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Antiproliferative effects of some novel synthetic solanidine analogs on HL-60 human leukemia cells *in vitro*

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ABSTRACT

There is increasing evidence of the direct antiproliferative effects of various steroidal structures, including cardenolides, steroidal alkaloids and sexual hormones. The aim of the present study was to characterize the antiproliferative effects of three synthetic solanidine analogs (**1**–**3**) on HL-60 human leukemia cells.

The three compounds exerted similar cytostatic effects (IC_{50} values: 1.27–2.94 μM after a 72-h exposure) and the most effective (**2**) was selected for further investigations. Incubation with compound **2** resulted in a marked chromatin condensation followed by a gradual increase in cell membrane permeability detected by Hoechst dye 33258–propidium iodide double staining. A flow cytometric analysis revealed a marked decrease in the G1 phase and substantial increases in the S and G2/M phases after 24-h incubation, while after 48 h the proportion of cells in the subG1 phase was increased significantly with a concomitant decrease in cells in the G1 and G2/M phases. Compound **2** at 6.0 μM significantly decreased the activity of ribonucleotide reductase and proved to be a potent antioxidant in the lipid peroxidation and DPPH assays (IC_{50} values: 2.0 and 13.1 μM, respectively). The antiproliferative effect of the test compound on the non-cancerous human lung fibroblast cell line (MRC-5) was significantly weaker than that on the leukemia cells. These results lead to the conclusion that compound **2** induces a marked disturbance in the cell cycle, which is, at least partially, a consequence of the inhibition of DNA synthesis.

1. Introduction

Plants are among the most varied and promising sources of new anticancer agents: during the biosynthetic processes in plants, enzymes differing significantly from those in animals and humans catalyze the production of unique compounds which have long been applied in traditional medicine worldwide for the treatment of tumorous diseases [1]. Numerous results have recently been published on the antiproliferative effects of molecules with steroidal structures. Steroidal alkaloids are found in several species belonging in the *Solanum*, *Dioscorea* or *Lycopersicon*, etc. genera. They are usually isolated in the form of glycoalkaloids, but the pharmacological activities of the original aglycones are also investigated. One of the most studied plant steroids is diosgenin, which exerts antiproliferative effects on many types of cancer cell lines, e.g. HER2-overexpressing breast cancer cells [2], human leukemia K562 cells [3], HEL cells [4], HT-29 human colon cancer cells [5], human osteosarcoma 1547 cells [6] and melanoma M4Beu cells [7]. The mechanism for the induction of apoptosis by diosgenin in the different cell lines includes the suppression of fatty acid synthase expression and the modulation of Akt, mTOR and JNK phosphorylation [2], the disruption of Ca^{2+} homeostasis [8], the inhibition of NFκB-regulated gene expressions [9], the activation of p53 and the modulation of caspase-3 activity [7]. Two other members of the spirosolane series are solasodine and tomatidine, both of which exhibit significant inhibitory effects on HeLa, MCF-7 and HT-29 cells by arresting the cell cycle in the G_0/G_1 phase [10]. However, their noteworthy apoptosis-inducing effects were not revealed by flow cytometry. Moreover, a double-blind, randomized, placebo-controlled, multicenter study demonstrated that a cream containing solasodine glycosides was effective in the therapy of basal cell carcinoma, with a cure rate of 66% at 8 weeks and 78% at the 1-year follow-up [11]. Tomatidine was found to be able to decrease multidrug resistance in carcinoma cell cultures by inhibiting the drug removal function of P-glycoprotein (ATP-binding cassette B1) in the cell membrane [12].

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Numerous pharmacological effects of the structurally different, pyrrolidine ring-containing solanidine have already been described, including its acetylcholinesterase-inhibitory activity [13], its ability to impair the cell membrane integrity by complexing membrane 3β-hydroxysterols [14], its cytotoxic properties [15] and its chemosenzitizing activity [12]. In view of these data, the solanidine skeleton can be regarded as a basis for the design of new potential anticancer agents. Two of our currently tested molecules are modified in ring E by containing an additional N-heteroatom (i.e. this is a pyrazoline ring instead of a pyrrolidine ring), and there is a lack of ring F as compared with solanidine. Moreover, ring E is substituted with different phenolic functional groups. One of our compounds is not a pyrazoline, but a pyrazolidine derivative, with an ester function group at position 3. These compounds were previously found antiproliferative against human adherent cell lines, but no data have been published on the mechanism of the effect [16].

The aim of the present investigation was to characterize the mechanisms of action of these compounds on HL-60 human leukemia cells. The compound found to be most effective in the cell growth-inhibition assays was subjected to double staining with Hoechst dye 33258 and propidium iodide (PI) and flow cytometric analysis. To analyze the effect of this test substance on the activity of DNA synthesis, a ribonucleotide reductase assay was performed. The antioxidant activities of the compounds were tested with the lipid peroxidation and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays. The selectivity of its cytotoxic activity was characterized by determining its antiproliferative effect against the MRC-5 human fetal lung fibroblast cell line.

2. Materials and methods

2.1. Chemicals

Androstene-fused phenylpyrazoline (**1**), 4-methoxyphenylpyrazoline (**2**) and 2′,4′-dinitrophenylpyrazolidine (**3**) derivatives were synthetized as described previously [17]. The chemical structures of the tested compounds are presented in Fig. 1. All other chemicals, if otherwise not specified, were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary, or Vienna, Austria).

2.2. Cell culture

The HL-60 human promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO_2. The non-cancerous MRC-5 human lung fibroblast cell line was purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK). Cells were cultivated in minimum essential medium supplemented with 10% FBS, 1% non-essential amino acids and an antibiotic–antimycotic mixture under general conditions. All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). Cell counts were determined with a CC-108 microcellcounter (Sysmex, Kobe, Japan). Cells in the logarithmic phase of growth were used in all the studies described below.

2.3. Antiproliferative assay

HL-60 cells (0.1×10^6 per mL) were seeded in 25-cm^2 tissue culture flasks and incubated with increasing concentrations of the three compounds at 37 °C under cell culture conditions. Cell counts and IC_{50} values were determined after 24, 48 and 72 h, using the CC-108 microcellcounter. The viability of cells was determined by means of trypan blue staining. Results were calculated as numbers of viable cells. The subsequent analyses were carried out only with the most effective compound.

Near-confluent MRC-5 human lung fibroblast cells were seeded into a 96-well plate at a density of 5000 cells/well. After an overnight incubation, the medium containing the test compound was added in six different concentrations, from 0.1 μM to 30 μM. After a 72-h incubation, cytotoxicity was assayed by the addition of 20 μL of 5 mg/mL MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) solution [18]. After a 4-h contact period, the medium was removed and the precipitated formazan was dissolved in 100 μL of dimethylsulfoxide (DMSO) during a 60-min shaking at 25 °C. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls. The highest DMSO concentration of the medium (0.3%) did not have any substantial effect on the cell proliferation.

Sigmoidal dose–response curves were fitted to the measured points, and the IC_{50} values were calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

2.4. Hoechst dye 33258–propidium iodide double staining

Hoechst staining was performed as described earlier [19]. HL-60 cells (0.4×10^6 per mL) were seeded in 25-cm^2 tissue culture flasks and exposed to increasing concentrations of compound **2** for 8, 24 or 48 h. Hoechst 33258 and PI were added directly to the cells to final concentrations of 5 and 2 μg/mL, respectively. After incubation for 60 min at 37 °C, cells were examined under a Leica DMR XA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters for Hoechst 33258 and PI. This method allows a distinction between early apoptosis, late apoptosis and necrosis. Cells were judged according to their morphology and the integrity of their membranes, which can easily be seen after PI staining. Cells were counted under the microscope and the number of apoptotic cells was given as a percentage.

2.5. Analysis of apoptosis and cell cycle by flow cytometry

For the measurement of cellular DNA content, flow cytometric analysis was performed: 24 and 48 h after treatment, HL-60 cells (10^6 cells/condition) were harvested, washed twice with cold PBS and fixed in 2.0 mL of cold 70% ethanol for 30 min on ice. After two washing steps in cold phosphate-buffered saline (PBS), RNAse A and PI were added to final concentrations of 50 μg/mL and 12.5 μg/mL, respectively, and the mixture was incubated for 60 min at room temperature. Cells were analyzed on a FACStar (Beckton-Dickinson, Mountain View, CA, USA). In each analysis, 20,000 events were recorded, and the percentages of the cells in the different cell cycle phases (subG1, G1, S and G2/M) were determined by using winMDI2.9. The subG1 fraction was regarded as the apoptotic cell population [20].

2.6. Determination of ribonucleotide reductase in situ activity

To analyze the effect of incubation with the solanidine analog on the activity of DNA synthesis, an assay was performed as described previously [21]. Logarithmically growing cells were seeded in 25-cm^2 tissue culture flasks (5×10^6 cells) and grown overnight under cell culture conditions. Subsequently, the cells were incubated with various concentrations of the test compound for 24 h. After incubation, the cells were counted, adjusted to 5×10^6 cells and pulsed with [^{14}C]cytidine (0.3125 μCi, 5 nM) for 30 min at 37 °C. They were subsequently collected by centrifugation and washed with PBS. Total DNA was extracted from 5×10^6 cells and the specific radioactivity of the samples was determined with a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA, USA).