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ANTIPROLIFERATIVE ACTIVITY OF 2-AROYL- AND 2-HETEROYL-1,1,3,3-TETRACYANOPROP-2-EN-1-IDES

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The influence of previously synthesized 2-aroyl-1,1,3,3-tetracyanoprop-2-en-1-ides on the growth of conditionally normal and tumor cells was studied in continuation of a search for new anticancer drugs. Cytotoxicities of the compounds were studied with respect to human tumor cell lines from the ATCC. All compounds were ineffective against melanoma and lung and ovary cancer cell lines and exhibited moderate activity in the other cases. The tested compounds exhibited highly selective effects because they were safe for conditionally normal skin fibroblasts.

Keywords: 1,1,3,3-tetracyanopropenide, carbonitrile, cancer, antiproliferative activity, MTT assay.

According to the World Health Organization, 18.1 million new cases of cancer and 9.6 million deaths from it were recorded in 2018 [1]. Modern chemotherapy uses drugs that typically have low efficacy and high toxicity. Therefore, the search for new antitumor drugs is a crucial problem of contemporary healthcare.

Polycarbonitrile compounds have been searched by us over many years for new biologically active compounds with antiproliferative activity as potential chemotherapy agents [2 – 5].

In continuation of efforts in this direction, the antiproliferative activity of previously synthesized 2-aroyl- and 2-heteroyl-1,1,3,3-tetracyanopro-2-en-1-ides **1a-j** [6 – 7] was studied.

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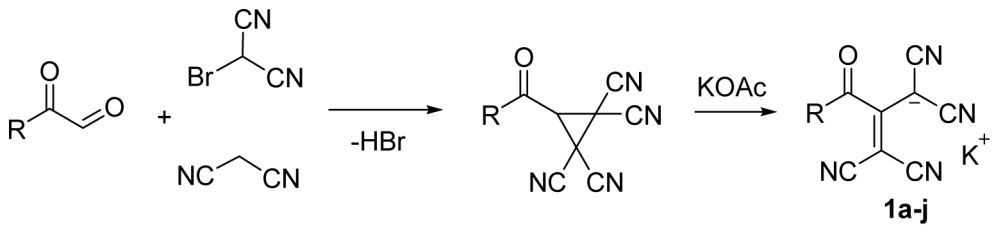
EXPERIMENTAL CHEMICAL PART

The studied compounds were prepared by the previously reported method.

Potassium 2-(4-methylbenzoyl)-1,1,3,3-tetracyanoprop-2-en-1-ide (1a). 4-Methylacetophenone (0.1 mol) and NaBr (5.0 g, 0.048 mol) were dissolved in DMSO (50 mL) in a 150-mL beaker. The mixture was heated carefully to 85°C with stirring and treated quickly with ~5 mL of conc. H_2SO_4. Foam formed because of the generation of Me_2S gas. The reaction temperature also increased. It was important to keep the reaction temperature in the range 100 – 115°C. When the reaction was finished (5 – 7 min), the bubbles of Me_2S rapidly disappeared and the mixture became viscous. The yellowish-orange oil that formed on cooling was dissolved in EtOH (50 mL). The resulting solution was used in



R = 4-$CH_3C_6H_4$ (**1a**), 2-$((CH_3)_2CHOOC)C_6H_4$ (**1b**), 3,4-$(OCH_3)_2C_6H_3$ (**1c**), 4-$NO_2C_6H_4$ (**1d**), 4-$CH_3OC_6H_4$ (**1e**), 2-thienyl (**1f**), 3-ClC_6H_4 (**1g**), 2-furyl (**1h**), 4-BrC_6H_4 (**1i**), Ph (**1j**).

the next step. Br_2 (16.0 g, 0.1 mol) was dissolved in distilled H_2O (600 mL) with stirring. Malononitrile (13.2 g, 0.2 mol) was dissolved in EtOH (50 mL). The mixture was poured into the Br_2 solution. A mixture containing 4-methylphenylglyoxal was added dropwise to the resulting solution with vigorous stirring for 30 min. The resulting white precipitate was filtered off, rinsed with chilled EtOH, and used in the next step without further purification.

3-(4-Methylbenzoyl)-cyclopropane-1,1,2,2-tetracarbonit rile (0.05 mol) was added to a mixture of KOAc (0.07 mol) and EtOH (20 mL) and stirred at 45 – 50°C until the solids dissolved. The resulting dark-yellow solution was filtered, cooled, and poured into KCl solution (10%, 75 mL). The mixture was left for 30 min at 5 – 10°C. The resulting precipitate was filtered off, rinsed with Et_2O, and dried in air [6].

Compounds **1c-j** were prepared analogously.

Potassium 2-[2-(isopropylcarbonyl)benzoyl]-1,1,3,3-tetracyanoprop-2-en-1-ide (1b). Ninhydrin monohydrate (1.96 g, 0.01 mol) was dissolved in DMF (15 mL) with gentle heating (50 – 55°C). The solution was treated with malononitrile (1.32 g, 0.02 mol) and Py (5 drops, ~0.1 g), stirred at 50 – 55°C for 5 – 7 min, and cooled to room temperature. The dark-brown solution was neutralized by adding H_2SO_4 solution (25%), cooled again, treated dropwise with Br_2 (1.6 g, 0.01 mol), stirred at room temperature for 10 min, and poured into distilled H_2O (150 mL). The resulting solid (1′,3′-dioxo-1′,3′-dihydrospiro[cyclopropane-1,2′-indene]-2, 2,3,3-tetracarbonitrile) was filtered off and recrystallized from *i*-PrOH—MeCN (3:1). A suspension of 1′,3′-dioxo-1′,3′-dihydrospiro[cyclopropane-1,2′-indene]-2,2,3,3-tetraca rbonitrile (0.27 g, 0.001 mol) and anhydrous KOAc (0.1 g, 0.001 mol) in *i*-PrOH (1.5 mL) was refluxed until the reaction was finished (TLC monitoring). The obtained yellow solution was quickly filtered and left to cool. The resulting precipitate was filtered off and recrystallized from *i*-PrOH—EtOH (1:1) [7].

EXPERIMENTAL BIOLOGICAL PART

Cytotoxicity of compounds was evaluated using the MTT proliferation assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Promega, USA) [8 – 10]. MTT reagent is converted by mitochondrial dehydrogenases of living cells into insoluble violet-colored formazan. The formazan crystals are readily dissolved by organic solvents such as *i*-PrOH or DMSO upon lysis of the cells. The activity of mitochondrial dehydrogenases and; therefore, the viability of the cells can be determined from the optical density of the formazan solution (500 – 600 nm). [11].

Cytotoxicity of the compounds was studied using human tumor cell lines from the ATCC (MCF7 breast adenocarcinoma; PC-3 prostate adenocarcinoma; A-498 kidney carcinoma; SNB-19 glioblastoma; M-14 melanoma; OVCAR-4 ovary adenocarcinoma; NCI-H322M non-small-cell bronchioloalveolar carcinoma; MDA-MB-231 metastatic breast adenocarcinoma; HCT-116 colon carcinoma) and conditionally normal primary human skin fibroblasts. The investigation included construction of a dose—effect curve and determination of the half-maximum growth inhibition concentration for tumor cells (IC_{50}) and conditionally normal cells (CC_{50}).

Human skin fibroblasts were cultivated in DMEM medium with added fetal bovine serum (10%), L-glutamine (2 mM), penicillin (100 μg/mL), and streptomycin (100 U/mL). Cells were inoculated in a 96-well plate with

TABLE 1. Half-maximum Cell Growth Inhibitory Concentration, Mean ± Standard Deviation

Compound	Cell line									
	MCF-7	HSF	M-14	PC-3	NCI-H322M	SNB-19	A-498	HCT-116	MDA-MB 231	OVCAR
1a	35 ± 1.84	>250 ± 3.54	>250 ± 3.52	19 ± 0.42	192 ± 1.51	22 ± 0.27	28 ± 0.34	37 ± 0.39	12 ± 0.25	>250 ± 3.03
1b	37 ± 1.02	>250 ± 2.09	242 ± 1.67	97 ± 1.01	49 ± 0.62	48 ± 0.32	35 ± 0.31	42 ± 0.51	33 ± 0.32	>250 ± 2.72
1c	25 ± 0.74	>250 ± 2.98	168 ± 1.34	22 ± 0.50	34 ± 0.35	18 ± 0.41	23 ± 0.28	56 ± 0.48	15 ± 0.30	>250 ± 3.11
1d	41 ± 1.35	>250 ± 3.12	191 ± 1.82	31 ± 0.53	29 ± 0.45	39 ± 0.50	22 ± 0.32	46 ± 0.32	31 ± 0.29	211 ± 2.08
1e	48 ± 1.01	>250 ± 3.35	248 ± 1.93	111 ± 0.99	59 ± 0.42	61 ± 0.58	49 ± 0.41	59 ± 0.53	47 ± 0.52	>250 ± 3.24
1f	57 ± 1.22	>250 ± 2.81	189 ± 2.02	35 ± 0.47	57 ± 0.55	46 ± 0.37	37 ± 0.32	78 ± 0.62	34 ± 0.36	>250 ± 3.13
1g	112 ± 0.94	>250 ± 2.66	240 ± 1.12	127 ± 1.12	71 ± 0.67	59 ± 0.45	64 ± 0.54	91 ± 0.38	63 ± 0.54	>250 ± 2.89
1h	36 ± 1.12	>250 ± 3.14	191 ± 1.24	35 ± 0.63	49 ± 0.53	73 ± 0.66	47 ± 0.39	115 ± 0.75	59 ± 0.49	>250 ± 3.10
1i	32 ± 1.31	>250 ± 3.04	178 ± 1.17	27 ± 0.48	36 ± 0.48	66 ± 0.57	101 ± 0.92	128 ± 1.02	44 ± 0.31	>250 ± 3.07
1j	72 ± 1.58	>250 ± 2.94	217 ± 1.55	98 ± 1.05	55 ± 0.52	79 ± 0.71	145 ± 1.07	151 ± 1.30	69 ± 0.63	>250 ± 2.93
Doxo-rubicin	0.13 ± 0.05	0.8 ± 0.01	0.14 ± 0.09	0.1 ± 0.06	0.15 ± 0.07	0.1 ± 0.02	0.1 ± 0.00	0.08 ± 0.03	0.2 ± 0.10	0.15 ± 0.07

3000 cells per well and cultivated in the presence of a phosphonium salt solution (1 μg/mL to 1 mg/mL) for 72 h at 37°C and 5% CO_2 [12]. Grown cells were rinsed with fresh medium and assayed by MTT as usual.

The reference drug was doxorubicin, currently one of the most efficacious and frequently used drugs.

A mixture of growth medium (9 mL) and MTT reagent (1 mL) (5 mg/mL in Hank's solution) was prepared in a reservoir for a multichannel pipette and used to fill a 96-well plate. The prepared reaction mixture (100 μL) was placed into each plate well and incubated in a CO_2 incubator for 3.5 h. Growth medium with the tested compounds was removed carefully with a vacuum aspirator, trying not to loosen the cells. When the incubation time was reached, the growth medium with the reagent was removed with a vacuum aspirator. Each plate well was treated with DMSO (100 μL) and incubated for 5 – 10 min. Violet color that appeared was detected on a Tecan plate reader at 555 nm (650 nm reference wavelength).

Results were processed using OriginPro 8 software. The percent surviving cells in each test well was calculated relative to wells with the positive control, the survival of which was taken as 100%. Next, cell viability expressed in percent was plotted as a function of the decimal logarithm of the concentrations of added compounds. The resulting curve was analyzed using the Fit Sigmoidal – DoseResp program in OriginPro 8. The logarithm of the concentration at 50% cell survival was found. The half-maximum growth inhibitory concentrations of conditionally normal and tumor cells (IC_{50}, μM, Table 1) were calculated.

The biological targets for cyano-containing drugs could be various structures in transformed cells. The target could be DNA molecules themselves and amino-acid residues such as serine and arginine because cyano groups tend to form H-bonds [13]. Blocking of the serine residue causes complete inhibition of enzymatic catalysis (e.g., acetylcholine esterase) and disruption of intercellular signal transduction, which leads eventually to the death of the tumor cells. Blocking the arginine residues disturbs nitrogen metabolism, which also negatively affects cell growth.

All compounds were ineffective against melanoma and lung and ovary cancer (three independent experiments). The other compounds were moderately active. Compound **1c** [potassium 2-(3,4-dimethoxybenzoyl)-1,1,3,3-tetracyanoprop-2-en-1-ide] was more active, possibly because of the larger lipophilicity of the radical. The proposed mechanism explaining the role of the carbonitrile in producing the activity is related to the formation of H-bonds with various amino-acid sequences in the enzymes [13].

An analysis of the results showed that the synthesized compounds, despite the weaker cytotoxicities for tumor cells, had highly selective action because they were safe for conditionally normal skin fibroblasts. This indicated that further searching in this compound class was advisable.

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