BIOPHYSICS AND BIOCHEMOSTRY

Neurotrophic Effects of Polyhydroxylated Steroids and Steroid Glycosides in Cultured Neuroblastoma Cells

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The effects of steroid compounds from Pacific Ocean starfishes were studied using cultured neuroblastoma C-1300 cells. Vital observations and examination of silver-impregnated preparations showed that the test substances in a concentration of 2-10 μM stimulate differentiation and improves survival of neuroblastoma cells under adverse conditions (similarly to neurotrophins). These substances in high concentrations (20-40 μM) had no effect or exhibited cytotoxic activity. The screening test allowed us to select several compounds for further studies of neurotrophic and neuroprotective properties

Key Words: *neuroblastoma; steroids; neurotrophic activity*

Endogenous steroid compounds, including sex hormones and neurosteroids, possess neurotrophic activity and improve survival of neurons in the central nervous system. They are used as protective drugs in the therapy of ischemic and neurodegenerative diseases [9,10]. A variety of reactions to mammalian steroid compounds limit their practical use [4]. Much recent attention is paid to the search for synthetic and natural analogues of these compounds with a narrower range of activity. Previous studies showed that polyhydroxysteroid glycosides from starfishes are capable of inducing differentiation of pheochromocytoma PC12 cells. These compounds are synergistic with nerve growth factor [7,8]. The bank of polyhydroxylated steroids and steroid glycosides from tropic and Far-Eastern starfishes was obtained at the Pacific Ocean Institute of Bioorganic Chemistry. Nine of these compounds were tested for the neurotrophic effect on cultured neuroblastoma C-1300 cells.

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MATERIALS AND METHODS

Neuroblastoma C-1300 cells not differentiating in response to neurogenic stimulation were obtained at the Institute of Human Morphology. Polyhydroxylated steroids were isolated from tropic and Far-Eastern starfishes at the Pacific Ocean Institute of Bioorganic Chemistry [1,2,5,6]. We used asterosaponin P1 (sodium salt (24*S*)-24-*O*-(α-3-*O*-methyl-*L*-arabinofuranoside)-5α-cholestane-3β,6α,8,15α,24-hexaol 5'-*O*-sulfate, PP1), 5α-cholestane-3β,4β,6α,7α,8,15α,16β,26-octaol (PP2) and 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptaol (PP3) from *Patiria pectinifera* starfish, lewisculoside G (3-*O*-(2,4-di-*O*-methyl-β-*D*-xylopyranosyl)-24-*O*-(α-L-arabinofuranosyl)-5α-cholestane-3β,4β,6β,8,1β,24-hexaol, HD1), 5α-cholestane-3β,4β,6β,7α,8,15β,24-hexaol (HD2), henricioside H1 ((24*S*)-3-*O*-(2,4-di-*O*-methyl-β-D-xylopyranosyl)-5α-cholestane-3β,

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4β,6β,8,15α,24-hexaol, HD3) and henricioside H3 ((24*R*, 25*R*)-3-*O*-(2,3-di-*O*-methyl-β-D-xylopyranosyl)-24-methyl-5α-cholest-4-en-3β,6β,8,15α,16β, 26-hexaol, HD4) from *Henricia derjugini* starfish, 24-ethyl-5α-cholestane-3β,5,6β,8,15α,28,29-heptaol 29-sulfate (CC1) from *Ctenodiscus crispatus* starfish, and mediasteroside M1 (24-*O*-[2-*O*-methyl-β-D-xylopyranosyl-(1→5)-α-*L*-arabinofuranosyl]-5α-cholestane-3β,6α,8,15β,24-pentaol, MM1) from *Mediater murrayi* starfish.

Neuroblastoma C-1300 cells were cultured in a CO_2 incubator (Sanyo) at 36°C, 5% CO_2/95% air, and 80% humidity. The culture medium contained 75% DMEM, 25% Iscove medium, 5% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. The medium was replaced at 2-3-day intervals. The media and reagents were from Sigma. For vital studies, the cells were placed in culture plates (5000 cells/ml). After 1 day the culture medium was replaced with a new medium containing 2% FBS and the test substances in concentrations of 2, 5, 10, 20, and 40 μM. The test substances were dissolved in DMSO. The final concentration of DMSO in the culture medium did not exceed 1%. The medium for control samples also contained 2% FBS and 1% DMSO.

The number of differentiated and undifferentiated cells in the medium was estimated daily for 4 days using an inverted microscope. The cells were counted in 4 fields of view (200 cells per field). Differentiated cells had processes with a length of not less than two diameters of the cell. Otherwise, differentiated cells had more than 3 processes.

Histological study was performed with cells layered on coverslips and put in culture plates. The strength of cell adhesion to glass is lower than that to plastic. Therefore, the cells were used in a higher concentration (10,000 cells/ml). Other procedures of culturing were similar to those described above. The test substances were added in a concentration of 10 μM. On day 5 of culturing the samples were fixed with bromoformol for silver impregnation by the method of Bodian. The samples were photographed with a Kodak DC120 digital camera (×64, not less than 10 fields of view). The count of differentiated neurons, total number of cells, mean length of processes, and number of processes and bifurcations per cell were estimated in the images (Image Tool software). The results were analyzed by means of MS Excel and Origin PRO 7.5 software. Statistical treatment involved Student's *t* test.

RESULTS

The culture of neuroblastoma is widely used to study neurotrophic properties of bioactive substances [5,11]. Vital study of the growth and development of neuroblastoma cells in the presence of the test substances showed that starfish polyhydroxysteroids and steroid glycosides in concentrations of 2-10 μM increased the number of differentiating cells. These substances in the same concentration improved survival and stimulated cell differentiation under conditions of infrequent medium replacement and high density of cells. The test substances in a higher concentration (20-40 μM) had no effect or exhibited toxic activity. Cell death was observed on day 3 after treatment with HD2, HD3, and HD1. The influence of these substances was observed starting from the 2nd day of culturing. We revealed changes in the number of differentiated cells and increase in the length and degree of branching of processes. These parameters were maximum on day 4 (Table 1).

Study of silver-impregnated preparations from neuroblastoma C-1300 cells showed that the test

TABLE 1. Number of Differentiated Neurons (% of total cell number) in Neuroblastoma C-1300 on Day 4 of Culturing with Test Substances in Various Concentrations (*M*±*m*)

Substance	2 μM	5 μM	10 μM	20 μM	40 μM
PP1	60.7±2.7*	60.5±4.5*	66.1±5.1*	46.5±17.7	40.0±17
PP2	58.4±8.7*	66.2±5.4*	62.7±7.9*	52.1±5.2*	32.7±7.8
PP3	72.0±8.8*	69.0±7.4*	64.0±3.9*	61.8±4.1*	37.6±12.2
MM1	69.4±2.8*	68.5±3.5*	66.2±1.3*	67.7±3.8*	41.3±5.7
CC1	69.3±2.7*	64.7±4.5*	67.2±5.6*	58.6±14.1*	53.5±5.5*
HD1	60.0±4.6*	62.0±4.6*	53.8±6.4*	25.0±9.0	0
HD2	66.8±8.1*	74.4±2.7*	47.0±13.0	10.4±5.9	0
HD3	65.1±2.7*	50.9±9.1*	40.7±8.9	19.6±1.7	0
HD4	56.3±4.4*	61.5±10.2*	59.8±3.8*	34.6±11.3	15.1±9.7

Note. Control, 35.51±2.17. *$p<0.05$ compared to the control.

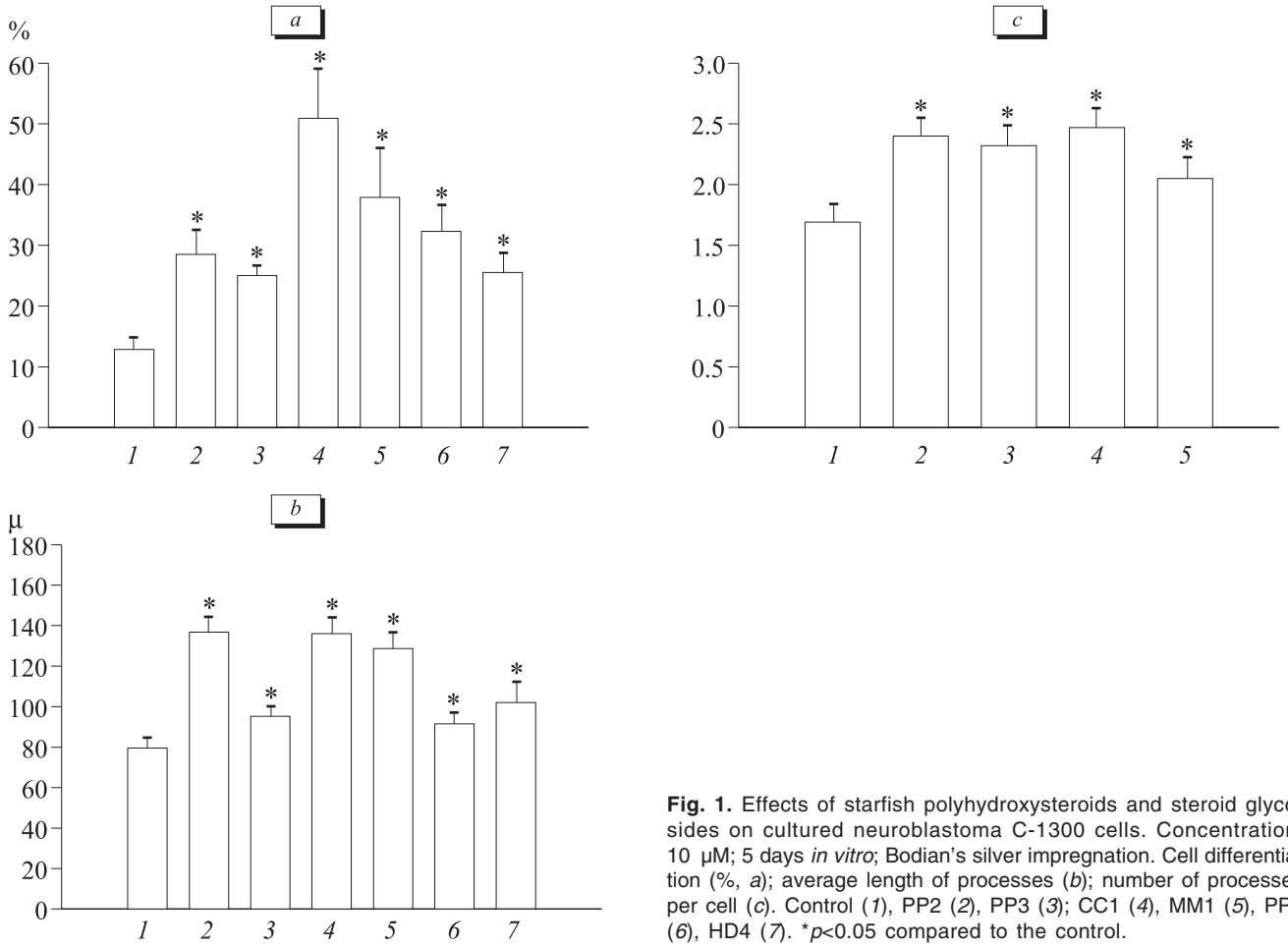

Fig. 1. Effects of starfish polyhydroxysteroids and steroid glycosides on cultured neuroblastoma C-1300 cells. Concentration, 10 μM; 5 days *in vitro*; Bodian's silver impregnation. Cell differentiation (%, *a*); average length of processes (*b*); number of processes per cell (*c*). Control (*1*), PP2 (*2*), PP3 (*3*); CC1 (*4*), MM1 (*5*), PP1 (*6*), HD4 (*7*). **p*<0.05 compared to the control.

substances increased the number of differentiated neurons (Fig. 1, *a*). Axons of cultured cells growing in the presence of steroid compounds were longer than in control samples (Fig. 1, *b*). CC1, MM1, and PP2 increased the mean number of cell processes by 2.4, 2.32, and 2.47 times, respectively, compared to the control (1.69; Fig. 1, *c*). These changes are consistent with visual formation of neuronal networks (Fig. 2). The increase in argentophilia of processes reflects maturation of the neurofibrillary apparatus. Cell density significantly decreased during culturing on coverslips with HD2, HD3, and HD1, which reflects decreased adhesive properties of cultured cells. However, the density of neuroblastoma cells cultured with CC1, MM1, PP2, and PP3 significantly exceeded the control.

Neurotrophic activity of the test compounds substances observed in our experiments is consisted with published data that polyhydroxysteroid glycosides from starfishes stimulate differentiation of pheochromocytoma PC12 cells. High activity was typical of not only glycosides, but also polyhydroxysteroids. It cannot be excluded that glycosidases of the test cells can convert glycosides into polyhydroxysteroids (active metabolites). Neurotrophic and neuroprotective properties of the test substances should be evaluated in further studies. CC1, MM1, PP2, and PP3 are of particular interest in this respect. These substances have a strong neurotrophic effect and do not exhibit toxic activity. It is necessary to evaluate whether activity of these natural compounds depends on their structural characteristics. The test substances hold promise to synthesize new medicinal preparations.

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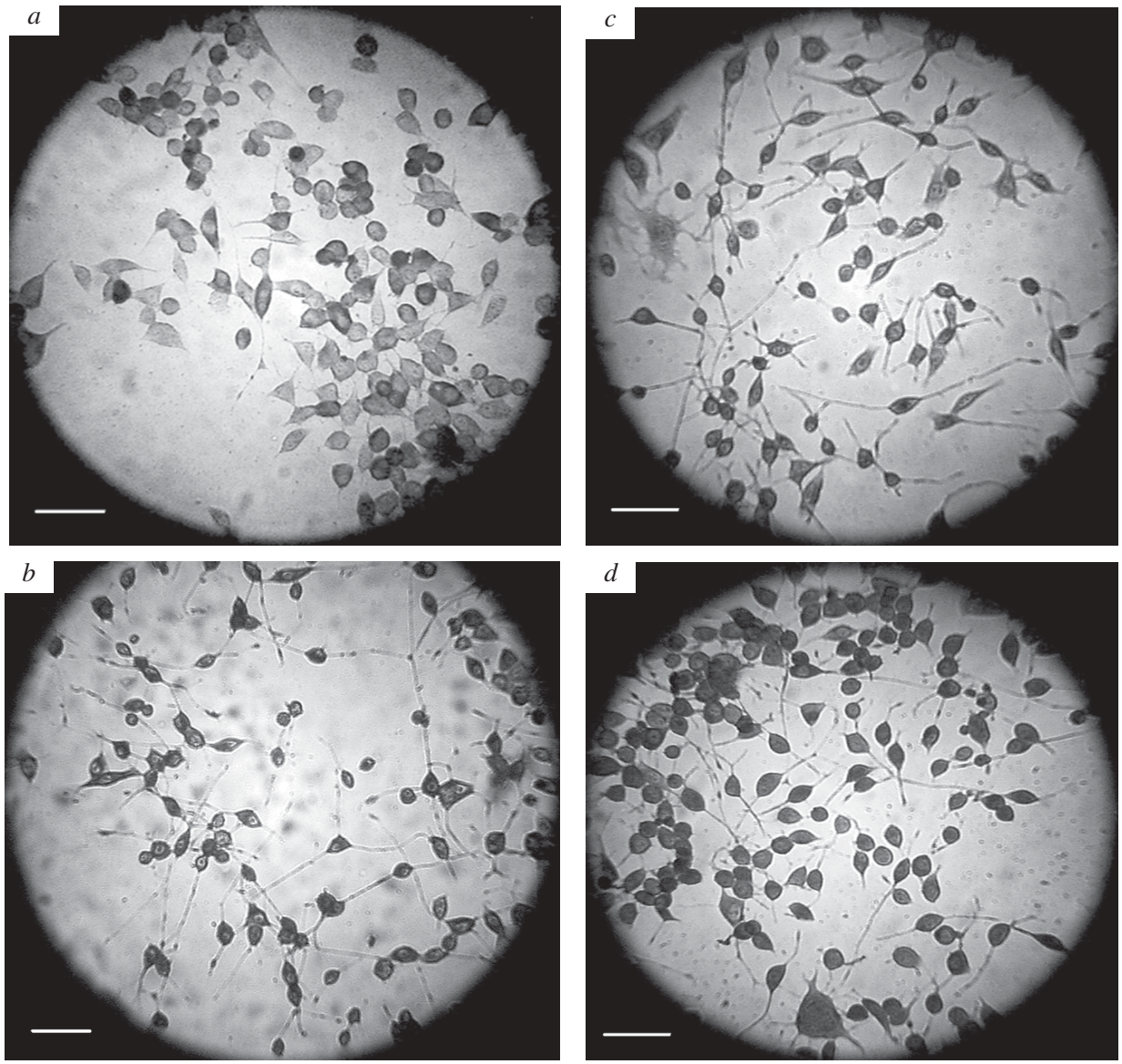

Fig. 2. Cultured neuroblastoma C-1300 cells under control conditions and after treatment with CC1, MM1, and PP2 in a concentration of 10 μM (5 days *in vitro*, Bodian's silver impregnation). Control (*a*), PP2 (*b*), MM1 (*c*), CC1 (*d*). Scale 100 μ.

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