Synthesis, characterization, anticancer evaluation and mechanisms of cytotoxic activity of novel 3-hydroxy-3-pyrrolin-2-ones bearing thienoyl fragment: DNA, BSA interactions and molecular docking study

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ABSTRACT

In order to make a progress in discovering a new agents for chemotherapy with improved properties and bearing in mind the fact that substituted 3-hydroxy-3-pyrrolin-2-ones belong to a class of biologically active compounds, series of novel 1,5-diaryl-4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones were synthesized and characterized by spectral (UV–Vis, IR, NMR, ESI-MS), X-ray and elemental analysis. All compounds were examined for their cytotoxic effect on human cancer cell lines HeLa and MDA-MB 231 and normal fibroblasts (MRC-5). Four compounds, 3-hydroxy-1-(p-tolyl)-4-(2-thienylcarbonyl)-5-(4-chlorophenyl)-2,5-dihydro-1H-pyrrol-2-one (D10), 3-hydroxy-1-(3-nitrophenyl)-4-(2-thienylcarbonyl)-5-(4-benzylxylophenyl)-2,5-dihydro-1H-pyrrol-2-one (D13), 3-hydroxy-1-(4-nitrophenyl)-4-(2-thienylcarbonyl)-5-(4-(benzyloxy)phenyl)-2,5-dihydro-1H-pyrrol-2-one (D14), and 3-hydroxy-1-(4-chlorophenyl)-4-(2-thienylcarbonyl)-5-(4-(benzyloxy)phenyl)-2,5-dihydro-1H-pyrrol-2-one (D15), that showed the highest cytotoxicity against malignant cells and the best selectivity towards normal cells were selected for further experiments. Results obtained by investigating mechanisms of cytotoxic activity suggest that selected 3-hydroxy-3-pyrrolin-2-one derivatives in HeLa cells induce apoptosis that is associated with S phase arrest (D13, D15, and D10) or unrelated to cell cycledistribution (D14). Additionally, to better understand their suitability for potential use as anticancer medicaments we studied the interactions between biomacromolecules (DNA or BSA) and D13 and D15. The results indicated that D13 and D15 have great affinity to displace EB from the EB-DNA complex through intercalation \[K_{sv}=(3.7 \pm 0.1) \times 10^{3} \text{M}^{-1}\] and \[K_{sv}=(3.4 \pm 0.1) \times 10^{3} \text{M}^{-1}\], respectively, an intercalative mode also confirmed through viscosity measurements. \[K_{a}\] values, obtained as result of fluorescence titration of BSA with D13 and D15 \[K_{a}=(4.2 \pm 0.2) \times 10^{5} \text{M}^{-1}\] and \[K_{a}=(2.6 \pm 0.2) \times 10^{5} \text{M}^{-1}\], support the fact that a significant amount of the tested compounds could be transported and distributed through the cells. In addition, by DNA and BSA molecular docking study for D13, D14 and D15 is determined and predicted the binding mode and the interaction region.

1. Introduction

One of the biggest health problem in our society and one of the primary targets in medicinal chemistry nowadays is cancer. Since the discovery of cisplatin and its use in clinical therapy, scientists have made a lot of effort to synthesize many platinum-based drugs that could be less toxic to healthy tissue [1–3] and to overcome the resistance of some tumors to cisplatin [4,5]. The main goal for developing non-platinum anticancer drugs is to overcome the limits of platinum drugs: severe toxicity on healthy tissue, narrow range of activity and after treatment acquired resistance [1–5]. The discovery of new nonplatinum drugs with less side effects is of the major interest in pharmaceutical
2.1. Synthesis and characterization

humanserumalbumin (HSA) [28]. There
drugs in the bloodstream. Therefore, it is important to investigate
that has the ability to transport a large number of endogenous and
[25,26], intercalation [27]). Bovineserumalbumin (BSA) istheprotein
via noncovalent associations (stacking [22–24], groove binding
covalentinteractions (interstrand/intrastrand cross-linking) [19–21]or
great interest due to their potential use as anticancer medicaments
on the interaction of small drugs with deoxyribonucleic acid are of
investigations showed that they have good antimicrobial activity [9–12]. Also,
these compounds have many other biological activities such as anti-
flammatory, antiviral, antifungal, and nootropic [13–15]. To sum up,
various biologically active natural products containing either 3-acyl-5-
hydroxy-3-pyrrolin-2-one or 3-acyl-3,4-epoxy-5-hydroxypyrrolidin-2-
were isolated, suggesting that the pyrrolidinone moiety is mostly
responsible for the biological activity of these compounds.

In order to discover new anticancer agents with improved properties
and bearing in mind previous facts, we studied three-component, the one-
pot reaction of acylpyruvic acid ethyl ester with an aromatic amine
and one previously reported. All the compounds are obtained in glacial
acetic acid as catalyst and solvent, by the procedure described earlier
[29] with minor corrections of reaction conditions.

All synthesized novel compounds were characterized by UV–Vis,
NMR (1H and 13C spectra of compounds D1-D15 are presented in the
ESI, Figs. S1–S30), MS spectroscopy and elemental analysis. For one,
compound D2 that crystallized from DMSO solution, crystal structure
was determined by using single-crystal X-ray diffraction analysis.

2.1.1. Crystal and molecular structures of D2

Single-crystal X-ray diffraction analysis has shown that the
D2 compound crystallized with two molecules of DMSO (Fig. 1). The
existence of two carbonyl groups (the C1–C1 and C5–O3) and one hy-
droxyl (the C2–O2H) is clearly confirmed. The central N1 five-mem-
bered ring is almost ideally planar (root-mean-square deviation of fitted
atoms is 0.012 Å). This ring forms a dihedral angle of 16.9(1)°
with neighboring C16–C21 phenyl ring. The C5–O3 carbonyl group is
directed between the N1 and S1 five-membered rings [the
C4–C3–C5–O3 and O3–C5–C6–S1 torsion angles are −16.1(3) and
−18.0(3)° respectively] approximately bisecting the dihedral angle
between these two heterocycles. With a dihedral angle of 89.2(1)° the
C10–C15 phenyl ring is almost perfectly placed in an orthogonal po-
position relating to the central N1 ring.

The C2–C3 bond with the bond length of 1.341(3) Å (Table S1) is
the shortest C–C bond in the whole molecule while the longest one is
C4–C10 (1.522(3) Å). The N1 is clearly an sp2 hybridized atom since
that the bond of sum angles around this atom is 359.9° and all N–C
bonds are coplanar. The N1 forms three N–C bonds with significantly
different bond lengths (Table S1). The shortest N1–C1 bond with an
interatomic distance of 1.369(3) Å probably enables electron π de-
localization across the N1–C1–O1 fragment.

The O2–H…O4 is only one classical intermolecular hydrogen bond
in the crystal structure of D2, however, it has quite short H…O distance
of 1.78(3) Å [O2–H = 0.80(3) Å, O2…O4 = 2.538(3) Å, O2–H…O4 = 159(3)°; symmetry code: (i) x + 1, y, z]. The C1–O1 and C5–O3
carbonyl groups form only very weak C–H…O hydrogen bonds with
H…O distance longer than 2.55 Å. Intermolecular C–H…π interactions
dominate in the crystal packing by their frequency. Thus in Fig. 1 one
can see that the methyl groups from two DMSO molecules are directed
to the centers of neighboring π systems.

2. Results and discussion

2.1. Synthesis and characterization

We used the three-component reaction between acylpyruvic acid
ethyl ester, (Z)-ethyl 2-hydroxy-4-oxo-4-(thiophen-2-yl)but-2-enoate
(A), and a mixture of differently substituted aromatic aldehyde (B)
and an aromatic amine (C) to synthesize fifteen 1,5-diaryl-4-(2-thien-
cylobonyl)-3-hydroxy-3-pyrrolin-2-ones (D1-D15), fourteen novel
and one previously reported. The investigations showed that they have good antimicrobial activity [9–12]. Also,
these compounds have many other biological activities such as anti-

chemistry [6]. Substituted 3-hydroxy-3-pyrrolin-2-ones belong to a
class of biologically active compounds. They can be obtained in the
reaction between ethyl-2-hydroxy-4-thienyl-4-oxo-2-butenoate with
4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones (Scheme 1). The fifteen 1,5-diaryl-4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones were exposed to
acetic acid as solvent, lead to the formation of 1,5-diaryl-4-(2-thien-
}

Scheme 1. General synthetic pathway towards 1,5-diaryl-4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones (D1-D15).
containing this fragment. Another significant influence of the substituents at R₁ was not observed except in case D10 (R₁ = Cl) where there is also improved antitumour activity, but to some lesser extent compared to compounds with benzyloxy group. Also, it was observed that the shift of the nitro group from meta- to para-position, as can be seen in compounds D13 and D14, had some influence to the cell growth inhibition. Some better antitumor activity is achieved when NO₂ group is in meta-position, as observed for D13 in both MTT and SRB assay. This different activity was also observed for some organic compounds in a recent study [32], and is most likely related to the steric effect of the compounds. Slight difference in the activity was observed in D15, when Cl is replaced in R₉, showing the positive change in antitumour activity comparing to D14.

There is no large amount of data on the antitumor effects of these types of compounds in the literature. Several studies indicate that their biological activity depends on the modifications of the heterocyclic ring. The 3-hydroxy-pyrrolin-2-one derivatives exhibited antitumor activity in vitro on three mouse tumor cell lines hepatoma (MG-22A), melanoma (V16) and neuroblastoma (Neuro2A) [33]. In vivo studies have shown that some pyrrolin-2-one derivatives had genotoxic effects on the mouse bone marrow [34]. There are also data on the antitumor effects of some diaryl-3-pyrroline-2-on derivatives on human malignant cells, such as U-937 histiocyte lymphoma cells [35]. Almost no data are available on the mechanism of action of these organic compounds, as well as the type of cell death they induce, so we examined whether cytotoxicity induced by tested compounds was due to apoptosis or cell cycle arrest.

2.2.2. The mechanisms of cytotoxic activity

To determine the type of cell death induced by selected 3-hydroxy-3-pyrrolin-2-one derivatives, Annexin V-FITC/7-AAD assay was performed. Flow cytometric analysis showed that all tested compounds induced apoptosis in HeLa cells, while the percent of necrotic cells was negligible. Considering that apoptosis, as opposed to necrosis, does not induce inflammation and injury of surrounding tissue, agents that induce this type of cell death can be envisaged as potential antitumor drugs [36]. In accordance with the results of MTT and SRB assay, flow cytometry showed that treatment of MDA-MB cells with 100μM concentration of all tested compounds resulted in the small percent of dead, predominantly apoptotic cells (Fig. 3).

Likewise, the effect of 3-hydroxy-3-pyrrolin-2-one derivatives on autophagy was different in these cell lines. While in HeLa cells compounds D13 and D15 induced marked and compound D10 moderate increases in the intensity of red fluorescence. Contrary, in MDA-MB 231 cells not only that compounds D13 and D15 had no influence on autophagy induction but compounds D10 and D14 even inhibited autophagy in certain extent (Fig. 4).

To find out the role which autophagy plays in 3-hydroxy-3-pyrrolin-2-one derivatives-induced cytotoxicity, cells were cotreated with autophagy inhibitor chloroquine (CQ). Results showed opposite effect of compounds D13 and D15 in HeLa and MDA-MB 231 cells (Fig. 5). While in HeLa cells inhibition of autophagy enhanced apoptosis in MDA-MB 231 cells resulted in the decrease of Annexin V-positive cells. In compounds D10 and D14, treated both HeLa and MDA-MB 231 cells autophagy inhibition had no effect on the induction of apoptosis.

Dual role of autophagy, a catabolic process by which cells degrade and recycle their own constituents, is well documented. Autophagy was first described as a mechanism for cell survival that maintains normal cellular function under conditions of intra- and extracellular stresses. However, autophagy may also activate the apoptotic program and promote cell death. For this reason, agents targeting autophagy may serve as a therapeutic advantage [37]. Our results showed that in HeLa cells autophagy induced by D13 and D15 has a cytoprotective role, since its’ inhibition enhanced apoptosis. In D13 and D15 treated MDA-MB 231 cells autophagy stayed on the basal level, therefore inhibition

2.2. Biological evaluation

2.2.1. Cytotoxic activity of 3-hydroxy-3-pyrrolin-2-on derivates

In the first screening stage, cytotoxic activity of newly synthesized compounds (D1-D15) against HeLa, MDA-MB 231 and MRC-5 was evaluated by MTT assay. Cells were treated with single dose (100 μM) concentration of tested compounds. Four compounds: D10, D13, D14, and D15 that showed the highest cytotoxicity against malignant cells and the best selectivity towards normal cells, were selected for further experiments (Table S3).

Cytotoxic effect of compounds D10, D13, D14, and D15 was further examined in a range of five concentrations on all three cell lines during 24 h and 48 h using MTT and SRB assays. The main difference between these two assays is that MTT measures metabolic activity, while SRB is based on measurement of cellular protein content and indirectly measures cell number, so we performed both tests to define whether tested compounds kill or affect metabolic activity of cells [30,31]. Generally, both MTT and SRB assay showed comparable values of cytotoxic effect for all tested compounds on all three designated cell lines. Results of both assays are presented in the form of comparative dose-response curves (Fig. 2).

Compounds D10, D13, D14, and D15 showed the cytotoxic effect on subjected tumor cell lines, being more potent for HeLa, than for MDA-MB 231. Calculated IC₅₀ values for MDA-MB 231 and non-transformed fibroblasts MRC-5 were above the highest concentration used in experiments (IC₅₀ > 100 μM). It was shown that all treatments on HeLa cells induced growth inhibition activity in dose and time-dependent manner. HeLa cells were more susceptible to tested compounds with moderate cytotoxic activity. Compound D13 exhibited the strongest cytotoxic effect after 48 h of exposure against HeLa cells with IC₅₀ values of 40.9 ± 5.9 μM and 54.35 ± 8.56 μM for SRB and MTT assays, respectively (Table S4).

From the above mentioned analysis, it can be noticed that when R₁ is benzyloxy group, activity of the compounds has been significantly improved as can be seen for three compounds (D13, D14, and D15)
of autophagy, as a homeostatic mechanism, resulted in alleviation of cell death. Although compound D10 enhanced autophagy in HeLa cells, cotreatment with CQ had no effect on apoptosis, showing that autophagy is not implicated in the cell death pathway. These results demonstrated that autophagy is not a part of the mechanisms of cytotoxicity induced by either tested 3-hydroxy-3-pyrrolin-2-one derivates.

To further reveal mechanisms underlying cytotoxicity induced by the tested compounds, cell cycle distribution was analyzed by flow cytometry. In multicellular organisms both apoptosis and process of cell cycle progression are essential for maintaining tissue homeostasis. Accumulated evidence revealed that these two processes share a set of regulatory molecules. Manipulation with cell cycle may prevent or induce apoptosis. Our results demonstrated that in HeLa cells treated with compounds D13, D15 and D10 arrested cells in S phase, indicating inhibition of DNA synthesis, while compound D14 had no effect on cell cycle progression. As expected, 100 μM concentration of the 3-hydroxy-3-pyrrolin-2-one derivates didn’t affect cell cycle progression in MDA-MB 231 cells (Fig. 6).
In conclusion, HeLa and MDA-MB 231 cell lines differ not only in histological type, but are also highly heterogeneous regarding mutations related to genes that control programmed cell death and cell cycle progression. This fact explains the different sensitivity of these cell lines to the tested compounds. Tested compounds apparently target molecules that are diversely expressed in HeLa and MDA-MB 231 cells. The data presented suggest that selected 3-hydroxy-3-pyrrolin-2-one derivatives in HeLa cells induce apoptosis that is associated with S phase arrest (D13, D15, and D10) or unrelated to cell cycle distribution (D14).

2.3. Fluorescence measurements

2.3.1. Fluorescence quenching on EB–DNA

Ethidium bromide (EB) is a compound known by intense fluorescence emission in the presence of DNA. Fluorescence emission, the consequence of strong intercalation between the base pairs of DNA and EB, can be quenched by the addition of the drug which can compete with EB to bind with DNA confirming that drug intercalates to base pairs of DNA [38,39]. Fluorescence quenching spectra (Fig. 7) of titration EB–DNA with D13 and D15, recorded in the range of 550–750 nm, show a decreasing trend with the increasing amounts of the compounds at 610 nm indicating that EB was partially replaced by compounds D13 and D15. The molar ratios of EB–DNA : D13 or D15 followed the order: 1 : 0 (control), 1 : 0.2, 1 : 0.4, 1 : 0.6, 1 : 0.8, 1 : 1, 1 : 1.2, 1 : 1.4, 1 : 1.6, 1 : 8, 1 : 2, in a total volume of 5.0 ml, pH 7.4, at 25°C with the incubation time of 6 h. Obtained results indicated that both compounds bind to DNA by an intercalative mode.

To better understand quantitatively the magnitude of the binding strength of the compounds with CT-DNA, the quenching constant (Kq) was calculated using Stern-Volmer equation [40] by examining the dependence of \( \frac{F_0 - F}{F} \) on [Q] (Fig. 7). Quenching constant for D13 and D15, presented in Table 1, indicate that both compounds have the capability to displaced EB from the EB–DNA complex by binding to DNA through intercalation [41].

2.3.2. Protein binding experiments

Based on the fact that the efficiency of drugs depends on their ability to bind to a carrier protein and knowing that bovine serum albumin (BSA) is the protein that has the ability to transport drugs in bloodstream we investigated the binding affinity of D13 and D15 to BSA. In the fluorescence quenching experiment, the fluorescence emission titration of BSA with compounds D13 and D15 was used for the investigation of binding properties by increasing the concentration of the quenchers, in the wavelength range of 300–500 nm (Fig. 8).

The molar ratios of BSA : D13 or D15 followed the order: 1 : 0 (control), 1 : 1, 1 : 2, 1 : 3, 1 : 4, 1 : 5, 1 : 6, 1 : 7, 1 : 8, 1 : 9, 1 : 10, in total volume of 5.0 ml, pH 7.4, at 25°C with the incubation time of 6 h. The fluorescence quenching data such as binding constant (Kb) and number of binding sites per BSA molecule (n) were described by using Eqn [42]:

\[
\log(F_0 - F/F) = \log K_b + n \log [Q]
\]

where F0 is the emission intensity in the absence of a quencher, F is the emission intensity in the presence of a quencher for the complexes D13 or D15–BSA and [Q] is the concentration of D13 or D15. The Kb value and n were obtained investigating dependence of the log \( (F_0 - F/F) \) versus log[Q] (Fig. 8). The obtained values of the Kb, given in Table 2, that are in the optimum range which is considered to be \( 10^{-4} - 10^{6} \) M\(^{-1}\) [43], indicate that both compounds have the appropriate ability for binding to BSA. The number of binding sites of D13 or D15 (n ≈ 1.5, Table 2) point that both D13 and D15 bind to BSA in the molar ratio of 1.5 : 1.
2.4. Viscosity measurements

The viscosity measurement of DNA after treatment with a varying concentration of ligands provides a reliable indication for the DNA binding mode. In general, this technique provides more definitive evidence for intercalative binding of particular substances to DNA, since changes in the DNA chain length are manifested by changes in the viscosity of the medium.

It is generally suggested that a classical intercalative binding mode causes a significant increase of DNA viscosity because the intercalative interaction requires the space of adjacent base pairs to be large enough to accommodate the bound small molecules and elongates the double helix. When electrostatic, groove or hydrophobic binding occurs in the binding process, no obvious changes of DNA viscosity are typically caused in the solution.

The relative specific viscosity ($\eta/\eta_0$) of DNA is firmly dependent on the length changes that may associate with the separation of DNA base pairs caused by intercalative interaction between DNA’s double helix and a small molecule. The influences of investigated ligands D13 and D15 on the viscosity of DNA are shown in Fig. 9. In our case, relative viscosity increase with a slope value of 0.52 (ligand D13) and 0.55 (ligand D15).

Upon addition of both ligands to DNA, the relative viscosity raises steadily, as in the case of the classical intercalator. The results indicated that the ligands bind with DNA through the intercalative mode since the viscosity increase of DNA is ascribed to the intercalative binding mode, causing the effective lengthening of the DNA. The slope of relative viscosity was similar for both ligands, pointing out to similar binding affinity towards DNA for investigated compounds.

2.5. DNA and BSA docking study

The method of moleculesimulation is an efficient way to determine and predict the binding mode and the interaction region. To elucidate the mode of interaction and binding affinity, docking studies have been performed on B-DNA and BSA in presence of D13, D14 and D15. The best-docked poses of the compounds with DNA dodecamer are displayed in Fig. 10, and calculated results for binding energies and docked inhibition constant are summarised in Table 3. The study revealed that ligands under investigation interact with DNA via an intercalation mode involving outside edge π–π stacking interactions between guanine and benzyl ring of the ligands. From the ensuing docked structures it is clear that all ligands fits well into the minor groove of the targeted DNA and it is mainly stabilized by van der Waals interaction as can be seen from Fig. 10. The relative binding energy of the docked structures was found to be $-51.035$, $-49.814$ and $-52.312 \text{ kJmol}^{-1}$ for D13, D14 and D15 respectively. The slightly lower binding energy for D14 can be consequence of one less electrostatic interaction of eNO$_2$ group. From Fig. 10 it can be noted the different orientation of D13 and D14 towards DNA during docking, compared to D15. Namely, for D13 and D14 docking is additionally stabilized by electrostatic interactions between eNO$_2$ and adenine, which cause different conformation after docking in contrast to D15. On the other hand, chlorobenzylring for D15 is outside of DNA helixes and not contribute to the binding affinity of ligand. The slightly higher binding energy for D15 can be attributed to one more π–π stacking interaction of the ligand with DNA.

The binding of ligands D13, D14 and D15 to BSA occurs at similar positions in domain I [44]. The essential driving force of D13, D14 and D15 binding to BSA is hydrophobic and a van der Waals force. Three hydrogen bonds have been observed in the D13-BSA system: oxygens

![Fig. 5. Influence of autophagy inhibition on apoptosis. Percent of apoptotic cells with (CQ+) or without (CQ-) cotreatment with autophagy inhibitor chloroquine determined by flow cytometric analysis of Annexin V/7-AAD double stained cells. Results are presented as average ± SD from three independent experiments.](https://example.com/fig5)

![Fig. 6. Cell cycle analysis. Graphs showing cell cycle distribution in untreated (control) and treated HeLa and MDA-MB cells. Results are presented as average ± SD of three independent experiments.](https://example.com/fig6)
Fig. 7. Top: Emission spectra of EB-DNA in the absence (black lines) and in the presence of compounds D13 and D15. [EB] = 50 μM, [DNA] = 50 μM; [D13] and [D15] = 0–100 μM; pH = 7.4; λ_{ex} = 500 nm. Bottom: Plots of F₀/F versus [Q].

<table>
<thead>
<tr>
<th>Compound</th>
<th>kₐ [M⁻¹ s⁻¹]</th>
<th>Kₛ [M⁻¹]</th>
<th>R</th>
</tr>
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<tbody>
<tr>
<td>D13</td>
<td>(3.7 ± 0.1) × 10¹¹</td>
<td>(3.7 ± 0.1) × 10³</td>
<td>0.989</td>
</tr>
<tr>
<td>D15</td>
<td>(3.4 ± 0.1) × 10¹¹</td>
<td>(3.4 ± 0.1) × 10³</td>
<td>0.998</td>
</tr>
</tbody>
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Fig. 8. Top: Emission spectra of BSA in the absence (black lines) and in the presence of compounds D13 and D15. [BSA] = 10.0 μM; [D13] or [D15] = 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM; pH = 7.4; λ_{ex} = 280 nm. Bottom: plots of log(F₀/F) versus log[Q].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kₛ [M⁻¹]</th>
<th>n</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>D13</td>
<td>(4.2 ± 0.2) × 10⁵</td>
<td>1.47</td>
<td>0.998</td>
</tr>
<tr>
<td>D15</td>
<td>(2.6 ± 0.2) × 10⁵</td>
<td>1.69</td>
<td>0.998</td>
</tr>
</tbody>
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from −NO₂ group makes two hydrogen bonds with Lys A:136 and Phe A:36 and one is formed between oxygen (from benzoxyl fragment) and Phe B:36. For ligand D14 interaction with BSA occurs via 4 hydrogen bonds, three the same like in D13 and one additional between −OH group and Lys B:136. In the case of D15, only one hydrogen bond is formed with Glu A:38 (Fig. 11). The formation of hydrogen bonds stabilizes the complexes of formed with Glu A:38 (Fig. 11). The formation of hydrogen bonds group and Lys B:136. In the case of D15, only one hydrogen bond is formed with Glu A:38 (Fig. 11). The formation of hydrogen bonds stabilizes the complexes of D13 and D14 with BSA significantly more than D15-BSA which is demonstrated by higher binding energy for D13 and D14. The interactions of all ligands were additionally intensified by π–π stacking interactions with lys amino acids (lys B:132 and lys B:136). Because the binding site is located deeply in the body of BSA instead of being on the surface of the protein, both ligands have to pass through the amino acid residues on the surface around the binding site to settle at the proper position in BSA. This process is against the binding process and increases the activation energy of the binding.

3. Conclusion

The three-component reaction between acylpyruvic acid ethyl ester and a mixture of differently substituted aromatic aldehyde and an aromatic amine, in glacial acetic acid as catalyst and solvent, is used for synthesis of fifteen 1,5-diaryl-4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones (D1-D15), fourteen novel and one previously reported. All synthesized compounds were characterized by UV–Vis, NMR, MS spectroscopy and elemental analysis. In addition, for compound D2, crystallized from DMSO solution, crystal structure was determined using single-crystal X-ray diffraction analysis. All the compounds were further tested for their cytotoxic effect on human cancer cell lines HeLa and MDA-MB 231 and normal fibroblasts (MRC-5). Compounds D10, D13, D14, and D15 showed cytotoxic effect on subjected tumor cell lines, being more potent for HeLa than for MDA-MB 231, but D13 exhibited the strongest cytotoxic effect after 48 h of exposure against HeLa cells with IC₅₀ values of 40.9 ± 5.9 μM and 54.35 ± 8.56 μM for SRB and MTT assays, respectively. All four compounds that showed the highest cytotoxicity against malignant cells and the best selectivity towards normal cells were placed under further experiments. Results obtained by investigating mechanisms of cytotoxic activity suggest that our tested compounds in HeLa cells induce apoptosis that is associated with S phase arrest (compounds D13, D15, and D10) or unrelated to cell cycle distribution (compound D14). In order to better understand their appropriateness for potential use as anticancer medicaments we studied the interactions between biomacromolecules (DNA or BSA) and D13 and D15. The obtained results indicated that D13 and D15 have great affinity to displace EB from the EB-DNA complex through intercalation [Kᵥ = (3.7 ± 0.1) and (3.4 ± 0.1) × 10⁹ M⁻¹, respectively] that is also confirmed via viscosity measurements experiments. Kᵥ values, obtained as result of fluorescence titration of BSA with D13 and D15 [Kᵥ = (4.2 ± 0.2) and (2.6 ± 0.2) × 10⁵ M, respectively], showed that a significant amount of the tested compounds could be transported and distributed through the cells. In addition, DNA and BSA molecular docking study for D13, D14 and D15 was further performed to elucidate the mode of interaction and binding affinity.

4. Experimental section

4.1. Materials

All solvents and substrate were purchased from Sigma. The (Z)-ethyl 2-hydroxy-4-oxo-4-(thiophen-2-yl)but-2-enoate (A) was synthesized according to the previously described methodology [45]. Melting-points (Mp) were determined on a Mel-Temp apparatus and are uncorrected. Thin-layer chromatography (TLC) was carried out on 0.25 mm Sigma-Aldrich coated silica gel plates (60F-254) using eluent DCM:EtOH (9:1) as a mobile phase and UV light for visualization. The IR spectra were recorded by a Perkin-Elmer Spectrum One FT-IR spectrometer on KBr pellet. The NMR spectra of D1-D15 were performed in DMSO-d₆ with TMS as internal standard on a Varian Gemini 200 MHz NMR spectrometer (¹H at 200 and ¹³C at 50 MHz). Mass spectrometry was performed by Waters Micromass Quattro II triple quadrupole mass spectrometer and MassLynx software for control and data processing, electrospray ionization in the positive mode was used. The electrospray capillary was set at 3.0 kV and the cone at 20 V, the ion source temperature was set at 120 °C and the flow rates for nitrogen bath and spray were 500 l/h and 50 l/h, respectively and the collision energy was 20 eV.

4.2. General procedure for synthesis of 1,5-diaryl-4-(2-thienylcarbonyl)-3-hydroxy-3-pyrrolin-2-ones

A mixture of 1 mmol of an aromatic aldehyde and 1 mmol of an aromatic amine dissolved in 3 ml of glacial acetic acid was heated to 50 °C. After reaching the temperature, 1 mmol of the (Z)-ethyl 2-hydroxy-4-oxo-4-(thiophen-2-yl)but-2-enoate, dissolved in 2 ml glacial acetic acid, was added dropwise. After 3–5 h of reaction time precipitation of the product occurred. The reaction is followed by thin-layer chromatography with CH₂Cl₂:CH₃CH₂OH=9:1 as a mobile phase. The IR spectra were recorded by a Perkin-Elmer Spectrum One FT-IR spectrometer on KBr pellet.
J = 8.4 Hz, CH(3)), 7.95 (dd, 1H, J = 4.9, 1.1 Hz), 8.05 (dd, 1H, J = 3.8, 1.1 Hz) ppm; 13C NMR (50 MHz, DMSO-d6): δ = 20.6, 21.2, 61.6, 120.2, 122.9, 127.9, 128.1, 128.5, 128.6, 129.3, 133.9, 134.6, 134.9, 135.2, 136.5, 144.1, 149.3 and 164.5 ppm; ESI-MS: m/z (%) = 375 [M]+. Calcd for C22H17NO3S (%): C 70.38, H 4.56, N 3.73; found: C 70.52, H 4.51, N 3.75.

4.2.3. 3-Hydroxy-5-phenyl-1-(4-hydroxyphenyl)-4-(2-thienylcarbonyl)-2,5-dihydro-1H-pyrrol-2-one (D3)

Yellow powder; yield: 72%; mp = 246 °C; IR (KBr): ν 3468, 3199, 1701, 1677, 1610, 1520, 1455, 1409, 1376, 1262, 1239, 1181, 1137, 1125, 996, 836, 824, 740, 714 cm⁻¹; 1H NMR (200 MHz, DMSO-d6): δ = 6.16 (s, 1H, CH), 6.61–6.74 (m, 2H, CH2), 7.07–7.39 (m, 8H, 8.4 Hz, CH(3)), 7.95 (dd, 1H, J = 4.9, 1.1 Hz), 8.05 (dd, 1H, J = 3.8, 1.1 Hz) ppm; 13C NMR (50 MHz, DMSO-d6): δ = 20.6, 21.2, 61.6, 120.2, 122.9, 127.9, 128.1, 128.5, 128.6, 129.3, 133.9, 134.6, 134.9, 135.2, 136.5, 144.1, 149.3 and 164.5 ppm; ESI-MS: m/z (%) = 375 [M]+. Calcd for C22H17NO3S (%): C 70.38, H 4.56, N 3.73; found: C 70.52, H 4.51, N 3.75.

Table 3
Docking parameters of D13, D14 and D15.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking score</th>
<th>Binding energy/kJ mol⁻¹</th>
<th>Docking inhibition constant μM</th>
</tr>
</thead>
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<tr>
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<td>−4.852</td>
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<td>1.144</td>
</tr>
<tr>
<td>D14</td>
<td>−4.551</td>
<td>−49.814</td>
<td>1.873</td>
</tr>
<tr>
<td>D15</td>
<td>−5.187</td>
<td>−52.312</td>
<td>0.684</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>BSA</td>
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</tr>
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</table>

Fig. 10. Molecular docking of D13 (A), D14 (B) and D15 (C) with DNA and a two-dimensional representation of interactions.
CH$_2$OH), 7.95 (dd, 1H, J = 4.9 Hz, 1.1 CH$_2$), 8.06 (dd, 1H, J = 3.8, 1.1 Hz), 9.47 (s, 1H, OH$_{Ar}$) ppm; $^{13}$C NMR (50 MHz, DMSO-d$_6$): δ = 115.4, 119.9, 125.0, 127.8, 127.9, 128.5, 128.6, 136.6, 144.2, 149.6, 155.4, 164.3 and 180.4 ppm; ESI-MS: m/z (%) = 377 [M$^+$].

Calcd for C$_{21}$H$_{15}$NO$_4$S (%): C 66.83, H 4.01, N 3.71; found: C 67.01, H 4.11, N 3.75.

4.2.4. 3-Hydroxy-5-phenyl-1-(4-chorophenyl)-4-(2-thienylcarbonyl)-2,5-dihydro-1H-pyrrol-2-one (D4)

White powder; yield: 81%; mp = 240 °C; IR (KBr): ν 3187, 1709, 1672, 1610, 1495, 1414, 1370, 1298, 1281, 1245, 1187, 1091, 987, 852, 836, 742, 725 cm$^{-1}$; $^1$H NMR (200 MHz, DMSO-d$_6$): δ = 6.32 (s, 1H, CH), 7.09–7.24 (m, 4H, CH$_{Ar}$), 7.29–7.42 (m, 4H, CH$_{Ar}$), 7.61–7.70 (m, 2H, CH$_{Ar}$), 7.96 (dd, 1H, J = 4.9 Hz, 1.1 CH$_2$), 8.02 (dd, 1H, J = 3.8, 1.1 Hz), 12.0 (s, 1H, OH$_{enol}$) ppm; $^{13}$C NMR (50 MHz, DMSO-d$_6$): δ = 61.4, 120.5, 124.3, 127.9, 128.6, 128.8, 129.6, 135.4, 136.2, 144.0, 148.9, 164.8 and 180.4 ppm; ESI-MS: m/z (%) = 396 [M$^+$].

Calcd for C$_{21}$H$_{14}$ClNO$_3$S (%): C 63.72, H 3.56, N 3.54; found: C 63.52, H 3.59, N 3.55.

4.2.5. 3-Hydroxy-5-phenyl-1-(4-bromophenyl)-4-(2-thienylcarbonyl)-2,5-dihydro-1H-pyrrol-2-one (D5)

White powder; yield: 84%; mp = 237 °C; IR (KBr): ν 3188, 1709, 1684, 1671, 1610, 1492, 1412, 1370, 1297, 1245, 1128, 986, 852, 833, 741, 724 cm$^{-1}$; $^1$H NMR (200 MHz, DMSO-d$_6$): δ = 6.31 (s, 1H, CH), 7.02–7.41 (m, 6H, CH$_{Ar}$), 7.41–7.68 (m, 4H, CH$_{Ar}$), 7.96 (dd, 1H, J = 5.0 Hz, 1.1 CH$_2$), 8.05 (dd, 1H, J = 3.8, 1.2 Hz) ppm; $^{13}$C NMR (50 MHz, DMSO-d$_6$): δ = 117.9, 120.5, 124.6, 127.9, 128.6, 131.7, 135.8, 136.2, 144.0, 148.9, 165.8 and 180.4 ppm; ESI-MS: m/z (%) = 440 [M$^+$].

Calcd for C$_{21}$H$_{14}$BrNO$_3$S (%): C 57.28, H 3.20, N 3.18; found: C 57.42, H 3.22, N 3.15.

4.2.6. 3-Hydroxy-5-phenyl-1-(4-iodophenyl)-4-(2-thienylcarbonyl)-2,5-dihydro-1H-pyrrol-2-one (D6)

White powder; yield: 72%; mp = 239 °C; IR (KBr): ν 3178, 1684, 1665, 1608, 1490, 1413, 1383, 1292, 1244, 1134, 983, 852, 826, 715, 698 cm$^{-1}$; $^1$H NMR (200 MHz, DMSO-d$_6$): δ = 6.30 (s, 1H, CH), 7.09–7.24 (m, 4H, CH$_{Ar}$), 7.30–7.35 (m, 2H, CH$_{Ar}$), 7.41–7.51 (m, 2H, CH$_{Ar}$), 7.59–7.69 (m, 2H, CH$_{Ar}$), 7.96 (dd, 1H, J = 5.0 Hz, 1.1 CH$_2$), 8.04 (dd, 1H, J = 3.8, 1.2 Hz) ppm; $^{13}$C NMR (50 MHz, DMSO-d$_6$):
δ = 61.2, 90.3, 120.5, 124.7, 127.8, 128.2, 128.6, 134.7, 135.3, 136.2, 137.5, 144.0, 148.8, 165.7 and 180.4 ppm; ESI-MS: m/z (%) = 487 [M]+. Calculated for C22H13ClNO3S (%) = 430 [M]+.

4.2.12. 3-Hydroxy-1-(4-chlorophenyl)-4-(2-thienylcarbonyl)-5-(4-bromophenyl)-2,5-dihydro-1H-pyrrrol-2-one (D12)

White powder; yield: 80%; mp = 230 °C; IR (KBr): ν = 3161, 1711, 1670, 1666, 1492, 1412, 1371, 1279, 1284, 992, 883, 759, 722 cm⁻¹; 1H NMR (200 MHz, DMSO-d₆); δ = 6.34 (s, 1H, CH), 7.18–7.28 (m, 3H, CH₃), 7.34–7.43 (m, 4H, CH₆), 7.66 (d, 2H, J = 9.0 Hz), 7.97 (dd, 1H, J = 4.9, 1.1 Hz), 8.09 (dd, 1H, J = 3.8, 1.1 Hz), 11.87 (br. s, 1H, OH_mol) ppm; 13C NMR (50 MHz, DMSO-d₆); δ = 21.1, 60.9, 120.2, 124.6, 128.9, 129.0, 129.2, 130.0, 130.2, 133.1, 135.1, 135.7, 144.3, 149.6, 165.0, 172.5 and 180.6 ppm; ESI-MS: m/z (%) = 475 [M]+. Calculated for C23H15BrCINO3S (%) = 513.13, H 2.76, N 2.95; found: C 52.22, H 2.74, N 2.96.

4.2.13. 3-Hydroxy-1-(3-nitrophenyl)-4-(2-thienylcarbonyl)-5-(4-benzoylphenyl)-2,5-dihydro-1H-pyrrrol-2-one (D13)

Yellow powder; yield: 73%; mp = 209 °C; IR (KBr): ν = 3159, 1681, 1666, 1666, 1496, 1414, 1383, 1243, 1172, 1091, 984, 846, 755, 715 cm⁻¹; 1H NMR (200 MHz, DMSO-d₆); δ = 4.92 (s, 2H, OCH₂), 6.28 (d, 1H, J = 4.5 Hz, CH₂), 6.82 (d, 2H, J = 8.5 Hz, CH₂), 7.16–7.52 (m, 10H, CH₆), 7.97–8.07 (m, 2H, CH₃), 11.68 (br. s, 1H, OH_mol) ppm; 13C NMR (50 MHz, DMSO-d₆); δ = 61.1, 115.0, 124.7, 128.2, 128.3, 128.6, 128.8, 129.0, 129.1, 129.5, 129.9, 132.3, 135.1, 135.6, 137.3, 144.0, 149.8, 156.0, and 180.9 ppm; ESI-MS: m/z (%) = 513 [M]+. Calculated for C₂₃H₁₇NO₅S (%) = 365.62, H 3.93, N 5.47; found: C 56.71, H 3.91, N 5.44.

4.2.14. 3-Hydroxy-1-(4-chlorophenyl)-4-(2-thienylcarbonyl)-5-(4-benzoylphenyl)-2,5-dihydro-1H-pyrrrol-2-one (D14)

Yellow powder; yield: 77%; mp = 242 °C; IR (KBr): ν = 3226, 1689, 1678, 1609, 1512, 1382, 1248, 1172, 988, 859, 753, 722 cm⁻¹; 1H NMR (200 MHz, DMSO-d₆); δ = 4.91 (s, 2H, OCH₂), 6.38 (s, 1H, CH), 6.83 (d, 2H, J = 8.6 Hz, CH₂), 7.20–7.35 (m, 8H, CH₆), 7.89–8.08 (m, 4H, CH₂), 8.18 (d, 2H, J = 9.2 Hz, CH₂) ppm; 13C NMR (50 MHz, DMSO-d₆); δ = 60.8, 69.4, 114.8, 121.2, 122.0, 124.4, 127.5, 127.9, 128.5, 128.8, 129.1, 134.9, 135.0, 135.6, 142.2, 143.6, 147.8, 147.9, 158.2, 165.3 and 180.4 ppm; ESI-MS: m/z (%) = 513 [M]+. Calculated for C₂₃H₁₇NO₅S (%) = 365.62, H 3.93, N 5.47; found: C 65.70, H 3.92, N 5.49.

4.2.15. 3-Hydroxy-1-(4-chlorophenyl)-4-(2-thienylcarbonyl)-5-(4-benzoylphenyl)-2,5-dihydro-1H-pyrrrol-2-one (D15)

Yellow powder; yield: 79%; mp = 205 °C; IR (KBr): ν = 3178, 1682, 1667, 1607, 1512, 1496, 1384, 1244, 1173, 1092, 985, 847, 756, 716 cm⁻¹; 1H NMR (200 MHz, DMSO-d₆); δ = 4.93 (s, 2H, OCH₂), 6.28 (s, 1H, CH), 6.83 (d, 2H, J = 8.8 Hz, CH₂), 7.21–7.39 (m, 10H, CH₂), 7.67 (d, 2H, J = 8.9 Hz, CH₂), 7.97 (d, 1H, J = 4.9 Hz, CH), 8.07 (d, 1H, J = 3.8 Hz, CH₂), 11.93 (s, 1H, OH_mol) ppm; 13C NMR (50 MHz, DMSO-d₆); δ = 61.2, 69.7, 115.0, 115.8, 120.8, 124.7, 128.1, 128.2, 128.3, 128.6, 128.9, 129.1, 129.5, 129.8, 132.3, 133.5, 135.6, 135.7, 137.3, 144.4, 149.0, 156.8, 165.0 and 180.8 ppm; ESI-MS: m/z (%) = 502 [M]+. Calculated for C₂₃H₁₇NO₅S (%) = 366.99, H 4.02, N 2.79; found: C 67.04, H 3.99, N 2.77.

4.3. X-ray crystal structure determination

Single-crystal X-ray diffraction data for D2 were collected at an Oxford Gemini S diffractometer equipped with a CCD detector, using monochromatized Mo Kα radiation (λ = 0.71073 Å). Data reduction and empirical absorption correction were performed with CrysalisPro [46]. The structure was solved by direct methods using SHELXS and refined on F² by full-matrix least-squares using SHELXL [47]. All non-
hydrogen atoms were refined anisotropically. One of two DMSO molecules is disordered over two positions of the S3 atom with occupation numbers of 0.653 and 0.347.

The H atom bonded to the O2 atom from hydroxyl group was located in difference Fourier map and refined isotropically. The rest of the H atoms were placed in geometrically calculated positions and refined using the riding model with Uiso values constrained to 1.2Ueq or 1.5Ueq of the parent C atoms. The PARST [48] and PLATON [49] software were used to perform the geometrical calculation, while ORTEP3 [50] and was employed for molecular graphics. Crystallographic details for structure analysis of the D2 compound are summarized in Table S2.

4.4. Cell lines and culture

Healthy human fetal lung fibroblasts (MRC-5) and two human cancer cell lines: cervix adenocarcinoma cells (HeLa) and breast adenocarcinoma cells (MDA-MB-231) were used for cytotoxicity screening of fifteen, previously synthesized, organic compounds. All cell lines were purchased from American Type Culture Collection (ATCC).

Maintenance of HeLa and MRC-5 were carried out using Dulbecco’s modified Eagle’s medium (DMEM), while MDA-MB-231 was maintained in Roswell Park Memorial Institute medium (RPMI-1640). Both media were supplemented with 10% heath-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), non-essential amino acids (0.1 mM), penicillin (100 IU/mL) and streptomycin (100 μg/mL). Cells were kept as a monolayer culture in 25 cm² plastic flask (Greiner Bio-One, Austria) under standard culture conditions of 37 °C, humidified air and 5% CO2. The media was changed every three days and cells were subcultured when necessary (0.05% trypsin – 0.53 mM EDTA). Depending on the type of experiment, cells were seeded in 96- or 24-well microtiter plates (Thermo Scientific, New York, NY).

4.5. Cytotoxicity assays

Effects of 3-hydroxy-3-pyrrolin-2-on derivatives (D1-D15) on HeLa, MDA-MB-231 and MRC-5 cells viability were evaluated by two colorimetric assays: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium bromide) and SRB (sulfurhodamine B). In all experiments, cells were plated in 96-well plates at the density of 3x10^3 cells per well and incubated to adhere overnight.

4.6. MTT assay

The MTT assay, based on the reduction of the yellow tetrazolium salt to a purple formazan dye by succinate-tetrazolium reductase, which exists in the mitochondrial respiratory chain and is active only in viable cells, was firstly described by Moshman [30]. After adherence, in the first stage (screening), medium was removed and cells were treated with synthesized compounds (D1-D15) in single dose at concentration 100 μM or media alone, and in the second stage, with selected compounds which exhibited the best inhibitory effect in screening stage, at concentration 1 μM, 3 μM, 10 μM, 30 μM, 100 μM or media alone. After 24 h and 48 h incubation with tested compounds, the medium was replaced with 100 μL of MTT (0.5 mg/mL PBS) and incubated for at least 2 h. Thereafter, colored formazan crystals were dissolved with 150 μL of DMSO. Absorbance was measured at 590 nm with a multiplate reader (Zenyth3100, Anthos Labtec Instruments GmbH, Austria). Cytotoxicity was calculated according to the formula: \(1 - \frac{A_{test}}{A_{control}} \times 100\). The IC50 values of compounds were calculated using Microsoft Office Excel free add-in ED50plus v1.0 software downloaded from: http://www.sciencegateway.org/protocols/cellbio/drug/data/.

4.7. SRB assay

Cytotoxicity was assessed by SRB assay as described by Skehan et al. [31] Effects of the selected 3-hydroxy-3-pyrrolin-2-on derivatives on cell viability was examined on HeLa, MDA-MB 231 and MRC-5 in concentration of 1 μM, 3 μM, 10 μM, 30 μM, 100 μM during 24 h and 48 h period of incubation. The method of plating and incubation was identical as it was in MTT until the end of incubation. Briefly, treatment was terminated by through fixing cells with 100 μL 10% trichloroacetic acid (TCA) for 1 h at +4 °C. This was followed by five washings with distilled water and staining using 0.1% SRB dissolved in 1% acetic acid at least for 15 min on room temperature. Subsequently, plates were washed four times with 1% acetic acid, air-dried and bound dye was solubilized with 150 μL 10 mM unbuffered Tris base (pH = 10.4) for 5 min on the gyratory shaker. Absorbance was measured at 510 nm with a multiplate reader (Zenyth 3100, Anthos Labtec Instruments GmbH, Austria). Cytotoxicity and IC50 were calculated according to the same formula as for MTT assay.

4.8. Flow cytometric analysis

HeLa and MDA-MB-231 cells were cultured overnight for adherence in 24-well plates at a density 1 × 10^5 per well. Cells were treated with the selected 3-hydroxy-3-pyrrolin-2-ones in concentrations corresponding to their IC50 values or in media alone (control) during 48 h at 37 °C in an atmosphere of 5% CO2 and absolute humidity. In some experiments, 20 μM chloroquine (CQ) was added to both untreated and treated cells 24 h after addition of tested substances. After incubation, the cell was collected and examined for the type of cell death and cell cycle perturbations induced by selected organic compounds.

4.9. Annexin V-FITC/7-AAD assay

According to the manufacturer’s instruction, Annexin V-FITC/7-AAD Kit (Beckman Coulter, USA) was used to determine a type of cell death induced by the selected 3-hydroxy-3-pyrrolin-2-ones. After designated treatment, both attached and detached HeLa and MDA-MB-231 cells were collected and washed in PBS. Afterward, cells were resuspended in 100 μL of ice-cold binding buffer (1 × 10^5/100 μL), stained with 10 μL of Annexin V-FITC and 20 μL 7-AAD and incubated in dark on room temperature for 15 min. Finally, Binding buffer (400 μL) was added to each tube and the samples were analyzed by flow cytometer Cytomics FC500 (Beckman Coulter, USA). Data for different cell populations (viable, necrotic, early and late apoptotic cells) were analyzed using Flowing Software (http://www.flowingsoftware.com/) and presented by dot plots.

4.10. Cell cycle analysis

After treatment, as previously described, perturbation of cell cycle was examined for HeLa and MDA-MB-231 cells. Briefly, cells were collected, washed in PBS and fixed overnight with 1 ml of ice-cold 70% ethanol at +4°C. Then, cells were washed, resuspended in PBS with the addition of RNase A (500 μg/ml PBS) and incubated for 30 min at 37 °C, followed by staining with 5 μL of propidium iodide (10 mg/ml PBS) during 15 min in dark. After incubation, samples were analyzed by flow cytometer Cytometric F500 and the data were analyzed using FlowJo Software. Results were presented by histograms.

4.11. Detection and quantification of acidic vesicular organelles (AVOs) with acridine orange

Acridine orange is cell permeable fluorescent dye that, excited by blue light, emits green fluorescence when it is localized in cytoplasm and nucleus, but emits red fluorescence within compartments with low pH, lysosomes, and phagolysosomes. For autophagy detection, treated and untreated (control) HeLa and MDA-MB 231 cells were trypsinized, washed in PBS and stained with acridine orange (1 mg/ml) for 15 min. Thereafter cells were washed in PBS and analyzed by flow cytometer Cytometric F500. The data were analyzed using Flowing Software.
Accumulation of acidic vacuoles (AVOs) was quantified as red/green fluorescence ratio, i.e. the proportion of mean red and green fluorescence intensity (FL3/FL1).

4.12. Viscosity measurements

The viscosity of aqueous DNA solution was measured in the presence of increasing amounts of D13 and D15 using Ubbelohde viscometer (SI Analytics GmbH, Mainz, Germany, type no. 525 03) by measuring the flow rate. Viscosimeter was filled with experimental liquid and placed vertically in a glass-sided thermostat maintained constant to ± 0.01 K, with a standard uncertainty of controlled temperature of ± 0.02 K. Flow time was recorded with a digital stopwatch with a standard uncertainty of ±0.001 s, after thermal equilibrium was obtained. All measurements were performed at 310.15 K. Results were obtained as the mean value of at least ten viscosity measurements, and the mean value of flow time was considered for further analysis. The viscosity values were calculated from the observed flow time of DNA containing solutions (t) corrected for that of buffer alone (t0), where n is the viscosity of DNA in the presence of ligand, n0 is the viscosity of DNA alone in the buffer solution, and R is molar ratio of ligands/DNA. The DNA concentration was fixed at 1·10 −5 mole/m³. The viscosity values were calculated from the observed flow time of DNA containing solutions (t) corrected for that of buffer alone (t0), where n is the viscosity of DNA in the presence of ligand, n0 is the viscosity of DNA alone in the buffer solution, and R is molar ratio of ligands/DNA. The DNA concentration was fixed at 1·10 −5 mole/m³.

The viscosity values were calculated from the observed flow time of DNA containing solutions (t) corrected for that of buffer alone (t0), where n is the viscosity of DNA in the presence of ligand, n0 is the viscosity of DNA alone in the buffer solution, and R is molar ratio of ligands/DNA. The DNA concentration was fixed at 1·10 −5 mole/m³.

4.13. Computational method

For the docking procedure, the crystallographic 3-dimensional structures of B-DNA dodecamer and bovine serum albumin (BSA) were obtained from the protein data bank, PDB ID: 1BNA and 3V03, respectively. The receptors were prepared using the protein preparation wizard by involving parameters like assigning bond orders to hydrogens, capping the termini and desolvation by deleting the crystallized free water molecules beyond 5 Å. The validity of the structure was checked and adjusted by adding missing residues and loops applying Prime included in the Schrödinger suite package [51]. The protonation and tautomeric states of residues were adjusted to match pH of 7 using PROPKA [52]. The DNA and BSA hydrogen bonds were optimized and minimized applying the force field OPLS 2005 [53]. The Glide module was used for generation of receptor-active pocket and this was further used for the docking studies [54].

Receptor and ligand docking studies were carried out using the ligand docking module from the glide application in the Schrödinger suite. This module is a grid-based method and gives scores based on the formation of favorable interactions between ligand and receptor. The prepared ligands were docked into the grid enclosed active pocket of the protein using the extra precision mode. Flexible ligand sampling was applied in the docking procedure. The receptor-ligand complex interactions were calculated based on the quality of geometric contacts and their energy. All poses were subjected to post-docking minimization. The best-enclosed structures for each ligand were determined, based on the model energy score which combines the energy grid score, the binding affinity, the internal strain energy, and the Coulomb-van der Waals interaction energy scores [55].

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.биоorg.2019.102954.