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Effect of new olivacine derivatives on p53 protein level

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Abstract

Background The p53 protein is a transcription factor for many genes, including genes involved in inhibiting cell proliferation and inducing apoptosis in genotoxically damaged and tumor-transformed cells. In more than 55% of cases of human cancers, loss of the essential function of p53 protein is found. In numerous reports, it has been shown that small molecules (chemical compounds) can restore the suppressor function of the mutant p53 protein in tumor cells. The aim of this study was to evaluate the potential anticancer activity of three newly synthesized olivacine derivatives.

Methods The study was performed using two cell lines—CCRF/CEM (containing the mutant p53 protein) and A549 (containing a non-mutant, wild-type p53 protein). The cells were incubated with olivacine derivatives for 18 h and then assays were carried out: measurement of the amount of p53 and p21 proteins, detection of apoptosis, cell cycle analysis, and rhodamine 123 accumulation assay (evaluation of P-glycoprotein inhibition). Multiple-criteria decision analysis was used to compare the anticancer activity of the tested compounds.

Results Each tested compound caused the reconstitution of suppressor activity of the p53 protein in cells with the mutant protein. In addition, one of the compounds showed significant antitumor activity in both wild-type and mutant cells. For all compounds, a stronger effect on the level of the p53 protein was observed than for the reference compound—ellipticine.

Conclusions The observed effects of the tested new olivacine derivatives (pyridocarbazoles) suggest that they are good candidates for new anticancer drugs.

Keywords Anticancer drug · p53 · Olivacine · Ellipticine · Pyridocarbazole

Introduction

In experimental oncology, attempts have been made to reconstruct the structure and function of the p53 protein for several years. This direction of research is promising because it gives hope for the development of new therapeutic strategies increasing the effectiveness of cancer therapies. The p53 protein is a transcription factor for many genes, including genes involved in inhibiting cell proliferation and triggering apoptosis in genotoxically damaged and tumor-transformed cells.

The main reasons for the loss of p53 function are point mutations in the TP53 gene. Mutations lead to substitution of amino acids in sections of p53 protein that directly bind to DNA or to exchange amino acids in fragments determining the maintenance of the spatial conformation of p53. The result of conformational mutations is the partial unfolding or aberrant folding of the p53 protein. As a result, these mutations make the protein more susceptible to attack by proteases, and inhibit protein binding to DNA and its physiological function as a transcription factor [1]. The reason for the loss of p53 function may also be post-translational modifications, including the processes of phosphorylation and acetylation as well as the proteolytic degradation process.

In normal cells, the p53 protein is present in very small amounts due to its rapid degradation in the ubiquitin–proteasome proteolytic system. The main regulator of p53 levels in normal cells is the MDM2 protein, which binds specifically to p53 and starts its ubiquitination (MDM2 has E3 ubiquitin ligase activity) [2]. The cellular level of p53 is regulated by negative feedback because the p53 protein stimulates transcription of the MDM2 gene. Abnormal p53 protein loses the ability to stimulate gene transcription for

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cell cycle regulation proteins and to induce apoptosis as well as to induce transcription of its negative regulator—MDM2 protein. A typical feature of most tumor cells is the significant increase in the content of abnormal p53, which does not function as a transcription factor (reduction of proteasomal degradation of p53 due to MDM2 deficiency) [2].

Reconstitution of the p53 function as a transcription factor should lead to inhibition of the cell cycle (induction of p21$^{\text{Cip1/Waf1}}$ transcription) and increased apoptosis of tumor cells [3–5]. In addition, this should increase the sensitivity of tumor cells to cytostatic therapy, because one of the causes of cancer insensitivity to cytostatics is the lack of signals inducing apoptosis from the abnormal p53 protein [4].

Attempts to reconstitute the p53 function include genetic engineering methods (inserting the normal TP53 cDNA into the tumor cells, most often with the adenoviral vector) and immunological methods (e.g., the use of antibodies binding to the C-terminal domain of p53 and forcing the conformation of this protein to near-native) [4]. In recent years, numerous in vitro experiments have been conducted with small molecules (chemical compounds) that bind to mutant p53 and are supposed to lead to the restoration of the proper function of the protein by changing the conformation. These compounds can also prevent improper folding of newly formed p53 protein (activity similar to molecular chaperones) by blocking ubiquitination sites in native p53 (stabilization of normal p53 prevents proteasomal degradation) [2, 4].

Ellipticine, olivacine, and numerous related pyridocarbazoles are small molecules with a proven effect restoring the function of p53 [2–5]. In cultures of tumor cells incubated with 9-hydroxy-ellipticine there was observed a significant increase in transcription of p21$^{\text{Cip1/Waf1}}$ and *Bax*, strong inhibition of cell proliferation, and increased apoptosis [2]. Detailed molecular analysis showed that the interactions with ellipticine were limited to the p53 protein with an incorrect conformation, while no such interactions with the native protein were found, both in normal cells and in tumor cells without the p53 mutation [2]. This very important observation indicates high selectivity of interactions between ellipticine and abnormal p53.

The subject of the study was the effect of olivacine derivatives on the p53 protein. The mechanism of action and antineoplastic properties of olivacine are similar to ellipticine and rely on direct interaction—DNA intercalation and inhibition of topoisomerase II (topo II) activity [6, 7]. It should be emphasized that some olivacine derivatives (e.g. compound S16020) exhibited a broad spectrum of anticancer activity, even more powerful than ellipticine and doxorubicin [8, 9].

Materials and methods

Tested compounds

The anticancer properties of three new olivacine derivatives were investigated. These compounds were synthesized in the Department of Organic Chemistry of Wroclaw Medical University. The compounds were presented in an earlier publication, where methods of synthesis and their anticancer potential are described [10]. This paper presents results for only three of the previously synthesized substances (numbered 12, 9 and 16) that showed the desired activity (their structural formulas are presented in Fig. 1c–e). As the reference compound from the group of pyridocarbazoles, ellipticine was used (Fig. 1a). Olivacine is difficult to purchase and therefore its isomer was used. Ellipticine has a similar chemical structure to olivacine (Fig. 1b) and shows similar activity.

The cytotoxicity of the tested compounds was assessed on BALB/3T3 fibroblast cells. Based on the results obtained after 18 h of incubation with the compounds, the concentrations at which 50% of the cells showed necrosis (TD_{50} values) were calculated. TD_{50} values were greater than 25 μM for all tested compounds. In our work a concentration range of up to 10 μM was chosen, i.e. non-toxic for normal cells [10]. The same work presents the cytotoxicity results of the tested compounds for cell lines used in this study. The evaluation of the effects of the tested compounds after 18 h incubation with the A549 and CCRF/CEM cells was performed using the SRB assay. Based on the results of the assay, IC_{50} values (half maximal inhibitory concentrations) were calculated. In the CCRF/CEM cell line, Compound 1 ($IC_{50} = 9.446 \pm 1.071$ μM) and Compound 3 ($IC_{50} = 6.169 \pm 1.965$ μM) showed greater inhibitory activity than ellipticine ($IC_{50} = 13.586 \pm 3.446$ μM). For Compound 2 ($IC_{50} = 13.681 \pm 0.771$ μM), a similar effect was observed as for ellipticine. In the A549 cell cultures, ellipticine activity was not observed in the SRB assay, while the tested compounds showed inhibitory activity (IC_{50} values: 10.665 ± 0.610 μM, 18.789 ± 1.032 μM, 10.391 ± 1.064 μM, respectively for Compounds 1–3).

Cell lines

The anticancer activity of the compounds was assessed on two different cell lines: acute human lymphoblastic leukemia cell line CCRF/CEM (ATCC: CCL-119; suspension cell line) and human lung adenocarcinoma A549 cell line (ATCC: CCL-185; adherent cell line). Both cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

Fig. 1 Structural formulas of tested and control substances: **a** ellipticine; **b** olivacine; **c** Compound 1; **d** Compound 2; **e** Compound 3
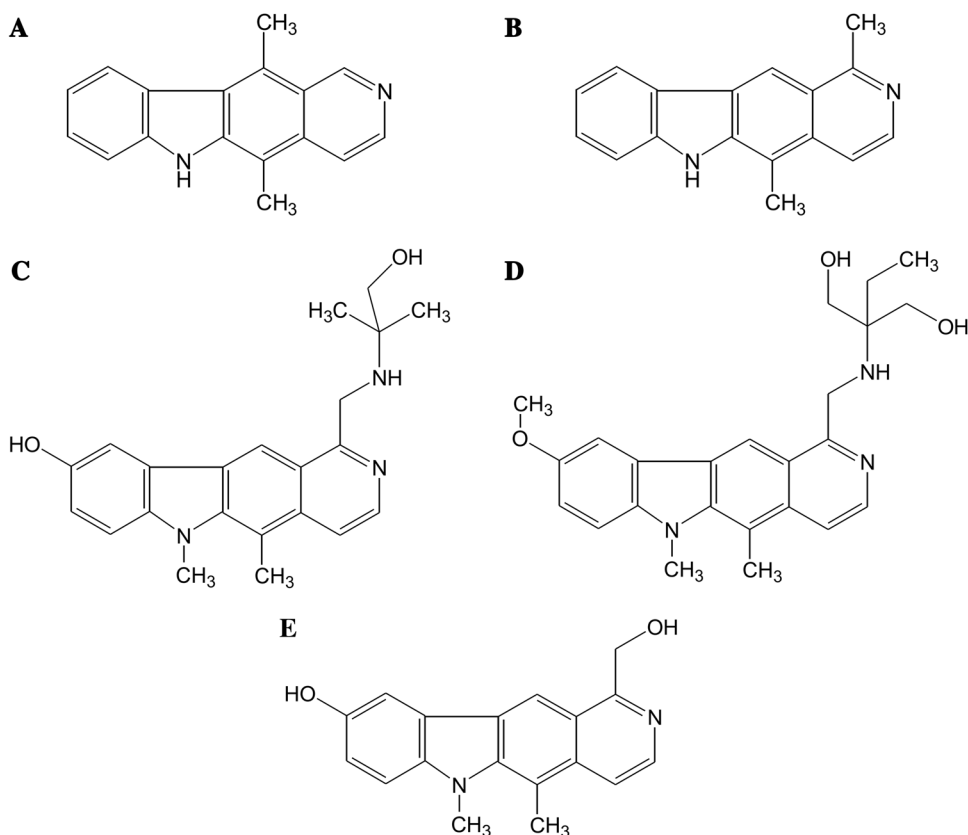
The cells were grown at 37 °C, 5% CO_2 in a humidified atmosphere in complete culture medium. The cells were passaged twice a week (in the case of the adherent line with trypsin–EDTA solution). The excess cells were used for compound testing or removed. Before assays, the adherent cells were suspended in MEM to neutralize the trypsin–EDTA, while the suspension cells were taken directly from the bottles. The cells were then centrifuged at 600 g for 10 min and resuspended in complete medium.

In the experiments 24-well or 96-well plates were used. In the 24-well plates, CCRF/CEM cells were seeded at a density of 5×10^4 cells/well and A549 cells at a density of 2×10^4 cells/well. Assays on 96-well plates were performed with CCRF/CEM cells seeded at a density of 1×10^4 cells/well and A549 at a density of 2×10^3 cells/well.

Culture medium

The culture medium recommended for a given cell type was used. The CCRF/CEM line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 200 mM L–glutamine, 10,000 units/ml penicillin, and 10 mg/ml streptomycin. For the A549 cell line, Eagle's Minimum Essential Medium (MEM) supplemented as above was used. The complete media thus prepared were stored at 2–8 °C for up to 2 weeks.

Detection of apoptosis

Cells were cultured in 24-well plates for 48 h. After this time the cells of the adherent line completely covered the surface of the wells. The tested compounds at concentrations of 0.1, 0.2, 0.5, and 1.0 µM were added for 4 or 18 h.

In the case of the adherent A549 cell line, medium from each well was harvested into appropriate pre-prepared centrifuge tubes (supernatant may contain dead and apoptotic cells). All wells were then rinsed with trypsin–EDTA solution (to neutralize the serum) and harvested suspension collected into appropriate tubes. In the next step, the cells were treated with trypsin–EDTA solution for 2 min and again collected into tubes.

For both cell lines, after harvesting the cells, the tubes were centrifuged at 600 g for 10 min. The cell pellet was resuspended in 100 µl of HEPES–NaOH buffer at pH 7.5, and then the mixture of fluorochromes Annexin V-FITC and propidium iodide was added and left in the dark for 10 min. The preparations were analyzed in the image-based cytometer Arthur (NanoEnTek Inc.).

Measuring the amount of p53 and p21 proteins

The measurement of the content of p53 and p21 proteins in cell lysates was made using the Roche p53 pan ELISA Kit

(Cat. No. 11 828 789 001) and Abcam Human p21 ELISA Kit (Cat. No. ab214658). According to the manufacturer's instructions the cell lysates and selective antibodies directed against p53 or p21 were added to antibody-coated wells. Tetramethylbenzidine (TMB) was used in the ELISA assay as a colorimetric substrate for horseradish peroxidase enzyme (HRP) conjugated with antibody. The absorbance was measured in a Victor2 microplate reader (PerkinElmer) at 450 nm and the levels of p53 and p21 proteins were calculated. The total amount of protein in the cell lysates was measured by the Bradford method using the Protein Quantification Kit-Rapid (Fluka). The final test results were expressed as nanograms of p53 or p21 per milligram of total protein content.

The ELISA experiments were performed for the tested compounds at concentrations of 0.2, 0.5, and 1.0 μM, and in the case of the A549 cell line also at concentrations of 5.0 and 10.0 μM.

Analysis of the cell cycle in flow cytometry

FITC BrdU Flow Kit (Becton, Dickinson and Company) was used to analyze the cell cycle. The cells were fixed, permeabilized and incubated with a fluorescently labeled antibody (FITC) directed against BrdU to determine the amount of BrdU incorporated into the cell DNA. BrdU (5-bromo-2′-deoxyuridine) is a thymidine nucleoside analogue that is incorporated into DNA during the S phase of the cell cycle. Simultaneously DNA staining with 7-aminoactinomycin D (7-AAD) binding to total DNA makes it possible to characterize the population of cells actively synthesizing DNA and their position in the cell cycle (G0/G1, S, G2/M).

The assay was performed using the CyFlow Space flow cytometer (Partec Germany). Analysis of the cell cycle was carried out with an incubation time of 18 h on the CCRF/CEM cell line with tested compounds at concentrations of 0.1, 0.5, 1.0, and 5.0 μM; and on the A549 cells with compounds at concentrations of 1.0, 5.0 and 10.0 μM.

Accumulation of rhodamine 123 (Rh-123)

Rhodamine 123 (Rh-123) accumulation assay was carried out to evaluate the effect of tested compounds on the transport functions of P-glycoprotein (P-gp).

After incubation of cells with tested compounds in 96-well plates for 18 h, the Rh-123 solution was added to a final concentration of 12.5 μM and incubated for 60 min. After this time, plates were shaken at 500 g for 10 min and the supernatant was removed. The cells were dissolved in 20 mM Tris–HCl (pH 7.7) containing 0.2% sodium dodecyl sulfate (SDS) to lyse the cells and release the intracellular fluorescent substrate. Fluorescence measurement was performed using a Victor2 microplate reader (ex. 485 nm/em.

538 nm). The compounds were tested at concentrations of 0.2, 0.5, 1.0, 5.0 and 10.0 μM for 18 h.

Statistical analysis

All test results were obtained in five independent experiments. Each ELISA experiment had three independent replicates, while the remaining tests had five replications.

Results of the evaluation of the effect of tested compounds on proliferation of A549 and CCRF/CEM cells were referred to the results for control cultures (without tested compounds) (E/E_0). Standard statistical methods were used to assess the significance of the obtained results. The analyses were carried out using non-parametric tests due to the lack of normal distribution of results.

A collective assessment of the p53-induced antitumor activity of the tested compounds for all performed assays was carried out using multiple-criteria decision analysis (MCDA). The MCDA was performed according to literature procedures [11–13]. The assay results were calculated according to the formula: $b = (exp-obs)^2/exp$ (exp—expected value; obs—observed value). The highest results (potentially anticancer) were considered as expected values. The result of the weighted sum determined as $MCDA = \Sigma\ 1/b \times weight$ gave the final result of the MCDA analysis for each tested compound. The weight of each assay was arbitrary and reflects the potential clinical relevance for anticancer activity.

Correlation coefficients were calculated using a nonparametric measure of rank correlation—Spearman's rank correlation coefficient.

Results

Detection of apoptosis

One of the goals of restoring the function of mutant p53 is to increase apoptosis. Calculated concentrations of tested compounds that cause an increase in the frequency of apoptosis by 50% after 18 h are shown in Tables 1 and 2, for CCRF/CEM and A549 cell lines respectively. The results for ellipticine are not shown due to its strong green fluorescence (in low concentrations) interfering with the emission of FITC.

The obtained results confirm the pro-apoptotic effect of all three tested compounds in cultures of CCRF/CEM cells (Table 1, Fig. 2a). After 18 h of incubation, the highest cytotoxic and proapoptotic effect was shown by Compound 3.

For the A549 cell line, after 18 h of incubation with each tested compound, a significant increase in the level of apoptotic cells was also found (Table 2, Fig. 2b). In the case of the A549 line (with wild-type p53) the effect was slightly weaker than for the CCRF/CEM line (with mutant p53).

Table 1 Calculated concentrations of tested compounds that cause an increase in the frequency of apoptosis by 50% in CCRF/CEM cells after 18 h; statistical significance compared to doxorubicin 1 μM (AC_{50} is the calculated concentration at which 50% of the cells are apoptotic)

Substance	AC_{50} (SD) [μM]	p
Compound 1	0.442 (0.062)	1.14×10^{-5}
Compound 2	0.520 (0.185)	5.64×10^{-5}
Compound 3	0.359 (0.109)	0.0003
Doxorubicin	1.005 (0.241)	–

Table 2 Calculated concentrations of tested compounds that cause an increase in the frequency of apoptosis by 50% in A549 cells after 18 h; statistical significance compared to doxorubicin 1 μM (AC_{50} is the calculated concentration at which 50% of the cells are apoptotic)

Substance	AC_{50} (SD) [μM]	p
Compound 1	1.034 (0.342)	0.0137
Compound 2	0.756 (0.377)	0.0123
Compound 3	0.596 (0.101)	0.0163
Doxorubicin	2.858 (0.975)	–

Amount of p53 and p21 proteins

Increased levels of p53 and p21 proteins were observed after incubation of CCRF/CEM and A549 cells with the tested compounds (Figs. 3, 4). The largest increase in p53 and p21 levels was observed for Compound 2, although the increase in the amount of both proteins also occurred after treatment of cells with the remaining tested compounds. In A549 cultures a larger amount of p53 was observed after incubation with each tested compound than with ellipticine.

Spearman's rank correlation coefficient was used to determine whether the increase in p53 protein level correlates with the amount of p21 in cells (Table 3).

Correlations between p53 and p21 protein levels (after treatment with tested compounds at concentrations of 0.2–1 μM or ellipticine) for both cell lines were strongly positive. In the case of the CCRF/CEM line, it was not possible to calculate correlation coefficients for higher concentrations (> 1 μM), because these concentrations had a pro-apoptotic effect. In A549 cell cultures, the correlation between p53 and p21 protein level after treatment with Compound 1 is strongly positive in the entire concentration range, while no significant correlation was found for other compounds.

Cell cycle

The effect of the increased level of p53 should be the inhibition of the cell cycle [14, 15]. In CCRF/CEM cells addition of Compound 1 resulted in a decrease in the number of cells in the S phase, and an increase in the G0/G1 phase and G2/M phase. Compounds 2 and 3 and ellipticine reduced the number of cells in the S phase and also stopped the cell cycle in the G2/M phase (Fig. 5).

In the A549 cell line, for all tested compounds and ellipticine, a decrease in the number of cells in the S and G0/G1 phases was also observed, as well as a significant increase in the number of cells in the G2/M phase (Fig. 6). The inhibition of the A549 cell cycle in the G2/M phase was particularly strong for Compounds 2 and 3 (for 10 μM concentrations by 54.7% and 67.4%, respectively).

Accumulation of rhodamine 123

The study also determined the effect of compounds on P-glycoprotein activity. Compounds 2 and 3 caused a significant increase in Rh-123 accumulation in cells (Fig. 7), which may
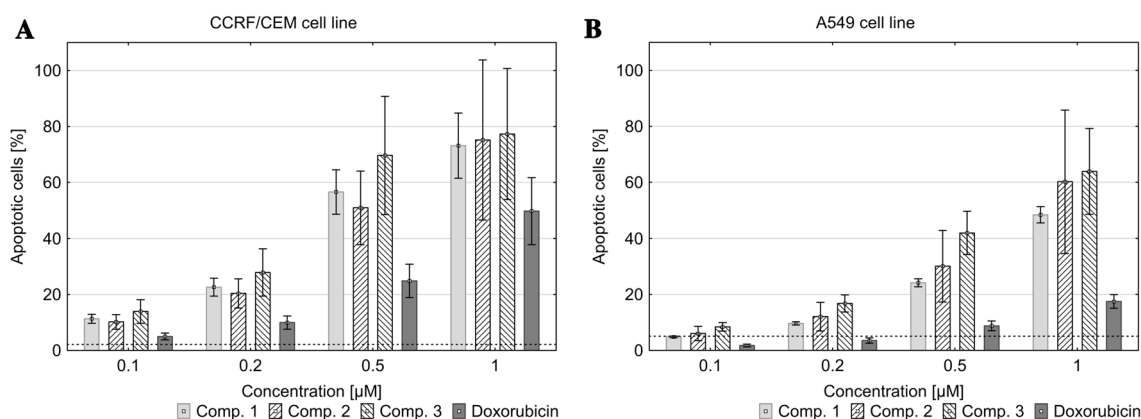

Fig. 2 Effect of tested compounds on the level of apoptotic cells after 18 h of incubation; **a** CCRF/CEM cell line (level in the control: $2.15 \pm 1.01\%$); **b** A549 cell line (level in the control: $5.10 \pm 1.42\%$)
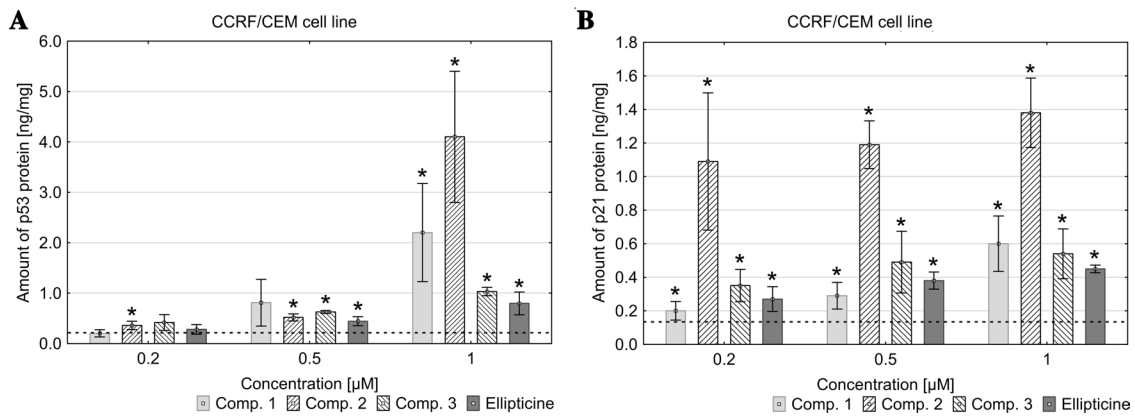
Fig. 3 Total content of p53 (**a**) and p21 (**b**) in CCRF/CEM cells after 18 h of incubation with tested compounds; statistical significance compared to the control; Kruskal–Wallis ANOVA with post-hoc test

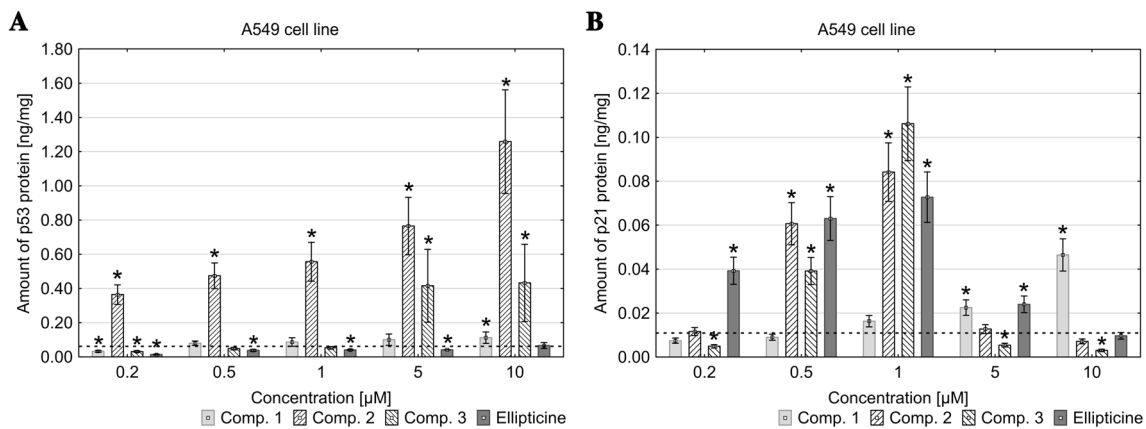(*$p < 0.05$); p53 content in the control: 0.22 ± 0.05 ng/mg; p21 content in the control: 0.13 ± 0.04 ng/mg

Fig. 4 Total content of p53 (**a**) and p21 (**b**) in A549 cells after 18 h of incubation with tested compounds; statistical significance compared to the control; Kruskal–Wallis ANOVA with post-hoc test

(*$p < 0.05$); p53 content in the control: 0.062 ± 0.006 ng/mg; p21 content in the control: 0.011 ± 0.004 ng/mg

Table 3 Correlations between p53 and p21 protein levels in CCRF/CEM and A549 cells determined for the respective concentration ranges of the tested substances (0.2–1 μM or 0.2–10 μM)

Substance	CCRF/CEM cells 0.2–1 μM concentrations	A549 cells 0.2–1 μM concentrations	A549 cells 0.2–10 μM concentrations
Compound 1	1.0 ($p < 0.001$)	0.95 ($p < 0.001$)	0.932 ($p < 0.001$)
Compound 2	0.833 ($p < 0.01$)	0.95 ($p < 0.001$)	−0.071 ($p = 0.8$)
Compound 3	0.917 ($p < 0.001$)	0.95 ($p < 0.001$)	−0.500 ($p = 0.058$)
Ellipticine	1.0 ($p < 0.001$)	0.95 ($p < 0.001$)	0.640 ($p = 0.82$)

explain the activity of both compounds against cell lines resistant to cytostatics by inhibiting P-gp [16].

Multiple-criteria decision analysis

A combined analysis of p53-induced anti-tumor activity after treatment with tested compounds was performed, based on: 1. level of p21$^{Cip1/Waf1}$ protein; 2. induction of apoptosis in cells; 3. accumulation of Rh-123 in cells. The results of individual assays were referred to the level of p53 in cells. The calculated assay results were compared in a multiple-criteria decision analysis (Table 4).

The multiple-criteria decision analysis showed that the highest result (the highest anticancer activity) was observed for Compound 3, both in CCRF/CEM cultures (with mutant p53) and A549 cultures (with wild-type p53). In the case of
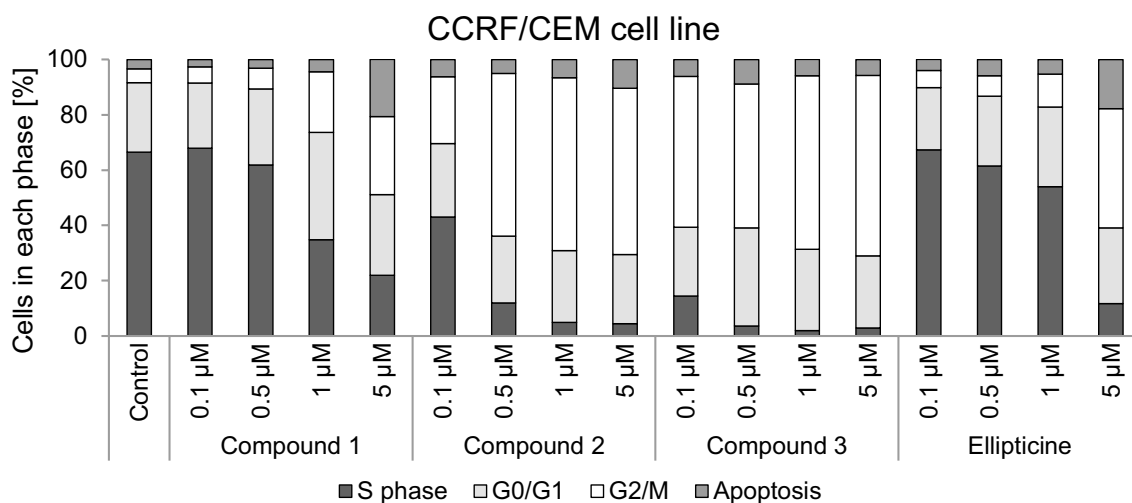
CCRF/CEM cell line

Fig. 5 Effect of tested compounds on the cell cycle in the CCRF/CEM cell line after 18 h of incubation
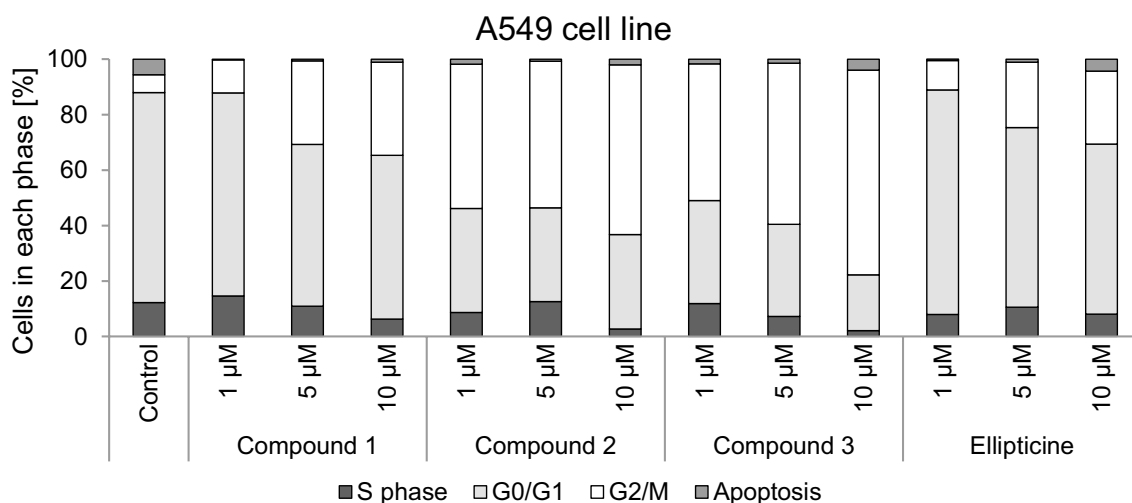
A549 cell line

Fig. 6 Effect of tested compounds on the cell cycle in the A549 cell line after 18 h of incubation
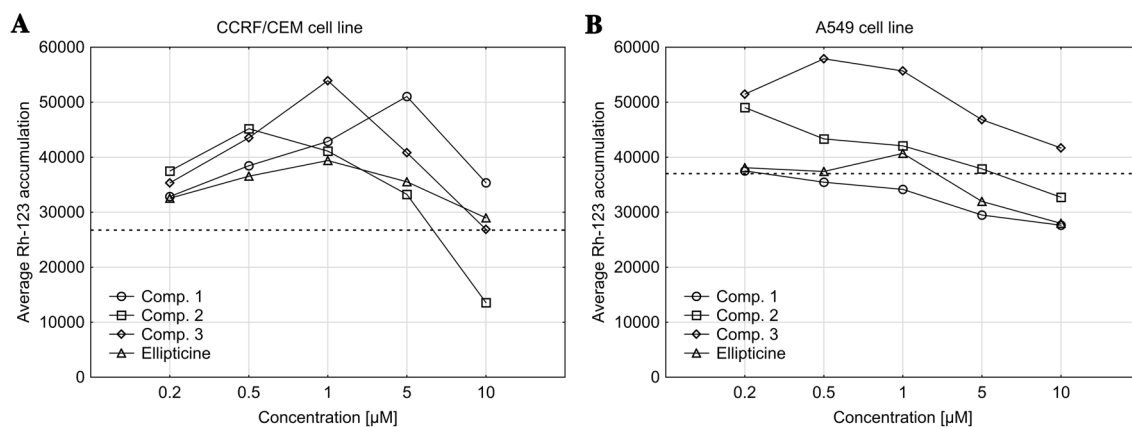

Fig. 7 Effect of tested compounds on accumulation of Rh-123 in cells after 18 h of incubation (expressed in conventional units based on mg of protein); **a** CCRF/CEM cell line (control = 26,582 ± 790); **b** A549 cell line (control = 36,913 ± 408)

Table 4 Results of multiple-criteria decision analysis of the effect of tested compounds on the level of p53 protein (antitumor activity) in CCRF/CEM and A549 cells

Cell line	Ratio	Expected effect	Weight of results	Comp. 1	Comp. 2	Comp. 3
CCRF/CEM	p21/p53	↑	0.3	1.125	4.905	5.314
	Apoptosis/p53	↑	0.5	1.819	0.044	3.875
	Rh-123/p53	↑	0.2	0.029	0.043	0.075
	Sum		1.0	2.973	4.992	9.264
A549	p21/p53	↑	0.3	0.318	0.308	2.788
	Apoptosis/p53	↑	0.5	0.005	0.002	0.724
	Rh-123/p53	↑	0.2	0.010	0.004	0.902
	Sum		1.0	0.333	0.315	4.414

Compound 2, the MCDA showed strong activity in CCRF/CEM cultures and very low in A549 cultures. Compound 1 showed the weakest effect among the compounds.

Discussion

The p53 protein, which is a transcription product of the *TP53* gene, is considered one of the most important factors inhibiting tumor growth, leading to inhibition of the cell cycle, to the intensification of apoptosis, and to increased chemosensitivity of cancer cells [17, 18]. p53 is an important modulator of the cellular response to oxidative, hypoxic, metabolic and genotoxic stress [18]. It affects many cell functions involved in the stress response—modifies cellular metabolism, influences autophagy and apoptotic processes, and acts as a multipotent transcription factor inducing or inhibiting the transcription of numerous target genes of this protein [18, 19]. An example of two areas (cytoplasmic and nuclear) of p53 activity is the effect on the induction of apoptosis in cell cultures exposed to genotoxic stress [20]. p53 forms a complex with the anti-apoptotic Bcl-xL protein, induces oligomerization and activation of the pro-apoptotic Bak protein, and increases the release of cytochrome c from the mitochondria. The p53 protein significantly increases apoptosis as early as 30 min after exposure of cells to a genotoxic agent [20, 21]. The effect in the cell nucleus is observed after 2 h from exposure to stress and is manifested by intensifying the transcription of factors enhancing apoptosis of the mitochondrial pathway [22].

The *TP53* gene shows the heterogeneity of the structure and diversity of the post-transcriptional and translational modifications of the transcription products, which determines the differences in the structure and function of the p53 protein. Numerous single nucleotide polymorphisms have been detected—more than 100 different haplotypes of *TP53* have been described [23]. Nine protein isoforms for this gene have been identified as a result of alternative mRNA splicing, and variability in translation initiation and post-translational modifications [24, 25]. The p53 isoforms differ in their main functions in response to DNA damage and cellular stress—for example, p53β isoform induces transcription of the pro-apoptotic *BAX* gene, but does not affect the expression of the p21 protein inhibiting proliferation, and the Δ133p53 isoform inhibits both BAX and p21 expression [25]. It should be emphasized that the various p53 isoforms are physiologically expressed forms of this protein, and therefore individual differences in the expression of isoforms of wild-type suppressor p53 protein cause differences in the function of this protein, including differences in susceptibility to cancer development [25].

In human tumors, the *TP53* gene has a high mutation rate. Mutations of this gene are found on average in more than 55% of cases of human cancers, and even more in some types of cancer, e.g. in pancreatic cancer and advanced ovarian cancer—75% and 96% (respectively) of clinical cases show the *TP53* mutation [26]. Over 80% of the described mutations of this gene are missense mutations that lead to increased expression of the p53 protein differing from the wild-type protein with one amino acid [20]. The protein product of such a mutant *TP53* gene loses its physiological function of inhibiting the cell division cycle and inducing apoptosis (functions of the tumor suppressor protein). The mutant gene is dominant to the wild-type gene in the second locus (dominant negative activity) and the protein gains new activities that enhance tumor growth [20, 27]. Changes in the p53 protein conformation caused by missense mutations facilitate the interaction with intracellular signal transduction pathways from oncogenes. The mutant p53 protein stimulates the intracellular signal transduction pathways of HRAS oncogene, PI3K/Akt/mTOR and p38-MAPK pathways, and in this way promotes carcinogenesis, stimulates the cell cycle of transformed cells, increases their survival, and intensifies tumor cell migration, invasiveness, and the ability to create metastases [24, 28]. The mutant protein also activates the NF-κB transcription factor, leading to increased transcription and synthesis of pro-inflammatory cytokines and, as a result, to the severity of chronic inflammation that enhances tumor progression [29–31], and to promotion of NF-κB target anti-apoptotic genes transcription.

Activation of NF-κB in tumor cells was observed in cases of missense p53 mutations, whereas no such effect was found in the absence of p53 protein. The function of the mutant p53 protein in the activation of NF-κB may explain the increased frequency of p53 mutations in malignant tumors, compared to the incidence of total p53 deletions [30].

Previous observations indicate that mutual regulatory mechanisms concern cells containing wild-type p53, whereas these interactions are significantly weaker in cells with mutant p53. For this reason, cancer cells containing the mutant p53 protein are much less sensitive to many anticancer drugs, including topo II inhibitors stabilizing topo II-DNA cleavage complexes [32, 33].

Experimental studies show that changes in the structure and function of the p53 protein due to missense mutations in the *TP53* gene are in some cases reversible, e.g. some functions of the protein can be activated at suitable temperatures. In cultures of the H1299 cells (human non-small cell lung carcinoma) containing A143 and V138 mutations, lowering the temperature of the culture to 32 °C led to conformational changes and restored the ability of the mutant p53 protein to inhibit the G1 cell cycle and induce apoptosis [34, 35]. It is possible to modify the tertiary structure of the mutant p53 protein to restore its tumor suppressor function. Numerous studies from the last two decades concern newly synthesized low molecular weight compounds that are considered to interact directly with the mutant p53 protein, forcing its folding and the conformation change to the extent that allows binding of promoter segments and activation of transcription of major target genes in tumor suppression, i.e. *BAX* and *p21*$^{Cip1/Waf1}$ [2, 36–38]. Studies in cell cultures have shown that the ellipticine and a large group of its derivatives bind to p53 proteins with missense mutations and reactivate p53 functions in the field of *p21*$^{Cip1/Waf1}$ and *MDM2* gene expression, and 9-hydroxy-ellipticine inhibits the G1 phase of the cell cycle and enhances apoptosis by inducing expression of BAX [2, 36, 39]. Analysis of data from the National Cancer Institute's anticancer drug screening program shows that in the panel of 60 cancer lines used by NCI some of the 112 ellipticine derivatives (N^2-alkyl-substituted ellipticiniums) have greater antitumor activity in cell lines containing mutant p53 than in lines with wild-type p53 [40]. Chemical syntheses of new derivatives of ellipticine and its isomer olivacine are still being carried out to obtain the most effective anticancer compounds against cells containing mutant p53 protein [41].

The variability of the structure and function of the *TP53* gene, the great diversity of p53 proteins (isoforms, polymorphisms, mutations), and the differences in mutant gene penetration in different conditions of the cell culture microenvironment do not allow for the assumption that the cell line in which the presence of the wild-type p53 was described or where a missense mutation of this protein has been identified

and determined will effectively meet the expected biological functions of the p53 protein. In the evaluation of new anticancer compounds, it is necessary to assess the direct interactions of the compounds with the p53 protein (leading to the partial reactivation of the mutant protein function) and the increase of the expression of the wild-type p53 protein. Therefore, it is appropriate to simultaneously evaluate the level of p53 protein in cell cultures and protein functions in cell cycle inhibition and influence on p21$^{Cip1/Waf1}$ protein level, on the rate of apoptosis, and on multidrug resistance mechanisms (the expression and functions of the P-gp membrane transporter). The cellular content of the p21$^{Cip1/Waf1}$ protein, the rate of apoptosis, and the inhibition of the P-gp transport function (increase in the accumulation of Rh-123 dye) were referred in our study to the cellular content of p53 in the same cultures. In the multiple-criteria decision analysis (MCDA), the activities of three tested olivacine derivatives were compared for selected anticancer effects induced by p53 protein: p21$^{Cip1/Waf1}$ protein level, induction of apoptosis, and inhibition of P-gp transport function in two tumor lines—CCRF/CEM (contains mutant p53) and A549 (contains wild-type p53). The results of MCDA indicate that Compound 2 selectively reactivates mutant p53 protein functions and may be considered in clinical cases of tumors with confirmed p53 mutation, whereas Compound 3 could be used for both wild-type p53 and mutant p53 tumors.

The observed effect of the tested compounds was the significant inhibition of the cell cycle, the increase in apoptosis and the number of dead cells, and increase of chemosensitivity of cancer cells. Our study shows that new pyridocarbazoles in the cultures of human tumor cells cause reactivation of mutant p53 functions and intensification of wild-type p53 functions, so they are potential candidates for future anticancer drugs.

Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest.

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