PQM-75: A N-benzyl-piperidine Acyl-hydrazone Derivative with Inhibitory Effects on Clonogenic Capacity and Cell Cycle Progression of HepG2 Cells

Cancer is a complex disease and a public health problem worldwide. Despite current advances in cancer therapy, many patients display unsatisfactory clinical responses to available drugs, which reinforces the continuous need for searching for new antitumor prototypes. Data from the literature have highlighted compounds containing acylhydrazone or benzyl-piperidine subunits for their significant antiproliferative activity on tumor cells. Herein, we report the evaluation of a series of N-benzyl-piperidinyl acylhydrazone hybrid derivatives (6a-k) for their cytotoxic profiles on A549 (non-small cell lung cancer) and HepG2 (hepatocellular carcinoma). As a result, derivatives PQM-75 (6i, (IC50 = 58.40 ± 1.87 µM) and PQM-88 (6k, IC50 = 59.58 ± 4.07 µM) showed the best antiproliferative activities against HepG2 and A549 cell lines, respectively. In addition, we demonstrated that compound 6i drastically reduced the clonogenic capacity of HepG2 cell cultures in comparison to control groups. We also observed an increased G2/M population in samples treated with 6i (at 60 µM) and a reduced frequency of cells in the S-phase. In addition, the frequency of cells in mitosis was higher in treated samples compared to control groups. In conclusion, derivative PQM-75 (6i) displays antiproliferative activity on HepG2 cells due to its ability to promote M-phase arrest.

Keywords: N-benzylpiperidine-acylhydrazones; antiproliferative activity; HepG2; cell cycle arrest.

1. Introduction

Cancer is a complex disease that arises from genetic and epigenetic alterations.1,2 According to the GLOBOCAN database, 19.3 million new cancer cases were estimated in 2020 and almost 10.0 million cancer deaths occurred this year.3 The global cancer statistics indicate that cancer remains a public health problem. Lung cancer is the leading cause of cancer death, followed by colorectal and liver cancer considering both sexes.3

Among the primary malignant tumor of the liver, hepatocellular carcinoma (HCC) is the most common and accounts for ~90% of cases. Unfortunately, up to 80% of HCC are diagnosed in an advanced stage when curative therapies, such as surgical resection or orthotopic liver transplantation, cannot be used. Thus, many patients with HCC have been treated by systemic therapy using kinase inhibitors, monoclonal antibodies, and immunomodulatory agents.4–6 Currently, sorafenib and lenvatinib have been used as first-line therapy for advanced HCC.4,7–9 Some second-line drugs have been used for patients previously treated with sorafenib,4,8,9 however there is no drug option for lenvatinib post-treatment.10 Although new drugs have been approved in the last decade for HCC treatment, clinical results are still unsatisfactory.5,10 The ineffectiveness of those treatments is associated, at least in part, with the resistance of tumor cells to the available drugs.11 In this scenario, many efforts have been employed to identify new antitumor drug candidate prototypes.

The acylhydrazone subunit has been considered a privileged structure due to its ability to interact with different biological targets including COX enzymes, tubulin, and TRPV1 and histamine receptors. It has been demonstrated that the NAH subunit is associated with good druggable properties such as chemical and plasma stability,12 with good druggable properties such as chemical and plasma stability. It is also an interesting structural subunit for the design of potential therapeutic candidates for cancer, such as acylhydrazone-combretastatin derivatives.13,14 Procaspase-Activating Compound 1 (PAC-1, Figure 1), is a recent example of an acylhydrazone
derivative with a significant ability to activate caspases and induce cell death, that has entered clinical trials in 2015 due to its promising antitumoral potential. However, despite the importance of this discovery, PAC-1 showed significant toxic effects on the nervous system, leading to the synthesis of several different analogues to overcome limitations in pharmacokinetics and toxicology.\textsuperscript{15,16} In another work, the acylhydrazone subunit was used in the design of new drug candidates structurally related to combretastatin A-4 (CA-4, Figure 1), which displayed significant antiproliferative activity and low cytotoxic effects against three normal cell lines. Further in vivo studies, showed that CA-4 was capable to inhibit the growth of lung carcinoma, revealing its promising antitumor properties.\textsuperscript{13} In addition, another CA-4 derivative, with an innovative structural acylhydrazone-bridged subunit was obtained by Do Amaral and co-workers,\textsuperscript{14} which showed an improved selectivity against tumor cells than CA-4.

Based on the above-mentioned findings supporting the use of acylhydrazone moiety as structurally interesting biophore fragments in the design of anticancer drug candidate prototypes, we decided to investigate the antiproliferative activity of a series of N-benzyl-piperidinyl acylhydrazone derivatives that have been studied by our research group.\textsuperscript{17} Data from the literature have reported the benzyl-piperidine moiety as a pharmacophoric subunit for the antiproliferative activity in embelin analogues and previous results obtained by our group (data not shown) evidenced that these N-benzyl-piperidinyl acylhydrazone-based compounds disclosed better antiproliferative activities than the natural embelin.\textsuperscript{18} Furthermore, both benzyl-piperidine and acylhydrazone subunits have been described as important pharmacophores for antiproliferative activity, but there are no examples in the literature for hybrid compounds with both subunits in the same structural scaffold. Thus, in the present work, a series of N-benzyl piperidinyl acylhydrazone hybrids (6a-k) was synthesized and their cytotoxic profiles were evaluated on A549 and HepG2 cell lines, which are representative of non-small cell lung cancer and carcinoma hepatocellular. The therapeutic proposals for these cancer types are still unsatisfactory motivating the search for new drug candidates.

2. Experimental

2.1. General

The synthesis of the N-benzyl piperidinyl acylhydrazone derivatives was carried out at the Laboratory of Research in Medicinal Chemistry (PeQuiM), at the Federal University of Alfenas (UNIFAL-MG). The antiproliferative evaluation was performed at the Laboratory of evaluation of antitumor prototype (LAPAN) from the Institute of Biomedical Sciences (UNIFAL-MG). Infrared spectra (IR) were obtained by Nicolet iS50 FTIR (Thermo Scientific USA) spectrophotometer coupled to Pike Gladi ATR at the Laboratory of analysis and characterization of drugs (LACFAR) at UNIFAL-MG. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were obtained on a Brucker AC-300 at the Nuclear Magnetic Resonance Laboratory of UNIFAL-MG. Internal standard tetramethylsilane (TMS) was used. The chemical shifts (\(\delta\)) were expressed in parts per million (ppm) and the coupling constants (\(J\), in Hertz (Hz)). The multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet), and m (multiplet). Analytical thin-layer chromatography

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Combretastatin A-4 (CA-4), CA-4 derivatives, and PAC-1 as examples of acylhydrazone-based compounds, and embelin and its N-benzylpiperidine derivative as examples of antiproliferative and cytotoxic ligands with potential anti-cancer properties}
\end{figure}
(TLC), which was performed to monitor the reactions, sheets of Merck silica gel 60 F254. Purifications by column chromatography (CC) were used flash silica gel (220-440 mesh, 0.035 mm - 0.075 mm), Sigma-Aldrich brand. The visualization of the substances was done in a UV lamp ($\lambda = 254$ or 365 nm). The melting bands were established in Mars equipment (PFM II) with crushed sample and packed in a capillary tube, without correction. In the analysis to obtain mass spectra, the samples were analyzed using an Agilent 1290 LC and an Agilent 6550 iFunnel Q-TOF LC/MS mass spectrometer. Electrospray ionization source operated in the positive mode with the parameters under the following conditions: Nebulization gas temperature 290 °C, capillary voltage 3500V, Nozzle Voltage 320 V, drying gas flow 14 mL/min, gas pressure 45 psi nebulization, auxiliary gas temperature 350 °C and auxiliary gas flow 12 mL/min. The quadrupole analyzer operated in the range of m/z 100 - 1500, 50V chipper, 750V octapo voltage, and acquisition of one high resolution spectral in the TOF up to 5 decimal places. All reagents used in the synthetic steps were purchased from Sigma-Aldrich or Acros, without further purifications.

2.2. Chemistry

The synthetic route for the preparation of the target compounds 6a-k used the commercial methyl 4-formylbenzoate (1) as starting material (Scheme 1). Methyl ester 1 was subjected to a reductive amination reaction with commercial racemic 3-hydroxypiperidine (2), in the presence of NaBH$_3$CN/ZnCl$_2$ to generate the N-benzylpiperidine ester 3.$^{19}$ In a subsequent step, ester 3 was reacted with hydrazine monohydrate via nucleophilic acyl substitution, leading to the key intermediate hydrazide 4.$^{20}$ In a final step, hydrazide 4 was reacted with a series of substituted benzaldehydes 5a-k$^{17}$ to furnish the desired N-benzylpiperidinyl acylhydrazone derivatives 6a-k in 42-66% overall yield.

2.3. Chemical preparation and characterization of synthetic intermediates and N-benzyl piperidinyl acylhydrazone derivatives 6a-k

All compounds were characterized by 1D NMR techniques in comparison to spectrometric data earlier obtained and published.$^{17}$ The purity of compounds was assessed by HPLC analysis and were all determined as higher than 97%. Chromatograms for purity testing of the final compounds were recorded on a Prominence model LC-10 HPLC (Shimadzu, Tokyo, Japan) equipped with an automatic injector (20 µL), and UV/VIS detector, and a C18 analytical column (Shimadzu CLC-ODS, 250 mm × 4.4 mm, inner diameter × 5 µm). The analyses were conducted in a two-phase isocratic system with a constant concentration of 90% methanol (A) and 10% acetonitrile (B). Assays were conducted at a flow rate of 1.2 mL/min.

2.3.1. Methyl 4-((3-hydroxypiperidin-1-yl)methyl) benzoate (3)

To a solution of 12.2 mmol of methyl 4-formylbenzoate (1) and 14.6 mmol 3-hydroxypiperidine (2) in 10 mL of methanol, was added 12.2 mmol of NaBH$_3$CN and 6.1 mmol of ZnCl$_2$. The reaction mixture was maintained at room temperature for 48h when TLC indicated the end of the reaction. Then, the solvent was evaporated, and the reaction mixture was resuspended in water, extracted with dichloromethane (3 x 10 mL) and the combined organic layers were washed with brine and dried over magnesium sulfate. After filtration, the solvent was removed, and the crude product was purified in CC to furnish ester 3 as a pale-yellow solid, in 90% yield.

![Scheme 1. Synthetic route of N-benzyl piperidinyl acylhydrazone derivatives 6a-k](image-url)
2.3.2. 4-((3-hydroxypiperidin-1-yl)methyl)benzohydrazide (4)

To a solution of 2.0 mmol of ester derivative (3) in 5 mL of ethanol, was added 11.7 mL of hydrazine monohydrate. The reaction mixture was maintained under reflux for 3 h when TLC indicated the end of the reaction. Then, the solvent was evaporated, and water was added to the reaction mixture, followed by extraction with dichloromethane (3 x 10 mL), washed with brine, and dried over magnesium sulfate. After filtration, the solvent was removed, and the crude product was purified in CC to furnish benzohydrazide 4 as a white solid, with a 94% yield.

2.3.3. General procedure for the preparation of N-acylhydrazone derivatives 6a-k

To a solution of 1.2 mmol of the substituted aldehyde 5a-k in 5 mL of ethanol and 2 drops of hydrochloric acid, 1.0 mmol of 4-((3-hydroxypiperidin-1-yl)methyl)benzohydrazide (4) was added. The reaction mixture was maintained at room temperature for 2 h when TLC indicated the end of the reaction. Then, the solvent was evaporated, and water was added to the reaction mixture, followed by extraction with dichloromethane (3 x 10 mL), washed with brine, and dried over magnesium sulfate. After filtration, the solvent was removed and the crude products were purified in CC, leading to the desired N-acylhydrazone derivatives 6a-k in 42–66% yields. All compounds were properly identified and characterized according to the literature data17.

2.4. Biological assays

2.4.1. Cell lines and treatment schedule

Tumor cell lines A549 (lung adenocarcinoma) and HepG2 (hepatocellular carcinoma), and non-tumor cell line CCD-1059Sk (fibroblast derived from human skin) were used in this study. Cell cultures were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium, Sigma, CA, USA) supplemented with 10% fetal bovine serum (Vitrocell, Campinas, Brazil). Cells were grown in a 37°C humidified incubator containing 5% CO2. Synthetized compounds were synthetized compounds were solubilized in DMSO for obtaining a stock solution, and aliquots were stored at -20°C until use. After attachment (24 h), the cells were treated with the substances for 48 h. The cell viability was not altered by vehicle (DMSO), which concentration did not exceed 0.5% (v/v).

2.4.2. Cell viability analysis

Cell viability was measured by MTS (dimethylthiazol carboxymethoxyphenyl sulfonyl tetrazolium) assay using CellTiter 96® Aqueous Non-Radiative Cell Proliferation assay (Promega) according to the manufacturer’s instructions. Briefly, the cells were seeded (1 x 10^4 cells/well for HepG2 cells; 5 x 10^4 cells/well) into a 96-well plate. Cell cultures were treated for 48 h with different compounds (at 60 µM for screening; or 0-240 µM range for dose-response curves). The analyses were performed in a spectrophotometer (490 nm). Cell viability rate was determined by comparing absorbance values of the treated with control samples. Experiments were performed in triplicate wells. Data are presented as the mean ± standard deviation (SD) from three independent experiments. The IC_{50} values were determined from non-linear regression using GraphPad Prism® (GraphPad Software, Inc., San Diego, USA). Cisplatin was used as a positive control.

2.4.3. Cell cycle analysis

Cell cycle analysis was performed according to Franken et al.21 Briefly, cells were treated for 48 h and then fixed with 75% ethanol at 4 °C overnight. Afterward, samples were rinsed twice with cold phosphate-buffered saline (PBS) and homogenized in dye solution (PBS containing 90 μg.ml^-1 propidium iodide (PI) and 3 mg.ml^-1 RNAase). DNA was quantified after 1h after staining. The analyses were performed by flow cytometry (Guava easyCyte 8HT, Hayward, CA, USA). Results are presented as mean ± standard deviations (SD) from 3 independent experiments.

2.4.4. Clonogenic assay

Clonogenic assay was performed according to Franken et al.22 Briefly, 500 cells were seeded in 35mm plates. Cells were treated for 24h and recovered in a drug-free medium for additional 15 days. Afterward, the colonies were fixed and stained with Crystal Violet. Only colonies containing more than 50 cells were analyzed using a stereo microscope (at 20X magnification). Assays were performed in triplicate and the data were presented as mean ± SD from 3 independent experiments.

2.4.5. Statistical Analysis

The results were tested for significance using a one-way analysis of variance (ANOVA) followed by a Dunnett post-test. The values were expressed as mean ± SD.

3. Results and Discussion

Compounds 6a-k were tested against A549 and HepG2 cells at 60 µM to select the substances with cytotoxic potential on tumor cell lines. Cell viability rates were reduced in HepG2 cultures treated with 6f or 6i, as well as in A549 samples treated with 6f, 6g, 6j, and 6k (Figure 2). Thus, dose-response curves were performed to determine IC_{50} values of these substances on HepG2 and A549 cell lines. In this step, a non-tumor cell line CCD-1059Sk (fibroblast derived from the human dermis) was included to determine selectivity indexes.

In general, the most active substances in the HepG2 and A549 cell viability assays exhibited low cytotoxicity on normal cell lines (CCD-1059Sk). The IC_{50} value for compound 6f on the CCD-1059Sk cell line was 193.50 ± 3.05 µM, and substances 6f, 6g, and 6j did not reduce the viability of CCD-1059Sk cells in the experimental
The most active substances on tumor cells were \( 6k \) (IC\(_{50} = 59.58 \pm 4.07 \) against A549) and \( 6i \) (IC\(_{50} = 58.40 \pm 1.87 \) against HepG2, Table 1), but showed smaller potencies than cisplatin on both tumor cell lines. However, both compounds \( 6k \) and \( 6i \) exhibited higher selectivity toward tumor cells in comparison to cisplatin, which displayed high cytotoxicity against CCD-1059Sk cells. Taking into account the comparative cytotoxic activities on the \textit{in vitro} screening, a carefully structure-activity relationship analysis evidenced that the sulfur atom in the SCH3 substituent on compound \( 6k \) seems to be decisive to modulate its cytotoxicity on A549 cells, once the OCH3 substituted analogue \( 6e \) was not active, as evidenced by the screening results. It is possible that higher polarizability and lower electron-withdrawing effect of the Sulfur atom and its consequent better electron releasing ability and basicity could explain this dissimilar activity.

Cytotoxic activity of \textit{N}-acylhydrazone derivatives on tumor cells has been described on different tumor cell lines including HepG2 and Huh-7 (hepatocellular carcinoma) and BCG-823 (gastric cancer). The \textit{N}-benzyl-piperidinyl acylhydrazone derivatives explored in this study represent an innovative chemical structural pattern concerning antitumor activity investigation. Therefore, the results obtained in this work could not be directly compared with literature data. However, it has been reported that benzyl-piperidinyl derivatives had higher cytotoxicity on tumor cells when compared to embelin, the parental natural product, suggesting that the benzyl-piperidine subunit could be an important pharmacophore for cytotoxic effect.

Based on IC\(_{50} \) values and that \( 6i \) contains a piperonyl group as substituent, in the next step, we sought to investigate more deeply the effect of compound PQM-75 (\( 6i \)) on HepG2 cells, considering that reduction of cell viability may have occurred due to the ability of this compound to inhibit cell proliferation and/or to induce cell death. Thus, clonogenic assay and cell cycle analysis were performed.

As a result, we observed that \( 6i \) significantly reduced, in a dose-dependent manner, the clonogenic capacity of HepG2 cells due to, at least in part, its ability to inhibit the proliferation of HepG2 cells. Antiproliferative activity of acylhydrazone derivatives has been described in both \textit{in vitro} and \textit{in vivo} models.

Furthermore, we sought to investigate whether compound \( 6i \) was acting in the inhibition of cell cycle progression. Thus, DNA content was quantified by flow cytometry. Cell morphological features were evaluated immediately before processing samples for cell cycle analysis. In control cultures, the cells exhibit polyhedral morphology and formed cellular aggregates (clusters) with a well-established cell-to-cell contact. Smaller cell clusters were observed in treated cultures compared to those observed in control groups, especially in samples treated with compound \( 6i \) at 60 \( \mu \)M (Figure 3C).

**Table 1.** IC\(_{50} \) values (\( \mu \)M) were determined by MTS assay at 48 h

<table>
<thead>
<tr>
<th></th>
<th>A549</th>
<th>HepG2</th>
<th>CCD-1059Sk</th>
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</thead>
<tbody>
<tr>
<td>PQM-66 (6f)</td>
<td>139.20 ± 6.78</td>
<td>124.36 ± 1.59</td>
<td>ND</td>
</tr>
<tr>
<td>PQM-67 (6g)</td>
<td>111.60 ± 5.52</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PQM-75 (6i)</td>
<td>ND</td>
<td>58.40 ± 1.87</td>
<td>193.50 ± 3.05</td>
</tr>
<tr>
<td>PQM-76 (6j)</td>
<td>120.50 ± 6.68</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PQM-88 (6k)</td>
<td>59.58 ± 4.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>cisplatin</td>
<td>21.75 ± 1.17</td>
<td>38.64 ± 2.40</td>
<td>24.70 ± 4.78</td>
</tr>
</tbody>
</table>

ND: not determined once viability rate was not sufficiently reduced to determine IC\(_{50} \) value
altered by treatment indicating that 6i inhibited proliferation in experimental conditions tested.

In the sequence, we determined the mitotic index to evaluate whether cell cycle arrest induced by 6i occurred due to inhibition of the G2/M transition of mitosis (M-phase) progression. Our data showed that there was a significant increase in the frequency of cells in mitosis in samples treated with 6i at 60 µM, as well as a significant increase in the percentage of cells in metaphase, compared to control cultures, which was indicative of the mitotic checkpoint activation. These findings suggest that compound 6i exhibits an antimitotic activity on HepG2 cells (Figure 3E) by inducing M-arrest. Considering that malignant cells display fast proliferation cycles and that mitosis apparatus has been an attractive target for anticancer therapies, our results are interesting and support the further investigation to evaluate the fate of HepG2 cells arrested at M-phase by treatment with compound PQM-75 (6i).

4. Conclusion

A series of N-benzylpiperidine acylhydrazone derivatives (6a-k) were investigated by their potential antiproliferative properties. The cytotoxic profile of these substances was evaluated on hepatocellular carcinoma HepG2 and non-small cell lung cancer A549 cell lines. Compounds PQM-75 (6i) and PQM-88 (6k) showed highlighted antiproliferative activities, reducing cellular viability in HepG2 and A549 cultures, respectively, with minimal effect on non-tumor CCD-1059Sk cells. Particularly, compound PQM-75 (6i) efficiently inhibited clonogenic capacity and induced M-phase arrest in the HepG2 cell line. Therefore, we reported for the first time the antimitotic effect of N-acylhydrazone derivatives on hepatocellular carcinoma cells. Our findings support further studies to investigate molecular targets associated with PQM-75 (6i).
with the antiproliferative activity of compound PQM-75 (6i) on HepG2 cells.

**Supplementary Information**

Supplementary information with NMR, IR, and mass spectral data are freely available at https://rvq.sbq.org.br/.

**Acknowledgments**

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**References**

2. Flavahan, W. A.; Gaskell, E.; Bernstein, B. E.; Epigenetic plasticity and the hallmarks of cancer. *Science* 2017, 357, 1. [Crossref] [PubMed]
4. Couri, T.; Pillai, A.; Goals and targets for personalized therapy for HCC. *Hepatology International* 2019, 13, 125. [Crossref] [PubMed]
6. Vogel, A.; Saborowski, A.; Current strategies for the treatment of intermediate and advanced hepatocellular carcinoma. *Cancer Treatment Reviews* 2020, 82, 101946. [Crossref] [PubMed]
15. Aziz, G.; Akselsen, Ø. W.; Hansen, T. V.; Paulsen, R. E.; Procapase-activating compound 1 indues a caspase-3-dependent cell death in cerebellar granule neurons. *Toxicology and Applied Pharmacology* 2010, 247, 238. [Crossref] [PubMed]
16. Roth, H. S.; Hergenrother, P. J.; Derivatives of Procapase-Activating Compound 1 (PAC-1) and Their Anticancer Activities. *Current Medicinal Chemistry* 2016, 23, 201. [Crossref] [PubMed]
20. Romeo, N. C.; Aguirre, G.; Hernández, P.; González, M.; Cerecetto, H.; Aldana, I.; Pérez-Silanes, S.; Monge, A.; Barreiro,

