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Comparative evaluation of *in silico* and *in vitro* pharmacological activity of some 5-nitrosalicylaldehyde-derived hydrazones

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***Abstract*—Aroylhydrazones derived from salicylaldehyde are compounds with interesting biological properties including a high anticancer activity. Salicylaldehyde benzoylhydrazone (SBH) is an unusually potent inhibitor of DNA synthesis and cell growth in a variety of cultured human and rodent cells. Various derivatives of SBH have been synthesized in order to discover new more effective antiproliferative agents.**

In the present paper we report the comparative evaluation of *in silico* and *in vitro* biological activity of three recently synthesized 5-nitrosalicylaldehyde-derived hydrazones, namely 5-nitrosalicylaldehyde benzoylhydrazone (5nSBH), 5-nitrosalicyl-aldehyde-4-hydroxy-benzoylhydrazone (5nShBH) and 5-nitro-salicylaldehyde isonicotinoylhydrazone (5nSIH). The molecular properties of the studied compounds, important for drug pharmacokinetics in the human body, were evaluated with the Lipinski's rule of five. The value of logP and the remaining parameters of drug similarity were calculated by the method based on group contributions. All 5-nitro-derivatives observed boundary conditions of the rule and theoretically can be successful oral drugs. The rule is used only as a first step in drug discovery, to find the lead candidates for further elaboration and quickly eliminate the compounds with poor pharmacological properties.

In order to examine the antiproliferative effects of the investigated compounds they were tested for in vitro cytotoxicity on a HL-60 acute myeloid leukemia and BV-173 chronic myeloid leukemia cell lines. 5-nitro-derivatives exhibited concentration dependent cytotoxic effect on the leukemia cell lines. The values of IC_{50} for 5-nitrosalicylaldehyde benzoylhydrazone are comparable with these for the referent cytotoxic drug cisplatin, whereas the IC_{50} values for 5nShBH and 5nSIH are higher. Based on the results of the MTT-dye reduction assay the compound 5nSBH deserves more detailed toxicological and pharmacological investigation for the development of new anticancer drug.

Keywords—nitro-salicylaldehyde benzoylhydrazone; Lipinski's rule; oral drugs; lipophilicity; cytotoxic activity

I. INTRODUCTION

Hydrazones represent an important class of biologically active drug molecules which has attracted the attention of medicinal chemists due to their wide range of pharmacological properties. Hydrazones demonstrate anti-inflammatory [1,2], analgesic [2], antituberculosis [3,4], antibacterial [5], antimicrobial [6], anti-HIV [6,7] and anticancer [6,8] activity.

Aroylhydrazones of the type R-CO-NH-N=CH-R′ have been investigated as iron chelators needed to treat Fe overload diseases such as β-thalassemia. It was found that compounds derived from salicylaldehyde are effective for chemotherapy of iron overload [9]. Previous studies have shown that these hydrazones are also very effective antiproliferative agents. Salicylaldehyde benzoylhydrazone (SBH) is an unusually potent inhibitor of DNA synthesis and cell growth in a variety of cultured human and rodent cells [10-11].

A number of substituted derivatives have been synthesized as potential drugs by many researchers in order to raise the therapeutic properties with minimal toxicity [12-14]. Investigations aimed developing new biologically active hydrazones effective as oral drugs [15]. Oral administration is the most preferable route for drug administration because of its non-invasive nature and the fact that it avoids the pain and discomfort associated with injections. One of the major limitations to this approach is that the newly discovered compounds tend to have high molecular weight and lipophilicity with low aqueous solubility, resulting poor oral bioavailability. A widely accepted measure of lipophilicity is log P and compounds demonstrating log P > 3.5 generally have poor aqueous solubility [16]. In general, decreasing lipophilicity will improve solvation potential by increasing solvent-solute interactions in aqueous media. A common approach to the reduction of lipophilicity is through the introduction polar groups.

This article presents the comparative evaluation of *in silico* and *in vitro* pharmacological activity of novel hydrazones derived from 5-nitrosalicylaldehyde and containing polar nitro-group. The molecules have more drug-like structures compared to the parent lead compound salicylaldehyde benzoylhydrazone and current investigation was carried out in order to establish their value as prospective drug candidates.

II. MATERIALS AND METHODS

A. Calculation of Molecular Characteristics

The molecular properties of the 5-nitrosalicylaldehyde benzoylhydrazone and its derivatives, important for drug pharmacokinetics in the human body, were evaluated with the Lipinski's rule of five (RO5) [17, 18] which states, that most "drug-like" molecules have log P ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 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 [19].

The value of log P and the remaining parameters of drug similarity were calculated by the method based on group contributions [20]. These have been obtained by fitting calculated log P with experimental log P for a training set more than twelve thousand, mostly drug-like molecules. The results were compared with the properties of salicylaldehyde benzoylhydrazone SBH.

B. Cell Lines and Culture Conditions

The cell lines used in this study, namely HL-60 (acute myeloid leukemia) and BV-173 (chronic myeloid leukemia), were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). 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. Cells were kept in log phase by supplementation with fresh medium after removal of cell suspension aliquots, two or three times a week.

C. 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 [21]. 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 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.

D. 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. In addition IC_{50} values were derived from the concentration-response curves using non-linear regression analysis.

III. RESULTS AND DISCUSSION

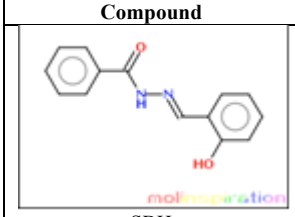
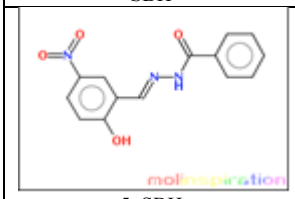
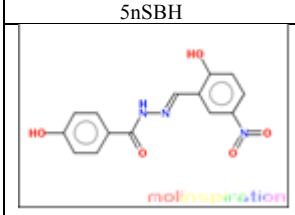
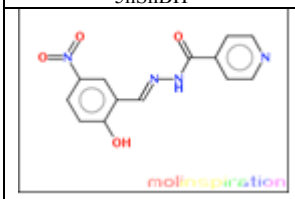
All compounds used in the present work, namely 5-nitro-salicylaldehyde benzoylhydrazone (5nSBH), 5-nitrosalicyl-aldehyde-4-hydroxybenzoylhydrazone (5nShBH) and 5-nitro-salicylaldehyde isonicotinoylhydrazone (5nSIH) were synthesized and characterized by Nikolova-Mladenova [22].

A. In silico Evaluation of Drug Likeness

Lipinski's rule is a rule for evaluation of drug-likeness of compounds, formulated by Christopher A. Lipinski, based on the observation that most medication drugs are relatively small and lipophilic molecules. The value of log P is one criterion used in medicinal chemistry to assess the drug-likeness of a given molecule and to predict the solubility of a potential oral drug.

The compounds and their characteristics used for evaluation of drug similarity on the bases of Lipinski's rule are presented in Table 1.

TABLE I. CHEMICAL STRUCTURE AND PARAMETERS OF EVALUATION OF NOVEL 5-NITROHYDRAZONE DERIVATIVES WITH LIPINSKI'S RULE OF FIVE

Compound	LogP	M_w	O,N	OH,NH
 SBH	3.039	240.262	4	2
 5nSBH	2.974	285.259	7	2
 5nShBH	2.495	301.258	8	3
 5nSIH	1.685	286.247	8	2

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

SBH has an average lipophilicity and value of log P equal to 3.039. The presence of the polar nitro group in salicylaldehyde moiety slightly decreases lipophilicity. Much more noticeable is the influence of the substituents on hydrazine ring. There is a clear trend – lipophilicity of hydrazone derived from benzhydrazide is the highest, it is lower in the derivative obtained from the 4-hidroxy-benzhidrazide and lowest for compound containing a pyridine nucleus.

It is important to note that the new hydrazones have suitable lipophilicity, values of log P between 1 and 3. The compounds are promising candidates to be further pursued as potential oral drugs. However, the rule cannot predict if a compound is pharmacologically active and we use it only as a preliminary screening.

B. In Vitro Cytotoxicity

The cytotoxic potential of the three novel 5-nitrosalicylaldehyde benzoylhydrazones against the human leukemic cell lines HL-60 (human promuelocytic leukemia) and BV-173 (pre-B cell lymphoma) 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] phenylpropanoic acid).

All of the tested 5-nitrohydrazone derivatives exhibited concentration-dependent cytotoxic effects after 72 h treatment of both HL-60 and BV-173 cells. The constructed concentration-response curves and the corresponding IC_{50} values obtained are shown in Figs.1-6 and in Table 2, respectively.

Each data point represents the arithmetic mean ± standart deviation (sd) of at least eight independent experiments. IC_{50} values were calculated as the concentrations of tested compounds causing 50 % decrease of cell survival.

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

Compounds	IC_{50} (µmol/l) ± sd	
	HL-60	***BV-173***
5nSBH	5.64 ± 1.09	2.39 ± 0.27
5nShBH	14.46 ± 2.14	7.62 ± 1.05
5nSIH	14.97 ± 2.41	10.19 ± 1.16
Cisplatin	4.70 ± 3.4	4.2 ± 2.1
Melphalan	18.5 ± 2.1	31.3 ± 3.9

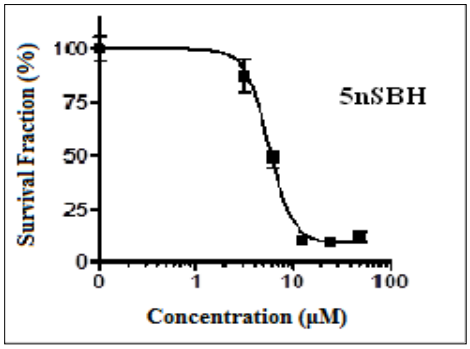


Fig. 1. Cytotoxic effect of 5nSBH as assessed by the MTT-dye reduction assay on HL-60 cells.

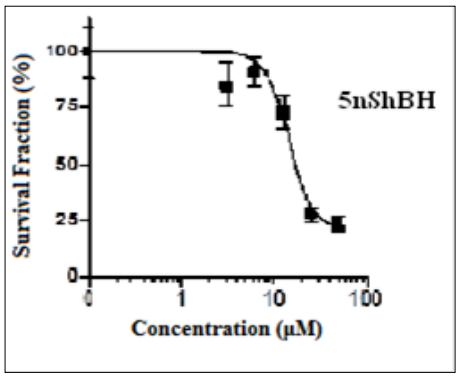


Fig. 2. Cytotoxic effect of 5nShBH as assessed by the MTT-dye reduction assay on HL-60 cells.

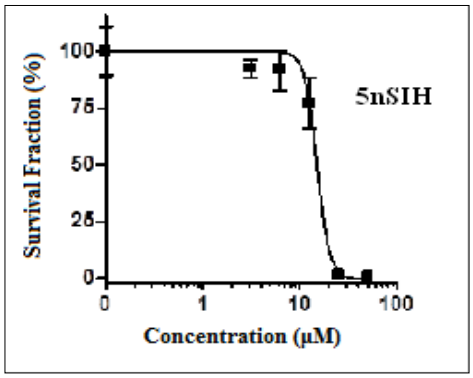


Fig. 3. Cytotoxic effect of 5nSIH as assessed by the MTT-dye reduction assay on HL-60 cells.

As evident from the results obtained, 5nSBH exerted the most pronounced cytotoxic effect against the myeloid HL-60 cells with IC_{50} value of 5.64 µmol/l (Fig. 1, Table II). The other two hydrazones under investigation were less active against HL-60 when applied at the concentrations 3.125-12.5 µmol/l. At the highest concentration of 50 µmol/l applied, 5nShBH showed profound maximal efficacy with less than 25 % viable cells and 5nSIH caused an almost absolute eradication of the malignant cells (Figs. 2 and 3). IC_{50} value for 5nSBH is comparable to this of Cisplatin, and the values of IC_{50} for hydrazones 5nShBH and 5nSIH are higher than this of Cisplatin, but lower to this of Melphalan.

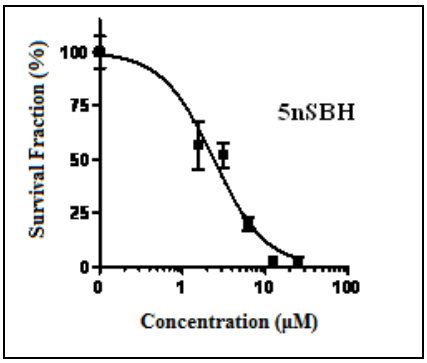


Fig. 4. Cytotoxic effect of 5nSBH as assessed by the MTT-dye reduction assay on BV-173 cells.

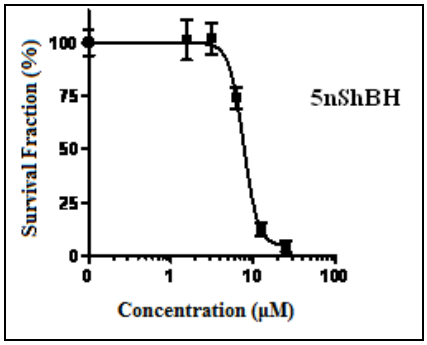


Fig. 5. Cytotoxic effect of 5nShBH as assessed by the MTT-dye reduction assay on BV-173 cells.

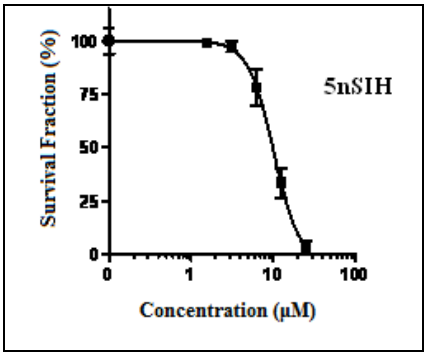


Fig. 6. Cytotoxic effect of 5nSIH as assessed by the MTT-dye reduction assay on BV-173 cells.

Leukemic cell line BV-173 exhibits high sensitivity to 5-nitrohydrazone derivatives. 5nSBH significantly reduced the percentage of viable cells by 44% even at the lowest applied concentration 1.56 µmol/l of the compound (Fig. 4, Table II). Hydrazones 5nShBH and 5nSIH have weaker suppressive action on the proliferation of cells at the low concentrations, but at a concentration of 12.5 µmol/l reduction of vital cells was 88 % and 67 % respectively (Figs. 5 and 6). All hydrazones have IC_{50} values lower than this of Melphalan as the value for 5nSBH is even less than this of Cisplatin.

The comparison of biological data amongst the novel hydrazones unambiguously indicates that 5nSBH is superior in terms of potency, causing 50 % inhibition of cellular viability at lower concentrations as compared to 5nShBH and the 5nSIH.

IV. Conclusion

The value of log P and the remaining parameters of drug similarity were calculated by the "rule of Lipinski". This rule is used as a first step in drug discovery to quickly find the lead candidates with encouraging bioavailability properties. 5-nitrohydrazone derivatives observed boundary conditions of the method and can be successful oral drugs according the rule.

The compounds were tested for in vitro cytotoxicity on two human leukemia cell lines HL-60 and BV-173. The analysis showed that all 5-nitro-hydrazones demonstrate high cytotoxic activity in micro molar concentrations against leukemia cell lines. Based on the results of the MTT-dye reduction assay the compound 5nSBH deserves more detailed toxicological and pharmacological investigation for the development of new anticancer drug.

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