological changes

The Romanowsky-Giemsa stain was used to identify abnormalities in morphology and pathological changes in RD cell line under influence of **6d** and **4h**.

The cells were pre-dispersed in 35 mm Petri dishes and then cultured for 24 h. Then suspensions of the test compounds were added at a concentration of 64 μM. One of the Petri dishes was used as a negative control, containing intact cells. Then the cells were cultured for 24 h, after which they were fixed with 10% formaldehyde. Then the Giemsa staining solution was added. Distilled water was used to remove the residue, and the samples were observed by the light-field microscope. Hoechst 33258 was used as a nuclear dye (Wuhan Servicebio Technology Co., Ltd., China). Cisplatin served as the positive control.

2.6.6. Annexin V-propidium iodide staining apoptosis testing

To determine the percentage of early and late apoptotic cells in RT-exposed cultures, conventional light and fluorescence microscopy was performed using Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI). The cells of the lines Rd and HEK-293 were seeded in 6-well plates the day before addition of the test substances **4h** and **6d** and then cultured for 24 h at 37 °C and 5% CO_2 humidified atmosphere. After 24 h, the culture medium in each well was replaced with the fresh one, and solutions of the test compounds were added at a concentration of 64 μM. After being cultured for 72 h at 37 °C and 5% CO_2 humidified atmosphere, the cells were washed with phosphate buffered saline (PBS), then stripped with 0.25% trypsin solution (Biolot, Russia), and centrifuged at 200g for 5 min. After centrifugation, and supernatant was removed. The cell precipitate was re-suspended in the 25 μl phosphate buffer. To the resulting suspension, 25 μl of freshly prepared dye mixture Annexin V-FITC/PI (G1511, Wuhan Servicebio Technology Co., Ltd., China) was added, pipetted, and incubated for 10 min in the dark. After that, 10 μl of the mixture was added to a cell counter slide (C100, RWD Life Science, China) for automatic counting.

The results were expressed as a percentage of the total number of cells. The Annexin V-FITC was used to identify early apoptotic cells (AnV+/PI-), propidium iodide was used to identify late apoptosis and necrotic cells (AnV-/PI+ и AnV+/PI+), and unstained cells (AnV-/PI-) were assessed as live.

2.6.7. Mitochondrial membrane potential assay

The dye JC-1 (G1515-100T, Wuhan Service bio Technology Co., Ltd., China) has been used to detect the mitochondrial membrane potential (ΔΨm).

The cells were pre-dispersed into wells of a 96-well plate at a seeding concentration of $4{\cdot}10^3$ cells per well the day before addition of the test substances. Then solutions of the test compounds were added in each well at a concentration of 64 μM. The cells with the compounds were cultured for 24 h. The cells were incubated with 0.5 mL JC-1 working solution at 37 °C and 5% CO_2 in a humidified incubator for 30 min.

The cells that were conditioned with 100 μM CCCP (Carbonyl cyanide 3-chlorophenylhydrazone) for 30 min at 37 °C were used as positive control. The cells were examined using a fluorescent microscope XDS-3FL4 (Optika, Italy).

2.6.8. Statistical analysis

Statistical data processing was carried out in the RStudio program (Version 1.4.1106 © 2009-2021 RStudio, PBC) using the R package (version 4.1.2). The cytotoxicity index (IC_{50}) was calculated by plotting dose-response curves us-

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ing the "drc" package [33]. Analysis of images from mitochondrial membrane potential was carried out by the «EBImage» package. Based on this package, the ratio of red pixels to green pixels was determined. Data were compared using a two-way analysis of variance (ANOVA), with Tukey's correction. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results and Discussion

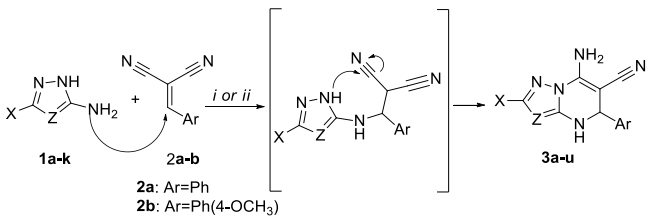
3.1. Chemistry

The synthesis of 2-substituted 7-amino-6-cyano-5-arylazolo[1,5-*a*]pyrimidines was carried out in 2 stages. By reacting the corresponding aminoazoles **1a-k** and arylidenemalononitriles [34] **2a-b** under basic conditions, 4,5-dihydro-7-amino-6-cyano-5-arylazolo[1,5-*a*]pyrimidines **3a–u** were synthesized in yields up to 80% – precursors of target compounds (Scheme 1, Table 1). This approach to the preparation of azolo[1,5-*a*]pyrimidines **3a–u** allows, by varying the dinucleophilic **1a–k** and dielectrophilic substrates **2a–b**, combining thechemical/pharmacophore substituents in the 2 and 5 positions of the azolo[1,5-*a*]pyrimidine framework.

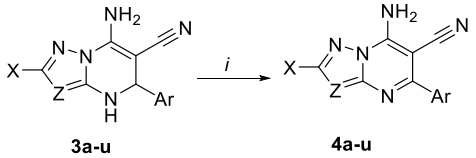
According to the proposed mechanism [14, 15] , this reaction proceeds sequentially in two stages under basic conditions: Michael addition involving a lone pair of electrons of the exocyclic nitrogen atom of aminoazole 1a-k and the electrophilic carbon atom of arylidene malononitrile 2a-b, followed by cyclization of the intermediate. Due to their extremely low solubility in water, 4,5-Dihydro-7-amino-5-arylazolo[1,5-*a*]pyrimidine-6-carbonitriles 3a-u were not considered as objects for further bioassays.

The resulting 4,5-dihydro derivatives 3a-u were oxidized using DDQ as an oxidizing agent to the target 7-amino-5-arylazolo[1,5-*a*]pyrimidine-6-carbonitriles 4a-u in yields up to 96% (Scheme 2, Table 1).

The structure of the given compounds (as well as regioselectivity of the previous reaction) was determined by the NMR, IR spectra and X-ray studies (see Supplementary materials for details).



Scheme 1 Reagents and conditions: i) 2a: Et_3N (1 eq.), i-PrOH, reflux, 2–10 h; ii) 2b: Et_3N (1 eq.), MeCN, reflux, 2–10 h; The interpretation of substituents X, Z and Ar is given in Table 1.



Scheme 2 Reagents and conditions: i) DDQ (1 eq.), MeCN, 25 °C. The interpretation of substituents X, Z and Ar is given in Table 1.

According to the X-ray diffraction data, the compound **4q** crystallizes in a centrosymmetric space group, in a general position. The general view of the molecule is shown in Figure 3. The bond lengths and bond angles in the molecule are close to the expected values. The heterocyclic fragment is almost flat; the plane of the phenyl ring of the benzyl fragment forms an angle of 73° with respect to the plane of the heterocycle, and the 4-MeOPh substituent is turned at an angle of –43° with respect to the plane of the heterocycle.

As an alternative to the organic oxidizing agent DDQ, using as the example the synthesis of compound **4b** (method B, Experimental part), we investigated the possibility of obtaining the target product by long-term bubbling of air through a solution of the corresponding dihydro derivative in trifluoroacetic acid with a yield of 80%. Using as the example the synthesis of compound **4u**, the possibility of obtaining the target aromatic derivative without isolating intermediate **3u** with a total yield of 50% was shown.

In the course of further studying the reactivity of the obtained aromatic derivatives **4** and improving the possible "pharmacophore" profile of the derivatives, a series of N7-acylated derivatives **6** were synthesized in yields of up to 87% (Scheme 3, Table 2).

Table 1 Reaction conditions and yields for compounds **3a–u** and **4a–u**.

Substituents			Letter	Yield, %	
X	Z	Ar		3	4
H	C-CN	Ph	a	60[a]	77[c]
H	CCO_2Et	Ph	b	65[a]	65[c] (80[d])
SMe	N	Ph	c	31[a]	94[c]
SEt	N	Ph	d	47[a]	93[c]
SPr	N	Ph	e	21[b]	73[c]
prop-1-yn-3-yl	N	Ph	f	54[a]	89[c]
SBn	N	Ph	g	54[a]	65[c]
thien-2-yl	N	Ph	h	50[a]	80[c]
furan-2-yl	N	Ph	i	72[a]	71[c]
pyridin-3-yl	N	Ph	j	80[a]	59[c]
5-nitro furan-2-yl	N	Ph	k	44[a]	87[c]
H	CCO_2Et	(4-OMe)Ph	l	25[b]	77[c]
SMe	N	(4-OMe)Ph	m	37[b]	88[c]
SEt	N	(4-OMe)Ph	n	25[b]	91[c]
SPr	N	(4-OMe)Ph	o	23[b]	94[c]
prop-1-yn-3-yl	N	(4-OMe)Ph	p	43[b]	96[c]
SBn	N	(4-OMe)Ph	q	52[b]	90[c]
thien-2-yl	N	(4-OMe)Ph	r	35[b]	82[c]
furan-2-yl	N	(4-OMe)Ph	s	41[b]	77[c]
pyridin-3-yl	N	(4-OMe)Ph	t	60[b]	61[c]
5-nitro furan-2-yl	N	(4-OMe)Ph	u	X	50[e]

Reagents and conditions: a) Et3N (1 eq.), i-PrOH, reflux; b) Et3N (1 eq.), MeCN, reflux; c) MeCN, DDQ (1 eq.), 0.5–1 h; d) TFAA, O_2/air, 2 h; X – did not obtained in pure form

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To carry out the transformations, the optimal method was to boil the corresponding 7-aminoazolo[1,5-*a*]pyrimidines 4 with an excess of the acylating agent (1.5 equivalents) in pyridine (Table 2).

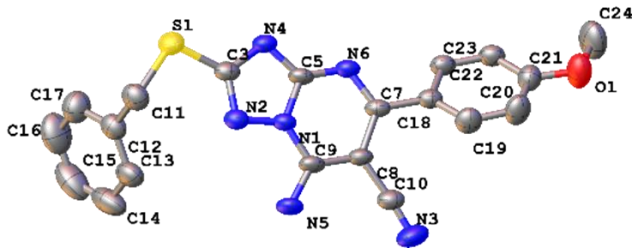


Figure 3 Crystal structure of **4q**. Thermal ellipsoids are given with 50% probability.

Table 2 Acylated derivatives of 7-amino-5-aryl-6-cyano-1,2,4-triazolo[1,5-*a*]pyrimidines.

№	X	Ar	R	Yield, %
6a	furan-2-yl	(4'-OMe)Ph	Ph	71
6b	furan-2-yl	Ph	Ph	20
6c	thien-2-yl	(4'-OMe)Ph	Ph	87
6d	furan-2-yl	Ph	furan-2-yl	44
6e	5-nitrofuran-2-yl	Ph	furan-2-yl	82
6f	thien-2-yl	Ph	furan-2-yl	45
6g	SBn	Ph	furan-2-yl	49
6h	furan-2-yl	(4'-OMe)Ph	furan-2-yl	77
6i	thien-2-yl	(4'-OMe)Ph	furan-2-yl	57

3.2. Bioassays

3.2.1. MTT Assay

Assessment of cytotoxic effect of 7-amino-5-aryl-6-cyanoazolo[1,5-*a*]pyrimidines 4 and their acylated derivatives **6** on cancer cell lines and normal line HEK293 was measured with the MTT assay. IC_{50} values were calculated from curves constructed by plotting cell and were summarized in Table 3.

The derivatives **4h** and **6d** revealed profound antiproliferative action against A172, HOS and Rd cancer cell lines (IC_{50} < 260 μM).

Highly significant cytotoxicity was shown by compounds **4g**, **6b**, **6g**, **6a** and **6c** against A172, Hos и Rd cancer cell lines (IC_{50} < 450 μM), but these compounds also showed significant cytotoxic potential against normal cell line HEK293.

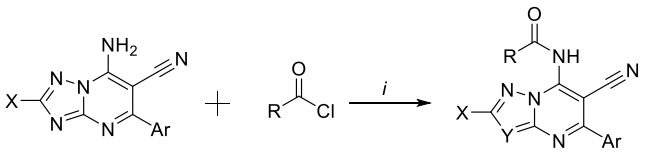
Compounds **4h** and **6d** show a high selectivity coefficient (Table 4), indicating that they selectively target rhabdomyosarcoma Rd.

Cytotoxic activity for **6h** was higher in cancer cells than in nontumorigenic cell lines. Compounds **6e** and **6f** were showed low anticancer efficacy against cancer cells.

For the compounds **4h** and **6d** with high-confidence activity data, with low IC50 values, the following characteristics were determined: cell death, proliferative activity, morphological changes in cells, mitochondrial potential.

3.2.2. Acridine orange (AO)-ethidium bromide (EB) double staining

The results from AO/EB double staining were shown in Figure 4a and 4b. The Rd cells exposed to compound **4h** showed apoptotic morphological changes and had a higher percentage of dead cells than the cells under the treatment with compound **6d** – 21.64% against 7.44%.



4g-k,r-s **5** **6a-k**

5a: R=Ph
5b: R=furan-2-yl

Scheme 3 Acylation of 7-amino-5-aryl-6-cyano-1,2,4-triazolo[1,5-*a*]pyrimidines. Reagents and conditions: i) RC(O)Cl (1 to 3 eq.), pyridine, reflux, 3h.

Table 3 Cytotoxicity (IC_{50} ± SE) of the main compounds on cell lines A172, Rd, HOS, HEK293, μM.

No.	Cell line			
	A172	Rd	HOS	HEK293
4h	178.8±16.1	28.6±5.8	182.3±33.7	>512
4g	249.6±30.6	90.0±11.5	197.7±14.1	416.1±38.2
4q	461.4±72.3	35.8±19.8	203.1±23.9	>512
6b	352.5±22.6	213.9±14.2	281.4±17.5	312.6±38.8
6d	256.5±25.0	28.9±7.2	91.5±18.2	502.7±27.6
6e	263.9±23.3	358.5±43.4	339.6±44.0	485.7±111.5
6f	376.3±28.5	349.2±15.9	475.9±37.1	436.9±24.6
6g	146.8±6.3	160.6±9.4	141.4±13.7	220.7±16.2
6a	165.1±5.3	187.5±14.4	175.9±10.3	265.3±40.5
6c	178.7±22.4	181.9±11.8	186.8±14.9	203.4±12.7
6h	324.1±47.9	448.0±24.9	470.9±38.9	728.0±89.5
Cisplatin	3.6±0.2	5.0±0.3	2.4±0.1	4.4±0.2

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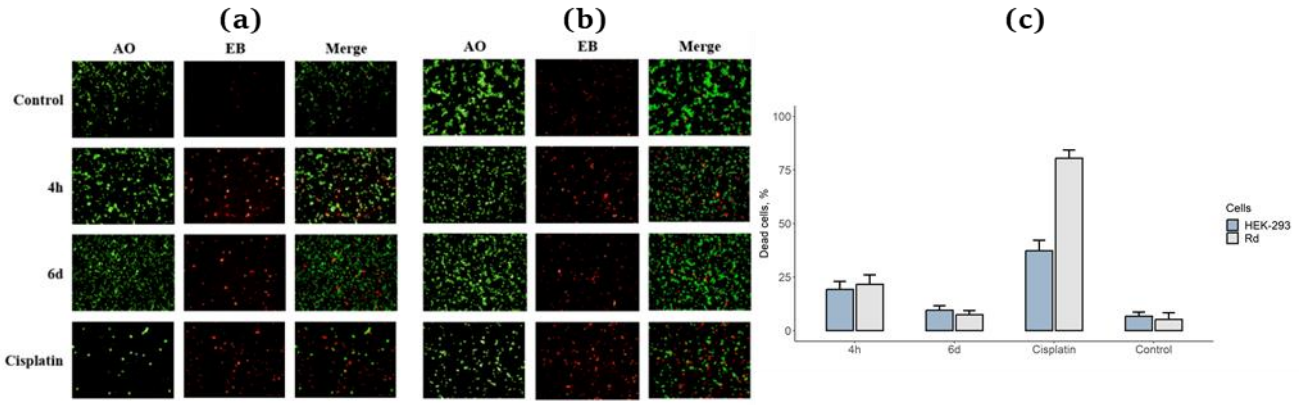

Figure 4 Staining of Rd (a), HEK293 (b) cell lines with a mixture of fluorescent dyes AO and EB. "Control" – intact cells, **4h**, **6d** and "Cisplatin" – cells after 72 hours of incubation with drugs and cisplatin at 64 μM concentration, respectively. Magnification 100x. (c) – percentage of cell death of Rd, HEK293 lines after 72-h incubation with compounds **4h**, **6d** and cisplatin (bar graph).

Table 4 Selectivity coefficient of the studied compounds.

No.	Cell line		
	A172	Rd	HOS
4h	2.9	**17.9**	2.8
4g	1.7	4.6	2.1
4q	1.1	14.3	2.5
6b	0.9	1.5	1.1
6d	2.0	**17.4**	5.5
6e	1.8	1.4	1.4
6f	1.2	1.3	0.9
6g	1.5	1.4	1.6
6a	1.6	1.4	1.5
6c	1.1	1.1	1.1
6h	2.2	1.6	1.5
Cisplatin	1.2	0.9	1.8

These data showed the absence of pronounced activity against Rd cell line. And, according to Figures 4b and 4c, compound **4h** turned out to be more toxic than compound **6d** against normal cell line HEK293 – the percentage of dead cells after 72 h of incubation with **6b** was 19.28%, and with **6d** it was 9.54%. In the control samples with intact cells, a low number of dead cells was observed due to pro-longed incubation of 72 h.

It is important to note that the data obtained as a result of staining with fluorescent dyes (AO and EB) differed from the experimental results of the MTT test (Table 3), in which high efficiency and selectivity of compound **4h** against Rd cancer cell was observed, while compound **4h** did not affect the viability in the control cell line HEK293. With double fluorescent staining, the effect was approximately the same on both lines, while the percentage of death cells did not exceed 25% (Figure 4c).

According to the obtained results, compound **4h** could express selective effects on Rd mitochondria, or had a cytostatic effect, causing a decrease in division activity without cell death, accompanied by a violation of the integrity of the cytoplasmic membrane. This may be the reason for causing a noticeable effect in the MTT test and an insignificant effect in double fluorescent staining of AO and EB.

3.2.3. Proliferative activity

According to the results shown in Figure 5, a significant number of red cells were observed in the control (intact) samples for both Rd (Figure 5a) and HEK293 (Figure 5b) cell lines, while cisplatin suppressed proliferation in both cases. Compound **4h** moderately inhibited cell division in both Rd and HEK293 cell lines, but did not completely block it. Compound **6d** showed no effect on the proliferative activity of rhabdomyosarcoma cells and human embryonic kidney cell.

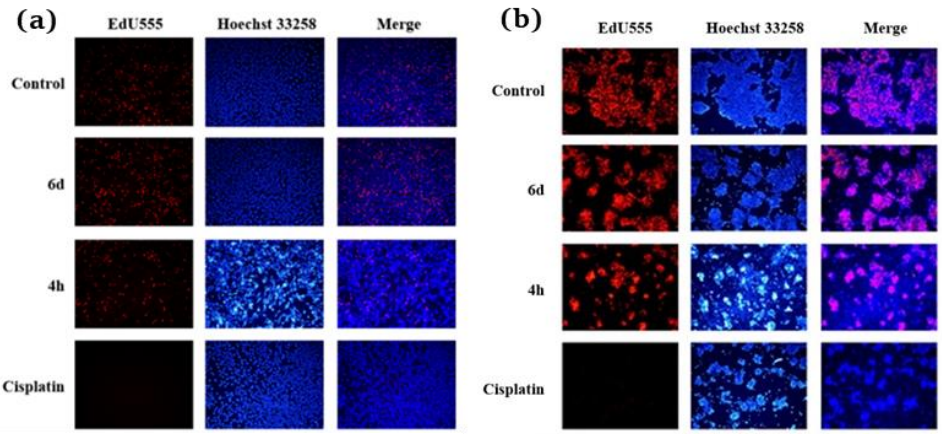

Figure 5 Staining of Rd (a) and HEK293 (b) cell lines with EdU-555 dye. "Control" – intact cells, **4h**, **6d** and "Cisplatin" – cells after incubation with compounds and cisplatin at 64 μM concentration, respectively. Magnification 100x.

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3.2.4. Morphological changes

Giemsa staining was conducted to observe morphology of Rd cell line after treatment with compound **4h** (Figure 6). The results suggested that there were multiple morphological changes: pyknosis, loss of plasma membrane integrity, changes in cell size and shape. Decrease in the percentage of the number of cells in the monolayer was also noticed.

Cisplatin, as a positive control, exhibited significant effects such as of debris, changes in cell shape and cell shrinkage. No significant morphological changes or reduction in the number of cells were observed in intact cells or in cells treated with compound **6d**.

3.2.5. Mitochondrial membrane potential assay

The cationic dye JC-1 was used to evaluating the effect of compounds **4h** and **6d** on mitochondrial membrane potential in Rd and HEK293 cell cultures.

Fluorescence microscopy-based mitochondrial membrane potential assay showed that the compound **4h** significantly decreased the mitochondrial membrane potential in Rd cell line. On the other hand, compound **6d** shows a similar, but less pronounced effect. It is necessary to point out that, although compound **4h** exhibited lower activity in proliferative assay and AO/EB staining compared with cisplatin, in that case we observe that compound **4h** significantly reduced mitochondrial membrane potential compared with cisplatin ($p < 0.001$).

Moreover, compound **4h** showed the same level of the effect of on mitochondrial membrane potential as cisplatin ($p = 0.53$). In additional, no significant drop in ΔΨm was observed in cells HEK293 (Figure 7b) after treatment with compound **4h** or cisplatin – the cells in all groups did not differ significantly between each other and intact cells ($p > 0.05$). These findings comported with the MTT test (Table 3) and were key to understanding pronounced effect obtained in the Rd cell line and its absence in the HEK293 line. We hypothesize that the antitumor properties of compound **4h** may be associated with selective inhibition of cell mitochondrial functionality, but without pronounced blocking of cell division, induced by cisplatin. So, our findings revealed that compound **4h** had a different mechanism of action compared with cisplatin.

3.2.6. Annexin V-propidium iodide staining apoptosis testing

To determine the presence of apoptosis in 4h-treated cells, cell suspensions were stained using the Annexin V-FITC protein, which specifically binds to phosphatidylserine, which appears in the outer leaflet during apoptosis. Propidiumiodide (PI) was used to detect dead cells in a population.

The percentage of early apoptotic RD cells observed under treatment with compound **4h** for 72 h were 33.3%; late apoptotic and necrotic cells were 6.3% and live cells population was 60% (Figure 8a).

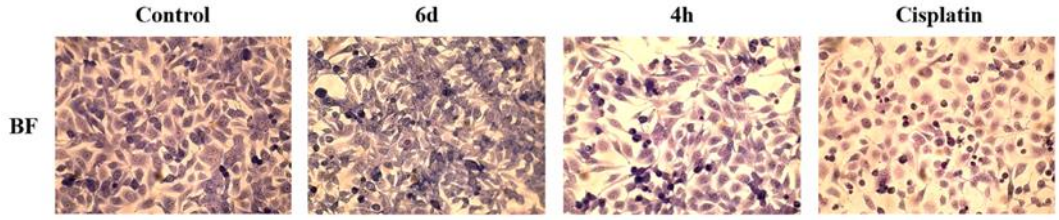

Figure 6 Staining of Rd cell line with Romanovsky-Giemsa dye after exposure to compounds **4h**, **6d** and cisplatin at 64 μM concentration. Magnification 200x.

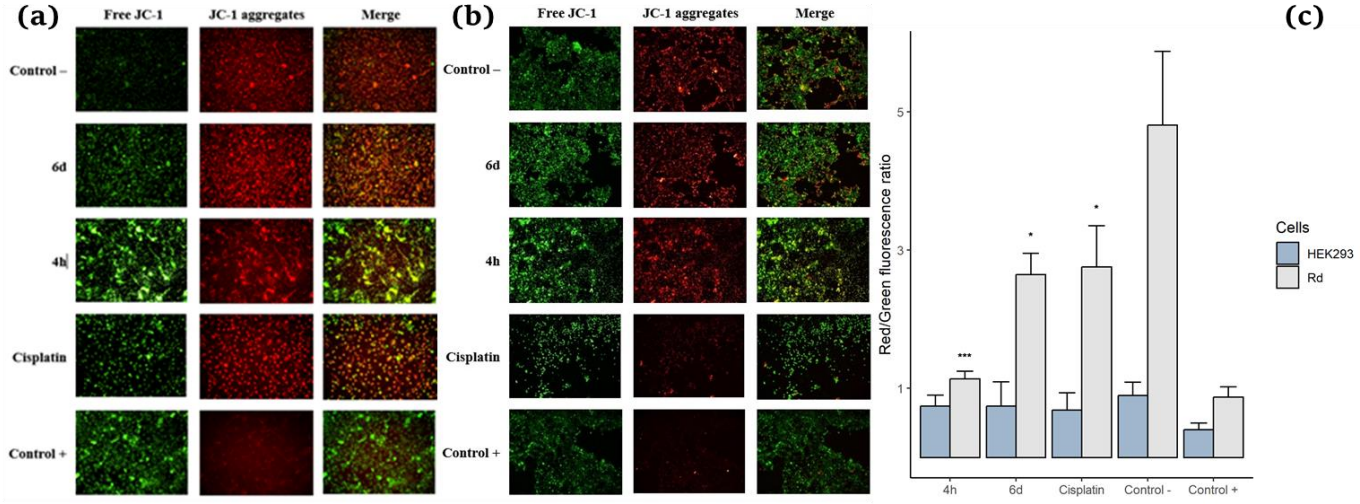

Figure 7 Staining of Rd (a) and HEK293 (b) cell lines with JC-1 dye. "Control -" – intact cells, "Control +" – cells treated with 100 μM CCCP for 30 min before staining, 4h, 6d and "Cisplatin" – cells after incubation with compounds at 64 μM concentration, respectively. JC-1, gives green (in case of decreased ΔΨm of mitochondria) or red (if ΔΨm is normal) fluorescence. Magnification 200x. (c) – ratio of red to green fluorescence on Rd, HEK293 cell lines after 72-h incubation with compound 4h, 6d and cisplatin (bar graph). * $p < 0.05$, *** $p < 0.001$ when compared with intact cells (Control -).

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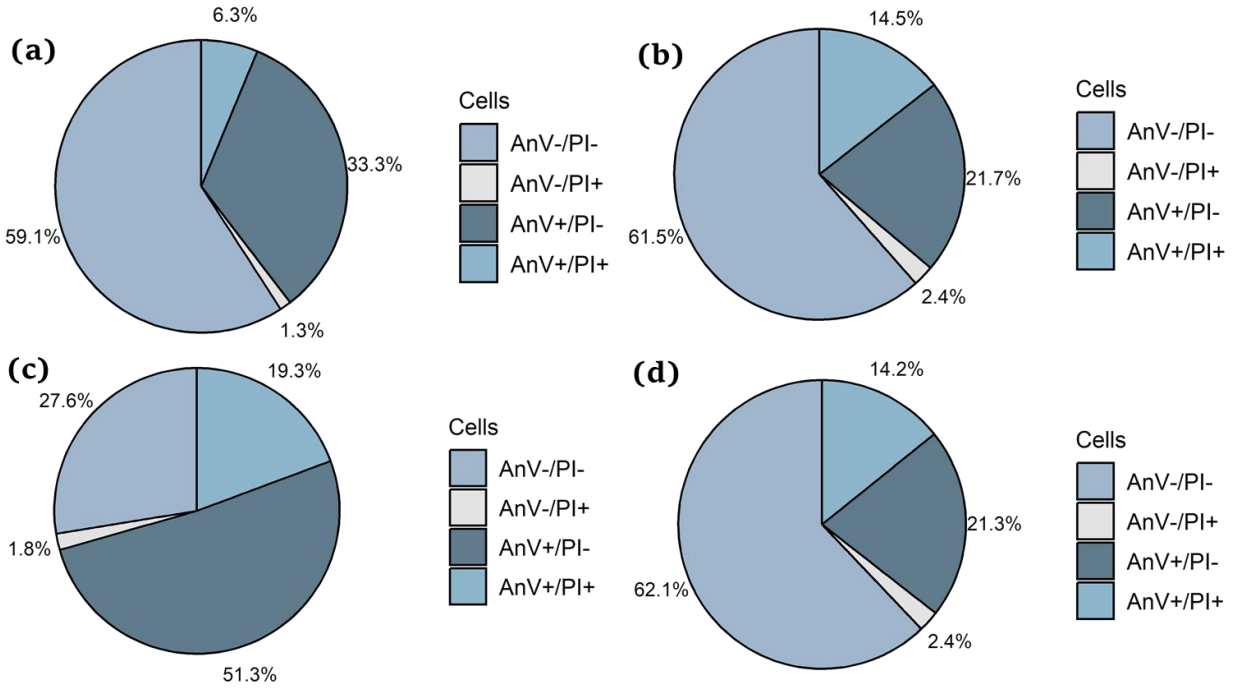

Figure 8 The percentage of cells in apoptosis and necrosis for the Rd and HEK293 lines (pie charts): (a), Rd and HEK293 line, respectively, after 72 h incubation with **4h** at 64 μM concentration (b), line Rd and HEK-293, respectively, after 72 h incubation with cisplatin at 64 μM concentration (c), (d).

Additionally, the early apoptotic, late apoptotic and necrotic, and live cells populations upon treatment by compound **4h** for HEK293 cell line were 21.7%, 14.5%, and 60%, respectively (Figure 8b).

The results of this experiment showed that compound **4h**, as well as cisplatin (Figures 8c and 8d), caused a moderate cytotoxic effect against both cell lines. It is interesting to note that percentages of cell death in the HEK293 line were almost identical under treatment with compound **4h** and cisplatin.

4. Limitations

Compounds **3a-u** have extremely low solubility in water and organic solvents (especially, DMSO), which makes it difficult to determine their bioactivity.

Cytotoxicity studies were limited to only three cancer cell lines, which may lead to speculation about the ineffectiveness of the proposed series of compounds despite the additional studies on their mechanism of action.

For a more complete understanding of the antiproliferative properties of 7-amino-5-aryl-azolo[1,5-*a*]pyrimidine-6-carbonitriles, further studies are needed on cell lines often used in the development of antitumor azoloazines with similar scaffold (for example, HCT116, PC9, Figure 2).

5. Conclusion

Thus, the new series of azolo[1,5-*a*]pyrimidines derivatives was synthesized, characterized, and evaluated for the cytotoxic activities against three human cancer cell lines (A172, Hos, and RD) and normal cell line HEK293. The results revealed that 7-amino-5-phenyl-2-(thiophen-2-yl)-pyrazolo[1,5-*a*]pyrimidine-6-carbonitrile **4h** has selective effect on mitochondrial network and function of RD cell line, caused a decrease in mitochondrial membrane potential and induced apoptotic cell death, without having any effect on cell division and cell membrane integrity.

Among the studied 7-amino-5-aryl-azolo[1,5-*a*]pyrimidine-6-carbonitriles and acylated derivatives, compounds with pronounced cytotoxic activity against rhabdomyosarcoma cells were found. As a result of in-depth studies of the antitumor activity of the compounds against the Rd cell line, it was suggested that the mechanism of their cytotoxic action is associated with a significant decrease in mitochondrial potential in neoplastic cells. The activity of 2-substituted 7-amino-5-aryl-azolo[1,5-*a*]pyrimidine-6-carbonitriles is not associated with a complete block of cell division, but is accompanied by the transition of cells to apoptotic death.

Supplementary materials

This manuscript contains supplementary materials (spectra of compounds), which are available on the corresponding online page.

Data availability statement

The data that supports the findings of this study (e.g. spectra of given compounds) are available in the supplementary materials of this article.

Author contributions

Conceptualization, I.I.B., V.V.M., S.K.K. and V.L.R.;
Formal analysis, V.L.R;
Funding acquisition, S.K.K. and V.L.R.;

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Investigation, I.I.B., D.D.C., O.A.K., E.A.F., M.D.T. and P.A.S.;
Methodology, I.I.B. and V.V.M.;
Project administration, S.K.K. and V.L.R.;
Software, I.I.B., P.A.S.;
Supervision, V.V.M., S.K.K. and V.L.R.;
Validation, I.I.B., V.V.M. and S.K.K.;
Visualization, I.I.B., V.V.M. and P.A.S.;
Writing – original draft, I.I.B., V.V.M., M.D.T. and A.E.C.;
Writing – review & editing, I.I.B., D.D.C., O.A.K. and S.K.K.
The experiments on the analysis of cytostatic activity were performed in the laboratory of primary bioscreening, cellular and gene technologies at UrFU by Vsevolod V. Melekhin, Maria D. Tokhtueva, Andrey A. Zonov.

Conflict of interest

The authors declare no conflict of interests.

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