Cancer cell growth inhibition by aroylhydrazone derivatives

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\textbf{ABSTRACT}

Hydrazones have versatile properties that make them promising for a range of possible applications. In this study, we examined a library of 17 aroylhydrazones derived from nicotinic acid hydrazide (1-12) and isonicotinic acid hydrazide (A-E) created by us for their biological activity. The antiproliferative activity of the compounds was investigated on non-tumour MCF-10A cells and cancer cell lines, MCF-7 and MDA-MB-231. Four compounds were selected as most active in cell growth inhibition of the tumour cell lines. These compounds, 5, 11, C and E, were tested on four additional cell lines: non-tumour BJ and cancer cell lines, HeLa, HepG2 and HT-29. Compounds 5 and E exhibited the highest selectivity index on cancer cell lines MDA-MB-231, HeLa and HepG2. High selectivity to MCF-7 cells was demonstrated with compound 5. Compound C was very selective to HepG2 cells as well as to MDA-MB-231 but to a lesser degree. Compound 11 showed selectivity against MDA-MB-231. The obtained results allow assessing the structure–activity relationship of the compounds and provide insight into the further development of this group of aroylhydrazones as more potent and selective anti-neoplastic agents.

\textbf{Introduction}

Over the last decades, hydrazones have been a subject of intensive investigations due to their versatile properties and possible applications in the field of analytical, organic and medicinal chemistry [1–3]. Due to the presence of an azomethine group (\(>C = \text{N-NH-CO}\)) hydrazones are considered as a special group of organic compounds, widely employed as ligands in coordination chemistry [4,5]. In addition, hydrazones and their metal chelates have a growing importance because of the wide spectrum of their biological activities such as analgesic [6], antimicrobial [7–10], anti-inflammatory [11], antifungal [12], anticonvulsant [13], antitubercular [14,15] and anticancer [2,10,16–21].

In particular, aroylhydrazones, which are easily formed by the condensation of aromatic hydrazides (arylamidrazides) with carbonyl compounds, are of special interest as they possess especially high and selective antiproliferative activities [22–24]. Among the aroylhydrazones, especially effective anti-proliferative agents are the hydrazones derived by condensation reaction of salicylaldehyde and different acid hydrazides. For example, 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 [25]. Various derivatives of SBH with a number of functional groups have been created in recent years, in order to discover new bioactive compounds with high antitumour activity and minimal toxicity [26–33].

In this regard, as part of our study on the analytical application of hydrazones and Schiff bases we prepared and characterized a series of differently substituted aroylhydrazones, both derived from nicotinic acid 1-12 (Figure 1) [34–41] and isonicotinic acid hydrazide A-E (Figure 2).

As a next step, this study aimed to evaluate the biological activity of these compounds. To characterize these analogues, we examined their: (1) antiproliferative activity; (2) selectivity to tumour cells. All compounds
were tested on non-tumour MCF-10A cells and on two cancer cell lines, MCF-7 and MDA-MB-231. Four compounds were selected as most active in inhibiting the growth of these tumour cell lines. These four compounds were tested on another four cell lines: non-tumour BJ and cancer cell lines HeLa, HepG2 and HT-29. These studies and the results obtained are important to resolve the structure–activity relationships that are essential for the development of more potent biologically active aroylhydrazones.

**Materials and methods**

**Chemistry**

All the reagents and chemicals used were of analytical and high purity grade. The solvents were purified and dried according to standard procedures. Salicylaldehyde was obtained from Merck (Germany), o-vanillin, nicotinic acid and iso-nicotinic acid hydrazides were purchased from Fluka (Switzerland); benzaldehyde, 2,4-dihydroxybenzaldehyde, 2-hydroxy-4-
chlorobenzaldehyde and 3,5-dichloro-2-hydroxy benzaldehyde purchased from Sigma (Switzerland).

All hydrazones were synthesized in the University of Zagreb, according to previously described procedures [29,34, 42–45]. They were obtained by the condensation reaction of equimolar amounts of the corresponding aroylhydrazide (nicotinic hydrazide for A-E) and differently substituted aldehydes or ketones. The reactions were carried out in dry alcohol (ethanol or ethanol) under argon atmosphere at 85 °C for 20 h. The solvent was evaporated and the solids were suspended in CH₂Cl₂, filtered (G-3), rinsed with CH₂Cl₂ (EtOH was used for 4, 5, 11 and 12), and dried at 50 °C over 24 h.

All obtained hydrazones were characterized by standard analytical procedures (UV/VIS, NMR, MS, HPLC, X-ray) [34,37–42]. The physico-chemical properties of the compounds 1–12 were investigated and reported [32,36,38,39,41].

Cell cultures

MCF-10A (human mammary epithelial cell line), MCF-7 (human breast adenocarcinoma cell line) and MDA-MB-231 (human breast adenocarcinoma cell line) were cultured in Dulbecco Modified Eagle’s medium (DMEM; Gibco, Austria) supplemented with 10% foetal bovine serum (Gibco, Austria), 100 U/mL penicillin (Lonza, Belgium) and 0.1 mg/mL streptomycin (Lonza, Belgium) under a humidified 5% CO₂ atmosphere at 37 °C. The complete growth medium for MCF-10A was additionally supplemented with: human insulin, cholera toxin, epidermal growth factor, hydrocortisone (Sigma Chemical Co.). The MTT assay is based on tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.). The MTT assay is based on the protocol first described by Mosmann [46]. In this assay, living cells reduce the yellow MTT to insoluble purple formazan crystals. The compounds were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the samples did not affect the viability of the cells. The assay was performed 72 h after treatment with the compounds. For this purpose, MTT solution was prepared at 5 mg/mL in PBS and was filtered through a 0.22 μm filter. Then 1 mL of MTT solution was added to 10 mL DMEM and 100 μL of this solution were added into each well, including the cell-free blank wells. Then the plates were further incubated for 3 h to allow MTT to be metabolized. The supernatant was removed and 100 μL/well DMSO/ethanol (1/1) was added. The plates were placed in a microtiter plate shaker for 10 min at room temperature to thoroughly mix the purple formazan into the solvent. An ELISA plate reader (TECAN, Sunrise TM, Grodig/Salzburg, Austria) was used for reading the results. Optical density (OD) was determined at a wavelength of 540 nm and a reference wavelength of 620 nm. Cell viability determined by MTT assay was expressed as:

\[
\% \text{ viability} = \frac{(\text{OD sample} - \text{OD blank control})}{(\text{OD control} - \text{OD blank control})} \times 100
\]

The effects of all tested hydrazones were compared to the activity of the referent cytostatic oxaliplatin (Ox), which is a drug widely used in the clinic [47,48].

Selectivity index (SI)

In the present study, the degree of selectivity of the synthetic compounds is expressed as selectivity index (SI). \(SI = \frac{IC_{50} \text{ of pure compound in a normal non-tumour cell line}}{IC_{50} \text{ of the same pure compound in a cancer cell line}}\), where \(IC_{50}\) is the half maximal inhibitory concentration of the compounds over cell viability [49].

Statistical analysis

Cell antiproliferative activity determined by MTT assay is expressed as percent of live cells versus the negative control group (untreated cells). The results are presented as mean values with standard deviation
The data were analysed for significance by one-way analysis of variance (ANOVA) and then compared by Bonferroni multiple comparison tests using GraphPad Prism Software, Version 3.00 (San Diego, CA, USA). Asterisks represent levels of statistical significance: $p \leq 0.001 (***)$, $p \leq 0.01 (**)$, and $p \leq 0.05 (*)$. Figure 3. Effect of compounds 5, 11, C and E on the growth of MCF-7, MDA-MB-231 and MCF-10A cells after 72 h of treatment. Cell antiproliferative activity determined by MTT assay is expressed as per cent of viable cells versus the control group (C, control) and is presented as mean ± SD ($n=6$), **$p < 0.01$, *$p < 0.05$, ANOVA-test. (±SD).
Table 1. Antiproliferative activity of the hydrazones on MCF-7, MDA-MB-231 and MCF-10A cells after 72 h of treatment (MTT-dye reduction assay).

| Compounds | IC50 of Mean ± SD (μmol/L) |
|-----------|---------------------------|
|           | MCF-7 | MDA-MB-231 | MCF-10A |
| 1         | 411.9 ± 158.1 | 327.2 ± 134.3 | 297.5 ± 71.99 |
| 2         | 166.9 ± 24.7  | 163.2 ± 39.42 | 153.9 ± 16.14 |
| 3         | 176.9 ± 19.08 | 104.8 ± 34.68 | 36.4 ± 7.53  |
| 4         | 128.8 ± 27.63 | 274.4 ± 53.38 | 63.35 ± 12.36 |
| 5         | 126.9 ± 32.03 | 107.8 ± 3.93  | 456.7 ± 29.22 |
| 6         | 238.5 ± 22.26 | 238.9 ± 60.33 | 108.3 ± 6.11 |
| 7         | 280.2 ± 106.2 | 386 ± 73.12   | 142.1 ± 39.85 |
| 8         | 927.1 ± 69.67 | 375.5 ± 69.44 | 253.3 ± 39.15 |
| 9         | 43.11 ± 4.25  | 56.34 ± 3.67  | 62.87 ± 8.16 |
| 10        | 70.67 ± 7.55  | 80.51 ± 13.03 | 56.54 ± 2.25 |
| 11        | 54.59 ± 3.94  | 35.29 ± 6.81  | 95.41 ± 17.11 |
| 12        | 52.98 ± 4.85  | 119.6 ± 32.56 | 22.96 ± 4.64 |
| A         | 74.74 ± 5.19  | 40.4 ± 3.7    | 62.34 ± 16.23 |
| B         | 63.42 ± 6.48  | 45.28 ± 2.39  | 41.7 ± 4.89 |
| C         | 174.5 ± 12.41 | 86.16 ± 5.37  | 215.7 ± 23.51 |
| D         | 152.5 ± 14.05 | 151.9 ± 13.03 | 87.85 ± 11.25 |
| E         | 314.8 ± 31.75 | 96.66 ± 4.58  | 560.9 ± 46.59 |
| Ox        | 10.84 ± 1.14  | 6.55 ± 2.06   | 10.94 ± 0.36 |

Table 2. Selectivity index of the hydrazones under investigation.

| Compounds | Selectivity index |
|-----------|------------------|
|           | MCF-10A/MCF-7 | MCF-10A/MDA-MB-231 |
| 1         | 0.722         | 0.909               |
| 2         | 0.922         | 0.943               |
| 3         | 0.206         | 0.347               |
| 4         | 0.492         | 0.231               |
| 5         | 3.619*        | 4.237*              |
| 6         | 0.454         | 0.453               |
| 7         | 0.507         | 0.368               |
| 8         | 0.273         | 0.675               |
| 9         | 1.458         | 1.116               |
| 10        | 0.800         | 0.702               |
| 11        | 1.748*        | 2.704*              |
| 12        | 0.433         | 0.192               |
| A         | 0.834         | 1.543               |
| B         | 0.658         | 0.921               |
| C         | 1.236         | 2.503               |
| D         | 0.576         | 0.578               |
| E         | 1.782*        | 5.803*              |
| Ox        | 1.009         | 1.670               |

*The selectivity index is defined as the ratio of the IC50 of a pure compound on a non-tumour cell line to its IC50 on a cancer cell line.

Results and discussion

A common route was used for the synthesis of the investigated arylhydrazones. The arylhydrazones 1–12 (Figure 1) and A–E (Figure 2) contain different substituents (e.g. 2-Cl, 3-Cl, -OH, NO2) at the salicylic aroyl part. We also varied the position of the pyridine N atom (e.g. at position 2 or 3), while keeping hydrazone spacer between the pyridine and salicylaldehyde moieties.

The antiproliferative activity of the compounds was tested over different cancer cell lines and non-tumour cells. The effects were compared to the activity of Ox, a referent cytostatic, which is widely used in the clinic [47,48].

The results of the antiproliferative study of the compounds 5, 11, C and E on non-tumour MCF-10A cells and cancer cell lines MCF-7 and MDA-MB-231 are shown in Figure 3. The cells were exposed to different concentrations (ranging from 4 to 2000 μmol/L) of the compounds for 72 h. The treatment of the cell lines with the compounds resulted in a concentration-dependent reduction in the number of the viable cells (Figure 3).

The antiproliferative activity of the compounds based on the half maximal inhibitory concentrations (IC50 values) is shown in Table 1. Compound 11 had a stronger inhibitory effect on the growth of the three cell lines compared to its parent compound. Compounds 5, C and E also showed promising results.

The main problem of the drugs in clinical use for cancer treatment is selectivity. The selectivity index (SI) is an important measure to identify substances with promising biological activity. As the SI demonstrates the relation between IC50 of the respective pure compound on a non-tumour cell line and its IC50 on a cancer cell line, the greater the SI value is, the more selective the compound is to tumour cells. An SI value of less than 2 indicates general toxicity of the pure compound [50]. Based on this, the SI data shown in Table 2 indicate that some compounds exhibit a high degree of antiproliferative selectivity. The substances 11, 5, C, E showed a higher cell growth inhibitory effect with the cancer cells MDA-MB-231 and MCF-7 in comparison with the non-tumour human mammary epithelial cell line MCF-10A. SI of compound 5 was 3.619 (MCF-10A/MCF-7) and 4.237 (MCF-10A/MDA-MB-231). High SI was found for compound E against MDA-MB-231 cells: 5.803. For comparison, the SI value of oxaliplatin (Ox) against MCF-7 and MDA-MB-231 were 1.009 and 1.670, respectively (Table 2).

In order to investigate more closely the antiproliferative activity of the compounds with the highest selectivity index, additional cell lines were used: HeLa, HepG2 and HT-29 (cancer cell lines) and BJ (non-tumour cell line). The IC50 values of compounds 5, 11, C and E are shown in Table 3. The highest cell growth inhibitory effect was observed with the substances 11 and C in the cell line HepG2. In this case, the IC50 value of 11 was 14.46 μmol/L and the IC50 value of C was 19.11 μmol/L (whereas the respective value of Ox was 2.39 μmol/L). These results are comparable with previously reported activities of arylhydrazones [32]. Hristova-Avakumova et al. [32] investigated the cytotoxic activity of 3-methoxy arylhydrazones on HepG2 (hepatocellular carcinoma cell line), HEK-293 (human
The results obtained with all tested substances from a library of aroylhydrazones showed that their biological activity depended particularly on the substituents at the salicylidene part of the molecule. The compounds of interest that showed selectivity for cancer cell lines have two hydroxyl groups in positions 2 and 5 (substances 5 and E), as well as the introduction of the nitro group in position 5 (substances 11 and C) increased the selectivity of the compounds for the cancer lines. However, the introduction of a chlorine atom at position 5 (substance 9) reduced the selectivity versus selectivity of substances 5 and 11. It should also be noted that the position of the nitrogen atom in the aromatic nucleus in nicotinic or isonicotinic residue was not relevant for the activity of the compounds tested.

**Conclusions**

The results obtained with all tested substances from a library of aroylhydrazones showed that their biological activity depended particularly on the substituents at the salicylidene part of the molecule. The compounds of interest that showed selectivity for cancer cell lines have two hydroxyl groups in positions 2 and 5 (substances 5 and E), as well as the introduction of the nitro group in position 5 (substances 11 and C). A modification that reduced the selectivity versus selectivity of substances 5 and 11 is a chlorine atom at position 5 (substance 9). The obtained results give insight into the structure–activity relationships of the tested group of compounds and lay the ground for further development of more potent and selective antitumour agents.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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