Design, molecular properties and *in vitro* cytotoxic activity of 3,5-dichlorosubstituted salicylaldehyde benzoylhydrazones

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Abstract—Salicylaldehyde benzoyl hydrazone (SBH) belongs to a class of hydrazones of the type R'-CH=N-NH-CO-R which possess a high antiproliferative activity. The common method for the synthesis of SBH is the Schiff base condensation between salicylaldehyde and benzhydrazide. Various derivatives of SBH have been designed in order to discover new more effective antiproliferative compounds. The inserting of halogen atoms in the molecules of different hydrazones strongly influences the biological activity of the compounds.

Novel 3,5-dichlorosubstituted salicylaldehyde benzoyl-hydrazone derivatives were designed by varying the type of the substituents at 4th position of hydrazide moiety. The molecular properties of the compounds, important for drug pharmacokinetics in the human body, were assessed with the Lipinski's rule of five. *In silico* evaluation of the value of logP (partition coefficient) and the remaining parameters of drug similarity, as well as the topological polar surface area and absorption percentage, were used to find the lead candidates with encouraging properties for further elaboration. Some of the investigated 3,5-dichloro substituted hydrazones were further tested for *in vitro* cytotoxicity on a K-562 chronic myeloid leukemia cell line by MTT-test. The bioassay results demonstrated that the compounds exhibit concentration-dependent cytotoxic effects at low micro molar concentrations. The values of IC_{50} are less, but comparable to these of Cisplatin and much lower to these of Melphalan. The results confirm that the compounds are potential candidates for future drug discovery study.

Keywords—3,5-dichlorosalicylaldehyde; hydrazones; Lipinski's rule of five; log P; cytotoxicity

I. INTRODUCTION

Hydrazones comprise a vast group of biologically active compounds which has attracted the attention of organic and medicinal chemists due to their extensive range of pharmacological properties, such as anti-inflammatory [1,2], analgesic [2], anti-tuberculosis [3,4], antibacterial [5], antimicrobial [6], anti-HIV [6,7] and anticancer [6,8] activity. Hydrazones derived by condensation of salicylaldehyde and different acid hydrazides possess especially high antiproliferative activities [9-11]. 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 [11].

Optimization and modification of known bioactive structures by including many active groups and pharmacophores is widely used manner for discovery of new potential drugs. "Drug-like" compounds are molecules which contain functional groups and/or have physical properties consistent with the majority of known drugs, and hence can be inferred as compounds which might be active biologically or might show therapeutic potential [12,13]. Various derivatives of salicylaldehyde benzoylhydrazone have been synthesized in order to discover new bioactive compounds with high antitumor activity and minimal toxicity [8-10], [14-16]. One of the main disadvantages of this approach is that newly synthesized compounds tend to have higher molecular weight, high lipophilicity and low aqueous solubility which results in poor bioavailability. Therefore drug-likeness can be considered as a delicate balance among molecular properties affecting pharmacodynamics and pharmacokinetics of molecules which affects their absorption, distribution, metabolism, and excretion in human body like a drug.

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 [17]. During the last years, the pharmaceutical chemists used some modern *in silico* tools in drug discovery to find the lead compounds and reduce the number of *in vivo* studies required [18]. *In silico* pharmacology enables the design of lots of compounds that can be screened against potential targets and determines the most capable ones. The appropriate molecular physicochemical properties include molecular weight, electronic distribution, lipophilicity, hydrogen bond donors/acceptors, solubility, viscosity and other related properties.

The membrane permeability and oral bioavailability of the compounds depend mostly on their lipophilicity. Widely accepted measure of lipophilicity is log P and compounds demonstrating log P > 3.5 usually have poor aqueous solubility [19]. Decreasing of lipophilicity will improve solvation potential by increasing solvent-solute interactions in aqueous media. In general, values of log P among 2 and 3 provide a

good balance between water solubility and lipophilicity of the compounds and ensure a good permeability and bioavailability.

This paper presents the evaluation of *in silico* biological activity of novel 3,5-dichlorosubstituted salicylaldehyde benzoylhydrazone derivatives designed by varying the type of substituents at 4th position of hydrazide moiety. The important molecular properties were calculated to reveal how the incorporation of different substituents affects the lipophilicity of the compounds. Furthermore some of the investigated 3,5-dichloro substituted hydrazones were tested for *in vitro* cytotoxicity on a K-562 chronic myeloid leukemia cell line by MTT-test.

II.   MATERIALS AND METHODS

A. Design of 3,5-Dichloro Substituted Hydrazones

The common method for the synthesis of SBH is the Schiff base condensation between salicylaldehyde and benzhydrazide. The investigated series of five compounds was designed by consecutively incorporation of chlorine atoms on 3rd and 5th position in salicylaldehyde and replacement of H-atom on 4th position in benzhydrazide with hydroxyl-group, methoxy-group, methyl-group or chlorine-atom. The structures of 3,5-dichloro substituted derivatives are present in Table I.

TABLE I.   STRUCTURES OF 3,5-DICHLORO –DERIVATIVE HYDRAZONES

№	Compound	R
1	**3,5Cl SBH** 3,5-dichlorosalicylaldehyde benzoylhydrazone	H
2	**3,5Cl ShBH** 3,5-dichlorosalicylaldehyde-4-hydroxybenzoylhydrazone	OH
3	**3,5Cl SmBH** 3,5-dichlorosalicylaldehyde-4-methoxybenzoylhydrazone	OCH₃
4	**3,5Cl SmtBH** 3,5-dichlorosalicylaldehyde-4-methylbenzoylhydrazone	CH₃
5	**3,5Cl SclBH** 3,5-dichlorosalicylaldehyde-4-chlorobenzoylhydrazone	Cl

B. Calculation of Molecular Properties

The molecular "drug-like" properties of 3,5-dichlorosalicylaldehyde derivative hydrazones, important for drug pharmacokinetics in the human body, were evaluated with the Lipinski's rule of five (RO5), formulated by the medical chemist Christopher A. Lipinski [20,21]. He concluded that a compound is more likely to be membrane permeable and easily absorbed by the body if it matches the following criteria:

- Its molecular weight is less than 500;

- The compound's lipophilicity, expressed as a quantity known as log P (the logarithm of the partition coefficient between water and 1-octanol), is less than 5;

- The number of groups that can accept hydrogen atoms to form hydrogen bonds (estimated by the sum of oxygen and nitrogen atoms) is less 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) is less than 5.

Improved extensions of the RO5 related to the calculations of the Partition coefficient (log P range from −0.4 to +5.6) were also applied [22].

The value of log P and the remaining parameters of drug similarity, as well as the topological polar surface area (TPSA), were calculated by the method based on group contributions [23]. These have been obtained by fitting the values of the calculated log P with experimental log P for a set of more than twelve thousand, mostly drug-like molecules. The percentage of absorption was estimated using the equation (1) according to [24].

$$\% \ ABS = 109 - (0.345 \times TPSA) \qquad (1)$$

The results were compared with the properties of salicylaldehyde benzoylhydrazone.

C. Cell Line and Culture Conditions

The cell line used in this study, namely K-562, was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). K-562 is human chronic myelogenous leukemia cell line, established from the peripheral blood of a 53-year-old woman with chronic myeloid leukemia (CML) in blast crisis in 1970. The cells were grown as a suspension-type cultures under standard conditions – RPMI 1640 liquid medium supplemented with 10 % fetal bovine serum (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 % carbon dioxide. Cell cultures were maintained in logarithmic growth phase by supplementation with fresh medium two or three times weekly.

D. Cytotoxicity Assessment (MTT-dye Reduction Assay)

The cytotoxic activity of the tested compounds was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mossman [25]. 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 1×10^5 cells per ml and after 24 h incubation at 37 °C they were exposed to various concentrations of the tested compounds for 72 h. For each concentration at least 8 wells were used. After the incubation with the test compounds 10 μl MTT solution (10 mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C and the MTT-formazan crystals formed were dissolved by adding 100 μl/well 5 % HCOOH in 2-propanol. The MTT-formazan absorption was determined using a microprocessor controlled microplate reader (Labexim LMR-1) at 580 nm.
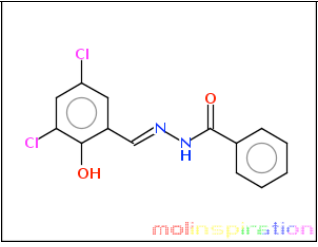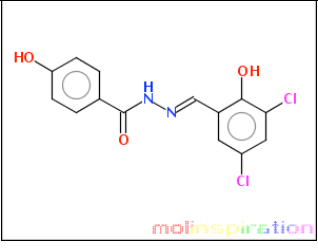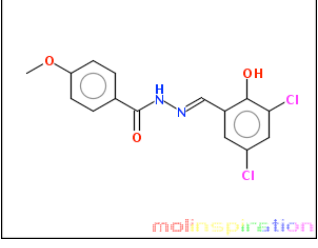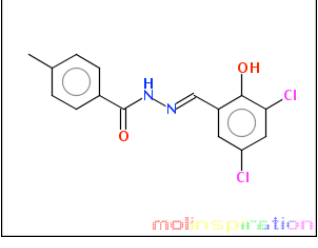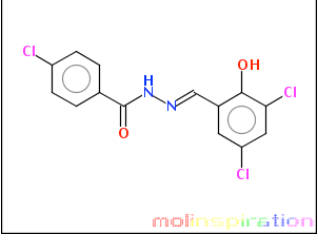
E. Data Processing and Statistics

The cell survival data were normalized as percentage of the untreated control (set as 100 % viability). The statistical processing of biological data included the Student's t-test whereby values of $p \leq 0.05$ were considered as statistically significant. IC_{50} values were derived from the concentration-response curves using non-linear regression analysis.

III. RESULTS AND DISCUSSION

A. In silico Evaluation of Drug Likeness

The compounds and their characteristics used for evaluation of drug similarity on the bases of Lipinski's rule are presented in Table II.

TABLE II.   CHEMICAL STRUCTURE AND PARAMETERS OF EVALUATION OF 3,5-DICHLOROSUBSTITUTED HYDRAZONES WITH LIPINSKI'S RULE OF FIVE

№	Compound	logP <5	MW	nO, N	nOH NH	nviola-tions	nrotb	atoms	volume	TPSA,Å² <140	% ABS
1		4.09	309.2	4	2	0	3	20	245.19	61.69	87.72
2		3.61	325.2	5	3	0	3	21	253.21	81.92	80.74
3		4.15	339.2	5	2	0	4	22	270.73	70.92	84.53
4		4.54	323.2	4	2	0	3	21	261.75	61.69	87.72
5		4.77	343.6	4	2	0	3	21	258.72	61.69	87.72

The value of log P is a measure of lipophilicity used in medicinal chemistry to assess the drug-likeness of a given molecule and to predict the solubility of a potential drug. Lipinski's rule is based on the observation that most medication drugs are relatively small and lipophilic molecules.

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 oxygen, nitrogen 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 [26]. The number of rotatable bonds (nrotb) describes the molecular flexibility which influences the oral bioavailability [27].

The calculations show that all 3,5-dichlorosalicylaldehyde derivative hydrazones observed boundary conditions of the "rule of Lipinski" and did not violate any of the listed criteria. Incorporation of various substituents in the molecule of SBH affects the value of log P, i.e. modify the lipophilicity of the compounds.

SBH possesses balanced lipophilicity and a value of log P is equal to 3.04. The inclusion of chlorine substituents in salicylaldehyde moiety increases the value of log P to 4.09. Much more noticeable is the influence of the substituents in the hydrazide ring. The inclusion of polar hydroxyl-group in the molecule of 3,5-dichlorosalicylaldehyde-4-hydroxybenzoyl-hydrazone decreases log P to 3.61, while the other three derivatives have higher lipophilicity. The compound 3,5-dichlorosalicylaldehyde-4-chlorobenzoylhydrazone which contains three chlorine atoms has the highest value of log P equal to 4.77.

All hydrazones demonstrate a TPSA of less than 140 Å², indicating a good permeability of the compounds in the cellular plasma membrane. The values are even lower than 90 Å² and thus the compounds are capable to penetrate the blood–brain barrier [28]. The percentage of absorption (% ABS) calculated ranged from 80.72 to 87.72.

Nevertheless of the small differences, all 3,5-dichlorosubstituted salicylaldehyde benzoylhydrazone derivatives have suitable lipophilicity with values of log P between 3.61 and 4.77 and potentially good permeability across cell membranes. However, the rule cannot predict the pharmacological activity of the compounds and we use it only as a preliminary screening.

B. In Vitro Cytotoxicity

Two of the hydrazones, 3,5-dichlorosalicylaldehyde benzoylhydrazone (3,5ClSBH) and 3,5-dichloro-salicylaldehyde-4-hydroxybenzoylhydrazone (3,5ClShBH) were synthesized and tested for *in vitro* cytotoxicity. The cytotoxic potential of the compounds against the human leukemic cell line K-562 (human chronic myeloid leukemia) was 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 referent agent Cisplatin and the clinically utilized antineoplastic drug Melphalan (2-amino-3-[4-bis(2-chloroethyl) amino] phenyl-propanoic acid).

The constructed concentration-response curves and the corresponding IC$_{50}$ values obtained are shown in Figs.1-2 and in Table III, respectively. 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.

The evaluation of the cell viability following 72 h treatment with 3,5-dichlorosubstituted salicylaldehyde benzoylhydrazone derivatives revealed that compounds exhibited concentration-dependent cytotoxic effects at low micro molar concentrations on K-562 cells.
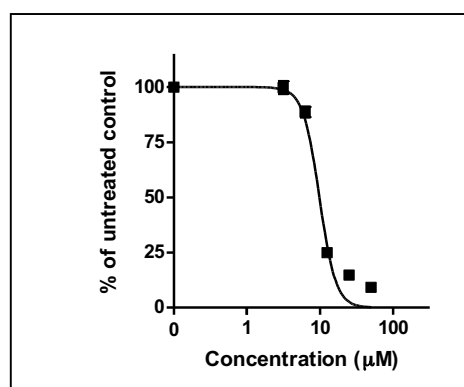

Fig. 1.   Cytotoxic effect of 3,5ClSBH as assessed by the MTT-dye reduction assay on K-562 cells.
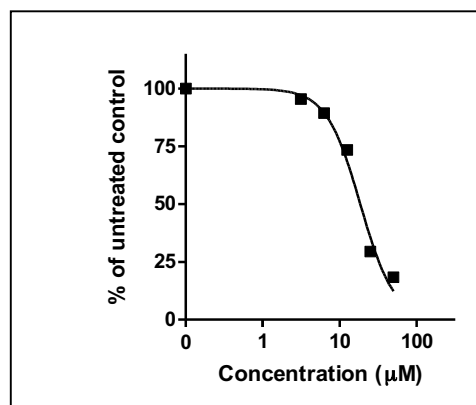

Fig. 2.   Cytotoxic effect of 3,5ClShBH as assessed by the MTT-dye reduction assay on K-562 cells.

TABLE III.   CYTOTOXIC ACTIVITY OF THE TESTED HYDRAZONES AFTER 72 H TREATMENT (MTT-ASSAY)

Compounds	IC_{50} (µmol/l) ± sd
3,5ClSBH	9.82 ± 1.7
3,5ClShBH	18.53 ± 2.2
Cisplatin	12.50 ± 2.4
Melphalan	28.2 ± 5.1

As evident from the results obtained, K-562 human leukemic cell line demonstrates high sensitivity to 3,5-dichloro-substituted hydrazones. The compound 3,5ClSBH exerted more noticeable cytotoxic effect with IC_{50} value of 9.82 µmol/l (Fig. 1, Table III). The IC_{50} values of the both hydrazones are much lower than this of Melphalan and for 3,5ClSBH even less than this of Cisplatin.

IV.   CONCLUSION

The values of log P and the remaining parameters of drug similarity were calculated by the "rule of Lipinski" for a series of five 3,5-dichlorosubstituted salicylaldehyde benzoyl-hydrazones. The rule was used as a first step in drug discovery to find the lead candidates with encouraging bioavailability properties. The results of *in silico* investigations indicated that all compounds observed boundary conditions of the method and have suitable lipophilicity and potentially good permeability across cell membranes.

The compounds were tested for *in vitro* cytotoxicity K-562 human chronic myeloid leukemia cell line. The analysis showed that 3,5-dichloro-hydrazones demonstrate high cytotoxic activity in micro molar concentrations against leukemia cell line. Based on the results of the MTT-dye reduction assay the compounds deserve more detailed toxicological and pharmacological investigations for the development of new anticancer drugs.

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