Original research article

Design and drug-like properties of new 5-methoxysalicylaldehyde based hydrazones with anti-breast cancer activity

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

Novel benzoylhydrazones were designed and synthesized by condensation of 5-methoxysalicylaldehyde and benzohydrazides with different substituents at 4th position. The structures of the new derivatives were confirmed by elemental and thermal analysis, mass, IR, \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectroscopy. The molecular properties of the compounds, important for drug pharmacokinetics and biodisposition in the human body, were assessed by the Lipinski’s rule of five. In silico evaluation of the LogP value and the remaining parameters of drug similarity, as well as the topological polar surface area and absorption percentage, were used only as a first step in the study. The investigated 5-methoxyderivative hydrazones were further tested for \textit{in vitro} cytotoxicity on three leukemic, two breast cancer and one non-tumor human cell lines using the MTT-dye reduction assay. The bioassay demonstrated that the compounds exhibited concentration-dependent antiproliferative activity at low micromolar concentrations against the used human cell lines. The solid tumor-derived breast cancer cell lines were generally more sensitive to the effects of the hydrazones with IC\textsubscript{50} values ranging 0.91 \textmu M–12.07 \textmu M. The results confirm that all compounds are more potent than the standard drug Melphalan and have appropriate properties as potential anti-breast cancer drug candidates.

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\section*{Introduction}

Cancer has severe health consequences and it is a leading cause of death. A defining feature of the cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. Cancer cells tend to form new cells more quickly than normal cells and this makes them a better target for chemotherapy drugs. Some of the anti-cancer drugs keep the cells from reproducing by damaging their DNA. Unfortunately, however, known drugs cause many untoward side effects and researchers are still trying to produce new ones to reduce toxic side effects, to tailor the drug to particular forms of cancer, and combat drug resistance.

Thousands of new biologically active compounds have been developed over the last decades. A significant part of them belongs to the group of hydrazones since many hydrazone derivatives have shown to exhibit anti-cancer properties (Becker et al., 2003; Chaston et al., 2004; Dandawate et al., 2014; Kalinowski and Richardson, 2005; Richardson and Milnes, 1997; Richardson et al., 2009; Savini et al., 2004). Especially effective anti-proliferative agents are the hydrazones derived by condensation reaction of salicylaldehyde and different acid hydrazides (Johnson et al., 1982; Kalinowski and Richardson, 2005; Lovejoy and Richardson, 2002; Richardson and Milnes, 1997). One of the compounds, salicylaldehyde benzoylhydrazone (SBH), has been shown to inhibit DNA synthesis and cell growth in a variety of cultured human and rodent cells (Johnson et al., 1982; Richardson and Milnes, 1997). Various derivatives of SBH with a number of functional groups have been created in order to discover new bioactive compounds with high antitumor activity and minimal toxicity (Chaston et al., 2004; Kalinowski and Richardson, 2005; Lovejoy and Richardson, 2002; Nikolova-Mladenova et al., 2011a,b). The substitutions slightly change the parent compound but give rise to a diversity of biological effects and various pharmacological and potentially therapeutic properties. The investigations demonstrated that the presence of methoxy group in salicylaldehyde results in derivatives with high antiproliferative and antioxidant activity (Hristova-Avakumova et al., 2015; Nikolova-Mladenova et al., 2011a,b).
Recently, the synthesis of some new 3-methoxy salicylaldehyde benzoylhydrazones and evaluation of their antiproliferative effect on a wide spectrum of human tumor cell lines was reported. All compounds exhibited excellent cytotoxic effects and 3-methoxysalicylaldehyde isonicotinoylhydrazone was found to be the most active cytotoxic agent against all tested cell lines, showing low micromolar IC_{50} values (Nikolova-Mladenova et al., 2011a). During the last years, the pharmaceutical chemists used some modern in silico tools in drug discovery to find new drug-like compounds. The development of novel "drug-like" compounds significantly increased the number of potential drug candidates requiring in vitro and in vivo evaluation but this is a long and costly process (DiMasi et al., 2003). During the last years, the pharmaceutical chemists used some modern in silico tools in drug discovery to find the lead compounds and to reduce the number of in vivo studies required (Thomas et al., 2006). In silico pharmacology enables the design of lots of compounds that can be screened against potential targets and determines the most capable ones. The "drug-like" molecules need to have appropriate physicochemical properties, namely molecular weight, electronic distribution, lipophilicity, hydrogen bond donors/acceptors, solubility and other related properties. The lipophilicity of the compounds affects mainly their membrane permeability and oral bioavailability. Widely accepted measure of lipophilicity is LogP and compounds demonstrating LogP > 3.5 usually have poor aqueous solubility (Dehring et al., 2004). Decreasing of lipophilicity improves solvation potential by increasing solvent-solute interactions in aqueous media. In general, values of LogP among 2 and 3 provide a good balance between water solubility and lipophilicity of the compounds and ensure a good permeability and bioavailability.

Materials and methods

Materials

5-Methoxysalicylaldehyde (1), benzhydrazide (2), 4-hydroxybenzhydrazide (3) and isonicotinoyl hydrazide (4) used for the preparation of the 5-methoxysalicylaldehyde benzoylhydrazone (5), 5-methoxysalicylaldehyde 4-hydroxybenzhydrazide (6) and 5-methoxysalicylaldehyde isonicotinoylhydrazone (7), were purchased from commercial sources (Merck, Sigma-Aldrich) and were used without any further purification. All other chemicals used were of analytical reagent grade.

The carbon, nitrogen and hydrogen contents of the new compounds were determined by elemental analyses on a "Euro Vector SpA" analyzer. The melting points were measured in open capillary tubes using a Büchi B-540 apparatus. The thermogravimetric analyses (TG, DTG and DTA) were performed on a "Perkin Elmer" Diamond DSC Calorimeter in air atmosphere with a heating rate of 10 °C/min. The thermal studies have been carried out in the temperature range 50–800 °C. Mass spectra were measured on a Thermo Scientific Q Exactive Plus mass spectrometer. The IR spectra were recorded on a Thermo Scientific Nicolet IS10 FT-IR Spectrometer using ATR technique in the range of 4000–400 cm⁻¹. The 1H NMR spectra were recorded on a Bruker Avance DRX 250 spectrometer in dimethyl sulfoxide DMSO-d₆ as solvent, chemical shifts (δ) are reported in parts per million (ppm), J values are given in Hz. Splitting patterns were indicated by the symbols: s (singlet), d (doublet), t (triplet) and m (multiplet).

Design of 5-methoxysalicylaldehydes

The common route for the synthesis of aroylhydrazones is the condensation of suitable aldehydes with acid hydrazides. The parent compound SBH was received by reaction of salicylaldehyde and benzhydrazide (Lyubchova et al., 1995). The investigated series of six compounds was designed by consecutively incorporation of a methoxy-group on 5th position in salicylaldehyde and varying the type of the substituents at 4th position in benzhydrazide. The structures of the 5-methoxyderivatives and SBH are present in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Comp</th>
<th>R</th>
<th>LogPₐ &lt;5</th>
<th>LogPₐ &lt;5</th>
<th>Mw &lt;500</th>
<th>O,N &lt;10</th>
<th>OH,NH &lt;5</th>
<th>Rotat Bonds</th>
<th>Volume</th>
<th>TPSA (Å² &lt;140</th>
<th>% ABS</th>
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<tr>
<td>5</td>
<td>C—H</td>
<td>3.07</td>
<td>3.18</td>
<td>270.29</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>243.66</td>
<td>70.92</td>
<td>84.53</td>
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<tr>
<td>6</td>
<td>C—OH</td>
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<td>2.84</td>
<td>286.29</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>251.68</td>
<td>91.15</td>
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<tr>
<td>7</td>
<td>N</td>
<td>1.78</td>
<td>2.28</td>
<td>271.28</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>239.50</td>
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<td>3.21</td>
<td>300.31</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>269.21</td>
<td>80.16</td>
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<tr>
<td>9</td>
<td>C—OC₆H₄</td>
<td>3.50</td>
<td>3.64</td>
<td>314.34</td>
<td>6</td>
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<td>80.16</td>
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<tr>
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<td>6</td>
<td>260.22</td>
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<td>84.53</td>
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<td>3.11</td>
<td>240.26</td>
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<td>3</td>
<td>218.12</td>
<td>61.69</td>
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</tr>
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</table>

a Values calculated by Molinspiration.  
b Values calculated by VCCLAB.
Calculation of molecular properties

The molecular “drug-like” properties of 5-methoxyisalicylaldehyde derivative hydrazones, important for drug pharmacokinetics in the human body, were evaluated with the Lipinski’s rule of five (ROS), formulated by the medical chemist Christopher A. Lipinski (Lipinski, 2004; Lipinski et al., 2002). He concluded that a compound is more likely to be membrane permeable and easily absorbed by the body if it matches the following criteria: molecular weight (MW) – smaller than 500; lipophilicity, expressed as a quantity known as LogP (the logarithm of the partition coefficient between water and 1-octanol) – lower than 5; the number of groups that can accept hydrogen atoms to form hydrogen bonds (estimated by the sum of oxygen and nitrogen atoms) – smaller than 10; the number of groups in the molecule that can donate hydrogen atoms to hydrogen bonds (usually the sum of hydroxyl and amine groups in a molecule) – smaller than 5. LogP value is used in medicinal chemistry to assess the drug-likeness of a molecule and to predict the solubility of a potential drug. Molecular volume and molecular topological polar surface area (TPSA) are also very useful parameters for prediction of drug transport properties. The polar surface area is defined as a sum of surfaces of polar atoms (usually oxgens, nitrogens and attached hydrogens) in a molecule. These parameters have been shown to correlate very well with the human intestinal absorption, monolayers permeability, and blood-brain barrier penetration. Molecules with a polar surface area of greater than 140 Å² tend to be poor at permeating cell membranes (Palm et al., 1997). The number of rotatable bonds (Rotat. bonds) describes the molecular flexibility which influences the oral bioavailability (Veber et al., 2002).

The value of LogP and the remaining parameters of drug similarity, as well as the TPSA, were calculated by the method based on group contributions (Molinspiration Cheminformatics, 2016). These have been obtained by fitting the values of the calculated LogP with experimental LogP for a set of more than 105 compounds (Lin et al., 2003). The remaining parameters of drug similarity were additionally figured out by another method developed on the basis of neural network ensemble analysis of about twelve thousand organic compounds (Tetko et al., 2005; VCCLAB, 2001, 2016). The percentage of absorption was estimated using the Eq. (1) according to (Zhao et al., 2002).

% ABS = 109 – (0.345 × TPSA)

Synthesis of 5-methoxyhydrazones

A solution of 5-methoxyisalicylaldehyde (0.005 mol) in 96% ethanol (30 ml) was added to the solutions of benzhydrazide (0.005 mol), 4-hydroxybenzhydrazide (0.005 mol) and isonicotinic hydrazide (0.005 mol) in 50% aqueous ethanol (80 ml). The mixtures were stirred for 30 min and white precipitates were formed. An extra 96% ethanol (100 ml) was added and the suspensions were stirred for 15 min at 50 °C. The resulting solutions were allowed to cool and left to stay for 24 h at room temperature. During this time crystals of the products 5, 6 and 7 were obtained, then filtered off. The solid hydrazones were dried for 2 days in a vacuum desiccator.

5-Methoxyisalicylaldehyde benzoylhydrazone, (5)

Yield: 76%; mp: 162–163 °C; Color: Pale yellow; IR (v cm⁻¹): 3370 (OH), 3223 (NH), 1633 (C=O), 1603 (C=N), 1573 (C─NH). ¹H NMR (250 MHz, DMSO-d₆) δ ppm: 3.75 (s, 3H, —OCH₃), 6.89 (d, 2H, J=6.75 Hz, ArH₃), 7.12 (s, 1H, ArH₇), 7.55 (m, 3H, ArH₉), 7.93 (d, 2H, J=8 Hz, ArH₈), 8.65 (s, 1H, CH=–N), 10.72 (s, 1H, NH), 12.10 (s, 1H, OH). ¹³C NMR (250 MHz, DMSO-d₆) δ ppm: 55.49 (OCH₃), 112.28, 117.24, 118.20, 118.93, 131.92, 132.87, 147.59 (CH=–N), 151.47, 152.12, 162.87 (C=O). HR ESI-MS m/z: 271.0734 [M+H]⁺. Calculated for C₉H₈N₂O₄: C, 62.49; H, 5.59; N, 9.72. Found: C, 62.28; H, 5.33; N, 9.50.

5-Methoxyisalicylaldehyde-4-hydroxybenzoylhydrazone, (6)

Yield: 82%; mp: 246–247 °C; Color: Bright yellow; IR (v cm⁻¹): 3350 (OH), 3300 (OH), 3215 (NH), 1634 (C=O), 1602 (C=N), 1577 (C─NH). ¹H NMR (250 MHz, DMSO-d₆) δ ppm: 3.72 (s, 3H, —OCH₃), 6.88 (m, 2H, ArH₃), 7.09 (s, 1H, ArH₇), 7.83 (d, 2H, J=8.75 Hz, ArH₈), 8.57 (s, 1H, CH=–N), 10.24 (s, 1H, O–H₃), 10.71 (s, 1H, N─H), 11.89 (s, 1H, O–H₇). ¹³C NMR (250 MHz, DMSO-d₆) δ ppm: 55.47 (OCH₃), 112.52, 115.07, 117.18, 117.86, 118.95, 123.27, 129.72, 146.91 (CH=–N), 151.39, 152.08, 160.84, 162.47 (C=O). HR ESI-MS m/z: 287.10223 [M+H]⁺. Calculated for C₉H₈N₂O₄: C, 62.93; H, 4.93; N, 9.79. Found: C, 62.74; H, 4.95; N, 9.63.

5-Methoxyisalicylaldehyde isonicotinoylhydrazone, (7)

Yield: 87%; mp: 195–196 °C; Color: Bright yellow; IR (v cm⁻¹): 3350 (OH), 3219 (NH), 1636 (C=O), 1603 (C=N), 1573 (C─NH). ¹H NMR (250 MHz, DMSO-d₆) δ ppm: 3.73 (s, 3H, —OCH₃), 6.90 (d, 2H, J=8.25 Hz, ArH₃), 7.17 (s, 1H, ArH₇), 7.84 (d, 2H, J=6 Hz, ArH₈), 8.67 (s, 1H, CH=–N), 8.79 (d, 2H, J=6 Hz, ArH₈), 10.51 (s, 1H, NH), 12.22 (br s, 1H, O─H). ¹³C NMR (250 MHz, DMSO-d₆) δ ppm: 55.48 (OCH₃), 111.77, 117.31, 118.64, 119.83, 121.49, 140.04, 148.27 (CH=–N), 150.33, 151.50, 152.16, 161.36 (C=O). HR ESI-MS m/z: 272.10267 [M+H]⁺. Calculated for C₉H₈N₂O₄: C, 58.13; H, 5.23; N, 14.53. Found: C, 58.38; H, 5.11; N, 14.67.

Cell line and culture conditions

In the present study, we performed a comparative evaluation of the cytotoxic activity of the newly designed 5-methoxy derivative hydrazones on six human cell lines, namely HL-60 (acute myeloid leukemia), SKW-3 (T-cell leukemia), BV-173 (chronic myeloid leukemia), MDA-MB-231 (ER-negative breast carcinoma), MCF-7 (ER-positive breast adenocarcinoma) and HEK-293 (non-tumor human embryonic kidney cell line). The solid tumor cell lines (MDA-MB-231 and MCF-7) were grown as monolayer adherent cultures in 90% RPMI-1640 supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, 1 mM sodium pyruvate and 10 mg/ml human insulin. The other cell lines were cultured in suspension-type cultures under standard conditions – RPMI-1640 liquid medium supplemented with 10% FBS and 2 mM l-glutamine, in cell culture flasks, housed at 37 °C in an incubator “BB 16-Function Line” Heraeus with humidified atmosphere and 5% CO₂. The cells were kept in log phase by supplementation with a fresh medium after removal of cell suspension aliquots, two or three times a week.

Tumor cell growth inhibition (MTT-dye reduction assay)

The tumor cell growth inhibitory effects of the tested compounds were assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann (Mosmann, 1983). The method is based on the reduction of the yellow tetrazolium salt MTT to a violet formazan via the mitochondrial succinate dehydrogenase in viable cells. In brief, exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μl/well) at a density of 1 × 10⁵ cells per ml and after 24 h incubation at 37 °C they were exposed to
Apoptosis assay

The apoptotic patterns of internucleosomal DNA fragmentation was detected in BV-173 cells using a commercially available ‘Cell- death detection’ ELISA kit (Roche Applied Science). The procedure was carried out according to the manufacturer’s instructions. In brief, exponentially proliferating BV-173 cells were exposed to equieffective concentrations of the tested compounds (1/2 IC₅₀ or IC₅₀) for 24 h. Afterwards cytosolic fractions of 1 × 10⁴ cells per test group (treated or untreated) served as an antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was carried out at 405 nm, by means of a microprocessor controlled microplate reader (Labexam LMR-1) at 580 nm.

Data processing and statistics

The cell survival data were normalized as percentage of the untreated control (set as 100%). The statistical processing of biological data included the Student’s t-test whereby values of p ≤ 0.05 were considered as statistically significant. In addition IC₅₀ values were derived from the concentration-response curves using non-linear regression analysis.

Results and discussion

In silico evaluation of drug likeness

A series of six new 5-methoxysalicylaldehyde derived hydrazones was designed by varying the type of the substituents at 4th position in benzhydrazide. The calculated values of LogP and the remaining parameters of drug similarity, as well as the TPSA, used for evaluation of drug similarity on the basis of Lipinski’s rule are presented in Table 1. The calculations show that all 5-methoxysalicylaldehyde hydrazone derivatives observed boundary conditions of the “rule of Lipinski” and did not violate any of the listed criteria. Incorporation of various substituents in SBH molecule affects the value of LogP, i.e. modifies the lipophilicity of the compounds.

LogP values resulted by both the methods are comparable. The small differences are supposed to be due mainly to a difference in the databases exploited by the distinct software. SBH possesses balanced lipophilicity that is unaffected by the insertion of methoxy-group in salicylaldehyde moiety. This is evident from the insignificant change in log P value of 5 in respect to SBH. Much more noticeable is the influence of the substituents in the hydrazide ring. The introducing of methoxy, ethoxy and methyl group in benzhydrazide nuclei increases the log P value and respectively the lipophilicity of hydrazones 8, 9 and 10. By contrast, the lipophilicity of the hydrazones 6 and 7, containing a polar OH-group or pyridine nuclei is lower. The replacement of benzene nuclei by pyridine nuclei notably reduces the LogP with more than 1 unit.

The changes in other molecular properties are also of potential importance. The molecular weight is the most convenient way to define the molecular size. The substituents slightly increase the molecular weight but all 5-methoxy derived hydrazones remain small drug-like molecules with Mw between 270 and 314. The molecular volume correlates with Mw and gives additional information about the actual three-dimensional proportions of the molecules. It takes into account all the available conformations of the molecule under physiological conditions and relates to the rotateable bonds in the molecule (Pajouhesh and Lenz, 2005). The highest volume and the highest number of rotateable bonds are present in 9 followed by 8 and least in 5, 6, 7 and 10. The rotateable bonds define the molecular flexibility and the ease by which the molecules pass over the membranes (Veber et al., 2002). According to the results above, hydrazones 5, 6, 7 and 10 have smaller molecules which suppose good solubility and permeability as compact molecule is easier to absorb than extended one.

The topological polar surface area is used to predict the blood- brain barrier penetration (Feng, 2002). It depends on the number of hydrogen bond donors and acceptors. Compounds with high hydrogen bond forming potential have minimal distribution through the blood brain barrier. Hydrogen bonds increase the solubility in water and impede the passive diffusion through the lipid bilayer membrane. They must be broken in order for a compound to permeate the lipid bilayer membrane. The 5- methoxy hydrazones contain small number of hydrogen donors (2–3) and acceptors (5–6) which is the confirmation for their balanced lipophilicity. All derivatives show a TPSA smaller than 140 Å², indicating a good permeability of the compounds in the cellular plasma membrane. Most of them, with exception of the hydrazone 6, demonstrate a TPSA lower than 90 Å² and thus are capable to penetrate the blood-brain barrier (Hitchcock and

Scheme 1. Synthesis of the 5-methoxy-substituted hydrazones.
The percentage of calculated absorption (% ABS) ranged from 77.55 to 84.53 which is an indication of good bioavailability.

Lipinski's rule is based on the observation that the most medication drugs are relatively small and lipophilic molecules with values of log P among 2 and 3 which provide a good balance between water solubility and lipophilicity of the compounds and ensure a good permeability and bioavailability. Nevertheless of the small differences, all hydrazones have suitable lipophilicity and potentially good permeability across the cell membranes. However, the rule cannot predict the pharmacological activity of the compounds and we use it only as a preliminary screening. Hydrazones 5, 6 and 7 are the most encouraging compounds for potential drugs therefore they were chosen for synthesis.

Chemistry

The new hydrazones 5-methoxy salicylaldehyde benzoylhydrazone (5), 5-methoxy salicylaldehyde 4-hydroxybenzoylhydrazone (6) and 5-methoxy salicylaldehyde isonicotinoylhydrazone (7) were synthesized by the Schiff base condensation in ethanol between 5-methoxy salicylaldehyde (1) and appropriate hydrazides – benzhydrazide (2), 4-hydroxybenzhydrazide (3) and isonicotinic acid hydrazide (4) according to the Scheme 1.

The hydrazones were obtained in excellent yields and their structures were determined on the basis of various analytical and spectroscopic techniques. The melting points and the elemental analysis of the hydrazones along with their HR ESI–MS, IR, 1H NMR and 13C NMR data are given in the Experimental section.

The elemental analysis suggests the molecular formulas of the hydrazones. The resulted composition was also verified by mass spectra and thermal studies of the obtained compounds. The HR ESI–MS spectra of the hydrazones showed protonated molecular ions [M+H]+ that coincide with the proposed molecular formulas. TGA and DTA data were used to determine the content of H2O molecule and to research the thermal decomposition of the compounds.

TG/DTG and DTA curves of hydrazones 5 and 7 are presented in Fig. 1. The thermal decomposition of the both hydrazones takes place in three stages. The first is the stage of dehydration and it comes between 60 and 133 °C and 70–115 °C, respectively. The experimental mass loss of 6.10–6.15% (calc. 6.25%) is due to the loss of one H2O molecule and it is accompanied by the DTG endo peak at 100 °C. Sharp endothermic peaks at 162 °C and 200 °C, respectively, in the DTA curves without a weight loss observed are result of the melting of the hydrazones 5 and 7. The second stage, which occurs in the temperature range of 220–350 °C, and the third step at about 400–600 °C correspond to decomposition of the compounds. The two exothermic peaks in the range of 300–700 °C in the DTA curves indicate decomposition of the compounds because of intensive burning. The decomposition finishes at about 590–620 °C, respectively.

TG/DTG and DTA curves of hydrazone 6 are presented in Fig. 2. The compound displays a thermal stability behavior up to 240 °C which shows that it is anhydrous. A sharp endothermic peak at 241 °C in the DTA curve without a weight loss is due to melting. In contrast to 5 and 7, the thermal decomposition of 6 is carried out in two steps. The first step starts between 250 and 360 °C and the second is in the range 360–630 °C. The DTA curve shows two exothermic peaks in the range of 300–640 °C and the TG curve indicates complete decomposition of 6 at 630 °C.

The DTA and TGA data confirmed that hydrazone 6 is anhydrous, whereas 5 and 7 contain one H2O molecule.

The structures of the newly synthesized hydrazones were found out through IR and NMR spectroscopy. IR spectra of the hydrazones show intensive band around 1602–1603 cm−1 assigned to the azomethine group C=N which proves the condensation between the aldehyde group of 5-methoxy salicylaldehyde and the amino group of hydrazides in formation of the Schiff base. The medium intensity peak around 3350–3370 cm−1 and the weak broad band at 3215–3223 cm−1 were assigned to the phenolic hydroxyl group and NH group, respectively. The intensive characteristic bands at 1633–1636 cm−1 in the spectra of the ligands were assigned to the frequency vibration of the carbonyl group C=O and suggest the existence of the ligands in keto form in solid state. Another important band at 1573–1577 cm−1 was attributed to ν(C−NH). The hydrazones were further studied by their 1H NMR and 13C NMR spectra in deuterated dimethyl sulfoxide. 1H NMR spectra revealed the presence of the aromatic protons in the region δ 6.88–8.79. The protons of methoxy group appeared as a singlet at δ 3.72 – 3.75. Signals for the protons of the characteristic for hydrazones azomethine group HC=N— were observed at δ 8.65, 8.57 and 8.67, respectively for 5, 6 and 7. The broad singlets around δ 11.89 – 12.22 were assigned to the protons of the hydroxyl group from the aldehyde ring. 13C NMR spectra demonstrated the signals corresponding to the carbon atoms of azomethine group at δ 147.59, 146.91 and 148.27 respectively for 5, 6 and 7. The peaks at δ 162.87, 162.47 and 161.36 were assigned to the C = O group.

In vitro tumor cell growth inhibition

The hydrazones 5, 6 and 7 were further tested for in vitro cytotoxicity. The cell growth inhibitory effects of the newly synthesized compounds against three human leukemic cell lines.
HL-60 (acute myeloid leukemia), SKW-3 (T-cell leukemia), BV-173 (chronic myeloid leukemia), two breast cancer human cell lines MDA-MB-231 (ER-negative breast carcinoma) and MCF-7 (ER-positive breast adenocarcinoma) and non-tumor human cell line HEK-293 (embryonic kidney) were studied using the standard MTT-dye reduction assay for cell viability. Throughout the screening investigation the data about the new compounds were compared with the clinically used antineoplastic drug Melphalan (2-amino-3-[4-bis(2-chloroethyl) amino] phenylpropanoic acid).

The tested compounds inhibited the growth of tumor cells in a concentration-dependent manner, which enabled the construction of concentration-response curves (Figs. 3 and 4). In addition the corresponding IC_{50} values were derived in order to allow a quantitative merit for assessment of the relative potencies of the compounds under investigation. The IC_{50} values obtained are summarized in Table 2. Each data point represents the arithmetic mean ± standard deviation (sd) of at least eight independent experiments. IC_{50} values were calculated as concentrations of the tested compounds causing 50% decrease of cell survival.

As evident from the results obtained, the three 5-methoxyhydrazones showed different sensitivity to the both human leukemic cell lines HL-60 and SKW-3. The compound 5 exerted the most pronounced cytotoxic effect with IC_{50} values of 5.29 and 3.38 μM, respectively (Fig. 3, Table 2). The other two hydrazones, especially compound 6, have higher IC_{50} values. All 5-methoxyhydrazones induced practically total eradication of the malignant cell population at concentrations 50–100 μM (Fig. 3). The evaluation of the cytotoxic activity of the 5-methoxyhydrazones in BV-173 cells revealed that all hydrazones produced comparable cytotoxic effects on BV-173 cells, as according to the IC_{50} values 7 being slightly more active (Table 2). The treatment of BV-173 cells with 5, 6 and 7 at concentrations above 10 μM resulted in almost total eradication of the viable cells (cell survival fractions lower than 4%).

All of the novel 5-methoxyderived hydrazones exerted profound growth inhibitory activity upon solid tumor breast cancer cell lines MDA-MB-231 and MCF-7. The 5 and 7 significantly reduced the percentage of viable MDA-MB-231 cells and exhibited similar cytotoxic effects with IC_{50} of 4.46–5.58 μM (Fig. 4, Table 2). The IC_{50} values are much lower than that of Melphalan.

The assessed cell growth inhibitory effects in MCF-7 cells exposed that this solid tumor cell line was the most sensitive to the tested compounds. The IC_{50} values are much lower than those of Melphalan. The hydrazone 5 demonstrated superior growth inhibitory activity on MCF-7 cells with IC_{50} values of 0.91 μM as compared to compounds 6 and 7 (Table 2). It caused 50% cell
event, characteristic for this type of cell death. The data show mono- and oligonucleosomal DNA-fragments, a key downstream indicator of apoptosis. The cascade activation of caspases was evaluated by an ELISA-assay to quantify the extent of apoptotic DNA-fragmentation.

After exposure to the tested compounds, we observed tumor-growth inhibitory activity and determined the selectivity indices. The most selective compounds among the arylhydrazones exhibited selectivity indices greater than 1, as compared to the reference anticancer agent. From these results, we concluded that human embryonal kidney. Evident from the selectivity indices presented in Table 3, the compounds are capable of differentiating malignant cell lines and HEK-293. Nevertheless, in all of the arylhydrazones, the selectivity indices were greater than 1, as opposed to the reference anticancer agent. The most selective of the series was compound 6.

Finally, to elucidate the mechanistic aspects of the observed tumor-growth inhibitory effects, we performed a parallel MTT-bioassay in the non-tumor cell line HEK-293, originating from human embryonal kidney. The IC50 values are much lower than those of Melphalan.

Furthermore, in order to assess the tumor-selectivity of the observed cell-growth inhibitory effects, we performed a parallel MTT-bioassay in the non-tumor cell line HEK-293, originating from human embryonal kidney. Evident from the selectivity indices presented in Table 3, the compounds are capable of differentiating malignant cell lines and HEK-293. Nevertheless, in all of the arylhydrazones, the selectivity indices were greater than 1, as opposed to the reference anticancer agent. The most selective of the series was compound 6.

Finally, in order to elucidate the mechanistic aspects of the observed tumor-growth inhibitory effects, we performed an ELISA-assay to quantify the extent of apoptotic DNA-fragmentation after exposure to the tested compounds. The cascade activation of specific nucleases during the apoptotic process degrades the higher order chromatin structure of DNA into histone-associated mono- and oligonucleosomal DNA-fragments, a key down-stream event, characteristic for this type of cell death. The data from Table 4 firmly indicate that the exposure to the series of hydrazones leads to the characteristic pattern of DNA fragmentation, indicating that their mode of action is at least partly mediated by the induction of programmed cell death through apoptosis.

Conclusions

A series of six new 5-methoxysalicylaldehyde derived hydrazones was designed by varying the type of the substituents at 4th position in the benzhydrazide moiety. The values of LogP and the remaining parameters of drug similarity were calculated by in silico methods. The results indicated that all hydrazones have suitable lipophilicity and potentially good permeability across cell membranes. Hydrazones 5, 6 and 7 showed the most encouraging properties and were chosen for synthesis. The compounds were further tested for in vitro cytotoxicity against five human cell lines, representative for some important types of human cancer, and one non-tumor cell line using the standard MTT-dye reduction assay for cell viability. The overall results revealed that all tested hydrazones reach 50% inhibition of the malignant cells proliferation at micromolar concentrations and thus could be considered as promising lead compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 [μM] ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>SKW-3</td>
</tr>
<tr>
<td><strong>5</strong></td>
<td>5.29 ± 1.2</td>
</tr>
<tr>
<td><strong>6</strong></td>
<td>13.80 ± 3.1</td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>6.68 ± 1.4</td>
</tr>
<tr>
<td>Melphalan</td>
<td>18.50 ± 2.1</td>
</tr>
</tbody>
</table>

**Table 2**

Antiproliferative activity of the newly synthesized 5-methoxyhydrazone derivatives and the reference drug Melphalan after 72 h continuous incubation (MTT-dye reduction assay).

**Table 3**

Selectivity indices of the tested compounds and the reference agent.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean IC50 values in cancer cell lines [μM]</th>
<th>IC50 in the non-tumor cell line HEK-293 [μM]</th>
<th>Selectivity indices</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5</strong></td>
<td>3.44</td>
<td>3.92 ± 0.8</td>
<td>1.14</td>
</tr>
<tr>
<td><strong>6</strong></td>
<td>9.61</td>
<td>17.11 ± 1.4</td>
<td>1.78</td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>5.81</td>
<td>8.81 ± 1.2</td>
<td>1.51</td>
</tr>
<tr>
<td>Melphalan</td>
<td>28.84</td>
<td>24.81 ± 2.9</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Calculated from the IC50 data in Table 2.

b Selectivity indices are calculated as ratio between the IC50 in the non-tumor cell line and the mean IC50 in the panel of tumor cells.
biologically active. The breast cancer derived cell lines showed far more pronounced sensitivity to 5-methoxyhydrazones as compared to the referent drug. Cytotoxicity data exposed that the hydrazones 5 and 7 are the most potent which implies that the lack of substituents in hydrazide moiety probably is a prerequisite for optimal activity of these compounds. Remarkable is the activity of all hydrazones on the solid tumor cell line MCF-7 as hydrazone 5 is 37-fold more active than Melphalan. The selectivity indices of differentiation between malignant and non-tumor cell lines were greater than that of the reference anticancer drug. Besides the MTT-bioassay the compounds were tested for induction of programmed cell death, and proved to induce concentration-dependent increase in the levels of histone-associated DNA fragments, characteristic for apoptosis.

Conflict of interests

The authors report no conflict of interests.

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References


