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ORIGINAL ARTICLE

Synthesis of new N,N′-bis[1-aryl-3-(piperidine-1-yl)propylidene] hydrazine dihydrochlorides and evaluation of their cytotoxicity against human hepatoma and breast cancer cells

Kaan Kucukoglu[1], H. Inci Gul[1], Rengul Cetin-Atalay[2], Yosra Baratli[3], Anne-Laure Charles[3], Murat Sukuroglu[4], Mustafa Gul[5], and Bernard Geny[3]

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Abstract

N,N′-Bis[1-aryl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides were synthesized by the reaction of 2 mols of 1-aryl-3-(piperidine-1-yl)-1-propanone hydrochlorides with 1 mol of hydrazine hydrate. Aryl part was C_6H_5 (**P1**), 4-$CH_3C_6H_4$ (**P2**), 4-$CH_3OC_6H_4$ (**P3**), 4-HOC_6H_4 (**P4**), 4-ClC_6H_4 (**P5**), 3-$CH_3OC_6H_4$ (**P6**), 4-FC_6H_4 (**P7**) and 4-BrC_6H_4 (**P8**). Except **P1**, all compounds were reported for the first time. The chemical structures were confirmed by UV, ^{1}H NMR, ^{13}C NMR and HRMS spectra. **P1**, **P2**, **P7** and **P8** against human hepatoma (Huh7) cells and **P1**, **P2**, **P4**, **P5**, **P6**, **P7** and **P8** against breast cancer (T47D) cells have shown cytotoxicity. **P1**, **P2** and **P7** had more potent cytotoxicity against Huh7 cells than the reference compound 5-FU, whereas only **P2** was more potent than the 5-FU against T47D cells. Representative compound **P7** inhibited the mitochondrial respiration at 144, 264 and 424 μM concentrations dose-dependantly in liver homogenates. The results suggest that **P1**, **P2**, **P7** and **P8** may serve as model compounds for further synthetic studies.

Keywords

Cytotoxicity, Huh7, hydrazone, Mannich bases, mitochondrial respiration, T47D

History

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and it ranks at third place in the list of cancer-related mortality[1,2]. As HCC is usually asymptomatic in the early stages, it is diagnozed at an advanced stage[3,4]. Furthermore, for most patients with HCC, surgery is the only curative treatment procedure, because HCC cells have high resistance to chemotherapeutic agents, so treatment options of HCC is very limited[5]. On the other hand, breast cancer is the leading cause of cancer death among women with approximately a million new cases each year[6]. Despite some therapy options such as surgery, chemotherapy, endocrine and radiation therapy in the treatment of breast cancer, several side effects and drug resistance to chemotherapeutic agents are often encountered problems in the course of therapy[7]. Therefore, it is necessary to develop novel approaches and discover new drug candidates that can be used in the treatment of HCC and/or breast cancer.

Mannich bases are synthesized by using a compound containing a reactive hydrogen atom, formaldehyde and a secondary amine in general. This process is known as the Mannich reaction[8]. They have various biological activities such as cytotoxic[9–13], anti-cancer[8], anti-inflammatory[14], anti-convulsant[15] and anti-fungal[16,17] activities. Their cytotoxic activities may be attributed to the α,β-unsaturated ketone, which is available in the chemical structure of the compound or is produced by deamination process *in vivo* or under simulated conditions *in vitro*[18–20] or inhibition of mitochondrial respiration[21,22].

In the 1920s, Warburg et al.[23] showed that tumour cells produce large quantities of lactic acid from glucose via the glycolytic pathway, even under aerobic conditions. It has been proposed that this so-called ''aerobic glycolysis'' and the consequent export of lactic acid leads to the low extracellular pH (pHe) characteristic of solid tumours. More recent works suggest that other metabolic pathways may also contribute to acid production[24,25]. Alternatively, the pHe of solid tumours may be independantly regulated *in vivo* because it is an intrinsic part of the tumour phenotype[26]. A theoretical model has proposed that a low pHe may confer survival and growth advantages for tumour cells at the expense of the surrounding normal cells[27] and *in vitro* studies have shown that a low pHe favours metastatic behaviour and production of angiogenic factors by human cells[28,29]. In any case, as the intracellular pH (pHi) is tightly controlled and maintained at neutral or slightly alkaline levels, the low pHe of solid tumours leads to a pH gradient (pHe–pHi) across the plasma membrane, which is the reverse of that found in normal tissue[30]. This reverse or negative pH gradient (Δ pH) can also have important consequences for the effective delivery of chemotherapeutic drugs from the blood stream to the tumour intracellular space[31].

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A large −Δ pH has been shown to be an important determinant for tumour cell uptake of the anti-cancer drug 5-fluorouracil[32,33], and a low pHe favours cellular uptake of other chemotherapeutic drugs that are weak acids, such as chlorambucil, or reduces uptake of weak bases such as mitoxanthrone[6,34,35].

The pH of tumours grown s.c. from 30 human tumour xenograft lines in rats were analysed with the use of H^+ ion-sensitive semi-microelectrodes prior to and following stimulation of tumour cell glycolysis by intravenous infusion of glucose. Without exception, all xenografts responded to the temporary increase in plasma glucose concentration by an accumulation of acidic metabolites, as indicated by a pH reduction to an average value of 6.43 from 6.83. This pH value corresponds to a ten-fold increase in H^+ ion activity in tumour tissues as compared to arterial blood[36]. If this is the case, tumour-selective activation of pH-sensitive anti-cancer agents, e.g. alkylating drugs, acid-labile prodrugs or pH-sensitive immunoconjugates may thus be feasible in a wide variety of human cancers.

Hydrazones are a special group of compounds in the Schiff base family among condensation compounds which are synthesized with aldehyde or ketones and amine derivatives in organic chemistry. A great attention has been focused on hydrazones for many researchers because of their various biological activities such as anti-microbial, anti-fungal, anti-inflammatory anti-tubercular, anti-convulsant, anti-tumoural and anti-viral activities[37].

Some polymeric micellar drug delivery systems having hydrazone structure were developed for the delivery of doxorubicin[38]. Hydrazone bond was included as it is cleavable under biologically relevant conditions under slightly acidic conditions (pH ca. 5, typical of the interstitial space of most solid tumours and of the endosomal environment) and is relatively stable under neutral conditions in blood plasma (pH 7.4)[39,40]. It was expected that this should minimize the release of toxic-free doxorubicin during delivery into tumour tissue and thus decrease side effects and increase the range of doses exploitable in therapy. In another study, the anti-cancer drug, adriamycin is conjugated to the core-forming segments through the hydrazone linkers that are stable under physiological conditions (pH 7.4), but cleavable under acidic intracellular environments in endosomes and lysosomes (pH 5–6)[41].

Considering above mentioned problems and the logic explained, the design and synthesis of hydrazones of some Mannich bases, N,N′-bis[1-aryl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides, were considered to develop new anti-cancer drug candidates. These compounds were designed as bi-functional alkylating agents and prodrug of Mannich bases, which can provide α,β-unsaturated ketones responsible for the cytotoxicity of them following hydrolysis of hydrazone in acidic environment of cancer cells to produce 2 mols of mono Mannich bases from 1 mol of hydrazone compound first. Then, they undergo deamination to produce α,β-unsaturated ketones which alkylate thiols. Another approach can be deamination of hydrazone first and then reaction of the product generated with cellular thiols by alkylation to produce cytototoxicity.

In addition, as it was previously shown that cytotoxic Mannich bases derived from styryl ketones inhibit mitochondrial respiration in rat liver cells[21,22]. Inhibition of mitochondrial respiratory chain by the representative compound **P7** was also tested in liver homogenate to elucidate the possible mechanisms of actions of the acid-sensitive hydrazones derived from mono Mannich bases.

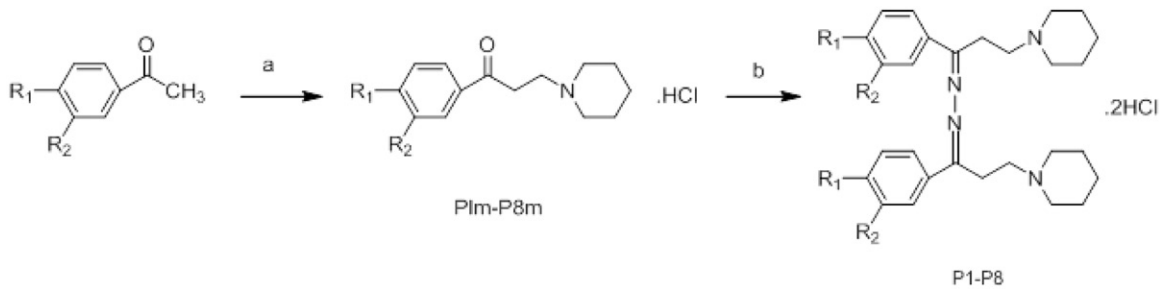
Experimental

Chemistry

Melting points were determined on an Electrothermal 9100 melting point apparatus (IA9100, Electrothermal, Essex, UK). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded employing a Varian 400 MHz FT spectrometer (Danbury, CT) for hydrazone derivatives **P1–P8**, while ^{1}H (60 MHz)-NMR spectra were recorded on a Varian EM-360 spectrometer for precursor mono Mannich bases **P1m–P8m**. NMR spectra of **P1–P8** were measured in MeOH-d_4 solutions using tetra methyl silane as an internal standard and chemical shifts are reported as parts per million (δ) while $CDCl_3$ was used for **P1m–P8m**. Coupling constants (*J*) are reported in Hertz. HRMS were recorded on a V6 Waters Micromass ZQ (Waters Corporation, Milford, MA) and UV spectra were taken on a Thermo Electron Heλios (α) (UVA 114903, Cambridge, UK) spectrophotometer for hydrazone derivatives. Synthetic starting material, reagents and solvents were purchased from Merck-Schuchardt (Hohenbrunn, Germany), Riedel-de Haën (Seelze, Germany), J.T. Baker Chemical Company (Phillipsburg, NJ), Fluka AG (Buchs, Switzerland), Acros Organics Chemical Co. (Fair Lawn, NJ) and Sigma-Aldrich Chemical Co. (St. Louis, MO).

*General procedure for the synthesis of precursor mono Mannich bases, 1-aryl-3-(piperidine-1-yl)-1-propanone hydrochlorides, (**P1m–P8m**)*

Except **P2m**, the mixture of corresponding acetophenone, paraformaldehyde and piperidine hydrochloride in 1:1.5:1 mol ratio was dissolved in ethanol. A few drops of HCl (37%) was added into this mixture. Reaction content was refluxed for sometime. Solvent was removed under vacuum. The residue obtained was crystallized from suitable solvent. Crystals formed were filtered, washed with diethyl ether, dried and crystallized. The amount of suitable ketone (mmol), heating period (h), melting point (°C), crystallization solvent and yield (%) of the compounds **P1m–P8m** (Scheme 1) were as follows: **P1m** (20 mmol, 9 h, 191–194 °C, methanol, 69%), **P2m** (8 mmol, 22 h, 182–187 °C, methanol-ether, 25%), **P3m** (30 mmol, 4 h,



Scheme 1. Synthesis of N,N′-Bis[1-aryl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides, **P1–P8**. Reagents and conditions: (a) paraformaldehyde, piperidine HCl, HCl (37%) and EtOH, 4–9 h, for **P1m**, **P3m–P8m**; acetic acid (99%), 22 h, for **P2m**; (b) Ethanolic acetic acid (3% w/v), hydrazine hydrate for **P1–P8**. $R_1 = R_2 = H$ (**P1**); $R_1 = CH_3$, $R_2 = H$ (**P2**); $R_1 = CH_3O$, $R_2 = H$ (**P3**); $R_1 = OH$, $R_2 = H$ (**P4**); $R_1 = Cl$, $R_2 = H$ (**P5**); $R_1 = H$, $R_2 = CH_3O$ (**P6**); $R_1 = F$, $R_2 = H$ (**P7**); $R_1 = Br$, $R_2 = H$ (**P8**).

215–218 °C, ethanol, 32%), **P4m** (30 mmol, 4 h, 224–226 °C, ethanol, 33%), **P5m** (20 mmol, 9 h, 196–198 °C, methanol-ether, 48%), **P6m** (30 mmol, 8 h, 152–155 °C, ethanol-ether, 45%), **P7m** (20 mmol, 8 h, 173–177 °C, methanol, 21%), **P8m** (20 mmol, 8 h, 205–207 °C, methanol, 41%). As precursor Mannich bases were registered in literatures with their melting points[42–45], NMR data of them was not reported here. In the case of **P2m**, the mol ratio of ketone, aldehyde and amine was 1:2:1. Acetic acid (99%, 30 ml) was used instead of ethanol. After refluxing 22 h, the solvent was evoporated *in vacuo* and diethyl ether was added into the residue and the mixture was cooled for one night at 4 °C. The precipitated compound was filtered, dried and crystallized from methanol-diethyl ether.

*General procedure for the synthesis of hydrazones, N,N′-bis[1-aryl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P1–P8**)*

A solution of hydrazine hydrate (1 mol ratio) in ethanol was added to a solution of mono Mannich base (2 mol ratio) in ethanolic acetic acid (3% w/v). The mixture was stirred at room temperature for 17–26 h. The precipitated compound was filtered, dried and crystallized from suitable solvent to give the corresponding hydrazone derivatives. The crystals formed were filtered and dried at room temperature. Experimental data of **P**-series hydrazone compounds, **P1–P8** (Scheme 1) are shown in Table 1.

*N,N′-bis[1-phenyl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P1**).* Yield: 57%. M.p. 185–189 °C. UV λ_{maks}^{MeOH} (log ε) 303 (4.22) nm. ^{1}H NMR (MeOH-d_4) δ 1.80–1.95 (m, 12H), 2.97 (m, 8H), 3.54–3.63 (m, 8H), 7.51–7.56 (m, 8H), 8.03–8.06 (m, 2H); ^{13}C NMR (MeOH-d_4) δ 21.39, 23.05, 23.74, 53.15, 53.5, 127.39, 128.92, 131.09, 136.05, 163.27. HRMS: 431.32 (M^+), 432.32 $(M+1)^+$.

*N,N′-bis[1-(4-methylphenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P2**).* Yield: 9%. M.p. 207–210 °C. UV λ_{maks}^{MeOH} (log ε) 313 (4.28) nm. ^{1}H NMR (MeOH-d_4) δ 1.85 (m, 12H), 2.41 (s, 6H), 2.99 (m, 8H), 3.52–3.63 (m, 8H), 7.35 (d, $J=7.7$ Hz, 4H), 7.94 (t, $J=8.4$ Hz, 4H); ^{13}C NMR (MeOH-d_4) δ 20.23, 21.41, 23.05, 23.61, 53.14, 53.67, 127.36, 129.53, 133.33, 141.77, 163.08. HRMS 459.35 (M^+), 460.35 $(M+1)^+$.

*N,N′-bis[1-(4-methoxyphenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P3**).* Yield: 72%. M.p. 214–217 °C. UV λ_{maks}^{MeOH} (log ε) 276 (4.38) nm. ^{1}H NMR (MeOH-d_4) δ 1.85–1.92 (m, 12H), 3.30 (m, 8H), 3.49–3.58 (m, 8H), 3.88 (s, 6H), 7.04 (d, $J=9.2$ Hz, 4H), 8.03 (d, $J=8.8$ Hz, 4H); ^{13}C NMR (MeOH-d_4) δ 21.43, 23.04, 32.38, 52.41, 53.59, 54.95, 113.86, 129.02, 130.51, 164.62, 195.17. HRMS 491.34 (M^+), 492.34 $(M+1)^+$.

*N,N′-bis[1-(4-hydroxyphenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P4**).* Yield: 64%. M.p. 219–221 °C. UV λ_{maks}^{MeOH} (log ε) 281 (4.36) nm. ^{1}H NMR (MeOH-d_4) δ 1.89 (m, 12H), 3.30 (m, 8H), 3.50–3.53 (m, 8H), 6.87 (d, $J=8.8$ Hz, 4H), 7.94 (d, $J=8.8$ Hz); ^{13}C NMR (MeOH-d_4) δ 21.43, 23.04, 32.21, 52.48, 53.57, 115.22, 127.92, 130.75, 163.20, 195.07. HRMS 463.30 (M^+).

*N,N′-bis[1-(4-chlorophenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P5**).* Yield: 48%. M.p. 184–189 °C. UV λ_{maks}^{MeOH} (log ε) 254 (4.32), 311 (4.06) nm. ^{1}H NMR (MeOH-d_4) δ 1.57–1.93 (m, 12H), 3.04–3.15 (m, 8H), 3.51–3.63 (m, 8H), 7.55 (d, $J=7.7$ Hz, 4H), 8.04 (d, $J=8.8$ Hz, 4H); ^{13}C NMR (MeOH-d_4) δ 21.41, 23.06, 32.93, 52.15, 53.64, 128.96, 129.78, 134.7, 139.99, 195.5. HRMS 499.24 (M^+), 501.24 $(M+2)^+$.

*N,N′-bis[1-(3-methoxyphenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P6**).* Yield: 88%. M.p. 187–192 °C. UV λ_{maks}^{MeOH} (log ε) 251 (3.94), 308 (3.67) nm. ^{1}H NMR (MeOH-d_4) δ 1.84 (m, 12H), 3.26 (m, 8H), 3.53–3.59 (m, 8H), 3.88 (s, 6H), 7.13 (dd, $J=8.3$, 2.6 Hz, 2H), 7.46 (t, $J=8.1$ Hz, 2H), 7.55 (t, $J=2.2$ Hz, 2H), 7.58 (td, $J=7.7$, 2.6 Hz); ^{13}C NMR (MeOH-d_4) δ 21.4, 23.03, 23.91, 53.19, 53.55, 54.96, 113.01, 116.38, 119.83, 130.1, 137.4, 160.44, 163.01; HRMS 491.34 (M^+), 492.34 $(M+1)^+$.

*N,N′-bis[1-(4-fluorophenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P7**).* Yield: 12%. M.p. 204–209 °C. UV λ_{maks}^{MeOH} (log ε) 306 (4.30) nm. ^{1}H NMR (MeOH-d_4) δ 1.80–1.86 (m, 12H), 2.99 (m, 8H), 3.52–3.60 (m, 8H), 7.27 (t, $J=8.8$ Hz, 4H), 8.11 (d, $J=8.8$ Hz, 4H); ^{13}C NMR (MeOH-d_4) δ 21.42, 23.05, 23.72, 53.18, 53.4, 115.91, 129.9, 132.37, 163.61, 166.11. HRMS 467.3 (M^+), 468.3 $(M+1)^+$.

*N,N′-bis[1-(4-bromophenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides (**P8**).* Yield: 14%. M.p. 195–199 °C. UV λ_{maks}^{MeOH} (log ε) 263 (4.13), 313 (4.30) nm. ^{1}H NMR (MeOH-d_4) δ 1.84–2.01 (m, 12H), 3.03 (m, 8H), 3.50–3.61 (m, 8H), 7.72 (d, $J=8.8$ Hz, 4H), 7.96 (d, $J=8.8$ Hz, 4H); ^{13}C NMR (MeOH-d_4) δ 21.39, 23.04, 32.84, 52.12, 53.66, 128.67, 129.82, 132.03, 135.04, 195.71. HRMS 587.14 (M^+), 589.14 $(M+2)^+$, 591.14 $(M+4)^+$.

Cytotoxic activity assay

Hydrazone compounds, **P1–P8** and 5-FU, which was used as positive control compound, were tested against human hepatoma (Huh7) and breast cancer (T47D) cells by NCI-60 SulforhodamineB Assay[46,47] to identify the compounds with growth-inhibitory activity. Briefly, T47D and Huh7 cells (5000 or 10 000) were inoculated into 96-well plates in 100 µl of standard DMEM medium (Gibco-Invitrogen Corp., Grand Island, NY) 24 h

Table 1. Experimental data of **P** series hydrazone compounds, **P1–P8** (Scheme 1).

Compound	Formula	MW	Reaction time (h)	Crystallization solvent	Yield (%)	Melting point (°C)
P1	$C_{28}H_{40}Cl_2N_4$	502.26	17	Ethanol	57	185–189*
P2	$C_{30}H_{44}Cl_2N_4$	530.29	23	Ethanol	9	208–210
P3	$C_{30}H_{44}Cl_2N_4O_2$	562.28	18	Methanol	72	214–217
P4	$C_{28}H_{40}Cl_2N_4O_2$	534.25	18	Ethanol	64	219–221
P5	$C_{28}H_{38}Cl_4N_4$	570.19	18	Ethanol	48	186–189
P6	$C_{30}H_{44}Cl_2N_4O_2$	562.28	26	Chloroform	88	190–192
P7	$C_{28}H_{38}Cl_2F_2N_4$	538.24	26	Chloroform/methanol	12	204–209
P8	$C_{28}H_{38}Br_2Cl_2N_4$	658.08	20	Ethyl acetate/methanol	14	195–199

*Reported melting point of **P1** was 172.5–175 °C[52].

prior to treatment with the compounds in increasing concentrations (2.5, 5, 10, 20, 40 μM). After 72 h of treatment, the cell culture medium was discarded and the cells were washed with 1× PBS ($CaCl_2$-, $MgCl_2$-free) (Gibco, Invitrogen). Then cells were fixed by gentle addition of 50 μl of ice-cold 10% (w/v) trichloroacetic acid (MERCK) for 60 min at 4 °C. After fixation, cells were washed with distilled water and dried in air. The cells were stained using 50 μl of a 0.4% (m/v) of sulforhodamine (Sigma-Aldrich) in 1% acetic acid solution at room temperature for 10 min. Extra unbound dye was washed five times with 200 μl of 1% acetic acid and air-dried. Sulforhodamine stain, which was bound to cellular proteins, was then solubilized by adding 200 μl of 10 mM Tris-base solution and the absorbance was acquired at 515 nm. Absorbance values of the compounds were normalized to their corresponding controls (DMSO). The IC_{50} values were calculated as previously described[48]. The IC_{50} values reported are the average of two independent determinations which differed by <10%.

Inhibition of mitochondrial respiration assay

Animals

Experiments were performed on nine adult male Wistar rats (8-week-old), housed in a thermo-neutral environment (22 ± 2 °C), on a 12:12 h photoperiod, and provided food and water *ad libitum*. This investigation was carried out in accordance with the Guide for the Care and Use of Laboratory published by the US National Institute of Health and approved by the institutional animal care commitee (NIH publication No. 85-23, revised 1996). Animals were submitted to a general anaesthesia with isoflurane and the liver was excised and immediately used for the study of mitochondrial respiration.

Study of mitochondrial respiration

Isolation of liver mitochondria. All operations were carried out at 4 °C. Liver was finely minced in ice-cold isolation buffer (50 mM Tris, 1 mM EGTA, 70 mM Sucrose, 210 mM Mannitol, pH 7.4 at 4 °C) and then homogenized with a Potter–Elvehjem device. The homogenate was centrifuged at 1300*g* for 3 min at 4 °C. The supernatant was centrifuged at 10 000 *g* for 10 min at 4 °C to sediment mitochondria. Finally, the mitochondrial pellet was washed twice and then suspended in 50 mM Tris, 70 mM Sucrose, 210 mM Mannitol, pH 7.4 at 4 °C. Protein content was routinely assayed with a Bradford assay using bovine serum albumin (BSA) as a standard[49]. Mitochondria were kept on ice until use.

Measurement of the mitochondrial respiratory chain complexes. Mitochondrial respiration was measured using a Clark-type electrode (Strathkelvin Instruments, Glasgow, UK) as previously reported[50]. Before oxygraph measurement, 3 ml of solution M (100 mM KCl, 50 mM Mops, 1 mM EGTA, 5 mM Kpi, 1 mg/ml BSA) was added to the oxygraph chambers for 10 min then, 0.15 mg of isolated liver mitochondria were introduced with 10 mM glutamate and 2.5 mM malate and the temperature was maintained at 25 °C. After that, succinate (25 mM) and adenosine diphosphate were added. Mitochondrial respiration in these conditions allowed determining the activities of the complexes I, II, III and IV (Figure 2). After that, the representative Fluor-bearing compound P7 was added at different concentrations.

Results are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by a Newman–Keuls post-test. (GraphPad Prism 5, Graph Pad Software, Inc., San Diego, CA). Statistical significance required a $p < 0.05$.

Results

In this study, hydrazone compounds **P1**–**P8** bearing a piperidine moiety were synthesized as presented in Scheme 1 for the first time, except compound **P1**. Experimental data of **P**-series of compounds are presented in Table 1. Compounds were obtained with the yield of 9–88%. The chemical structures of newly synthesized compounds were assigned on the basis of their spectroscopic data such as UV, ^{1}H NMR and ^{13}C NMR and HRMS. Spectral data are presented at the experimental section. Spectral data of the compounds were in accordance with the chemical structures of the compounds synthesized.

All the synthesized compounds having the chemical structure of N,N′-bis[1-aryl-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides were evaluated against the human hepatoma cells (Huh7) and breast cancer cells (T47D) in terms of cytotoxic activity. The cytotoxicity of the compounds and reference compound 5-FU are listed in Table 2 in terms of the inhibitory potencies (IC_{50}, μM).

Hydrazone compounds, **P1** (4.86 times), **P2** (10.08 times), **P7** (5.26 times) and **P8** (1.07 times, almost equal to 5-FU) had higher cytotoxic potency than the reference compound, 5-FU, against Huh7 cells. Of the compounds synthesized, only **P2**, a 4-methyl derivative, had more potent cytotoxic activity than 5-FU against T47D cells (1.45 times). The representative cytotoxic compound **P7** inhibited the mitochondrial respiration significantly at 144, 264 and 424 μM concentrations dose-dependantly in liver homogenates (Figure 1).

Table 2. Cytotoxic activity of hydrazones **P1**–**P8** (Scheme 1), against Huh7 and T47D cells (IC_{50}, μM).

Compound	Huh7	T47D
P1	8.66	19.90
P2	4.18	4.83
P3	*	*
P4	*	51.59
P5	*	114.93
P6	*	229.82
P7	8.01	10.05
P8	39.50	16.09
5-FU	42.12	7.0

5-FU, 5-Fluorouracil; *, no inhibition.

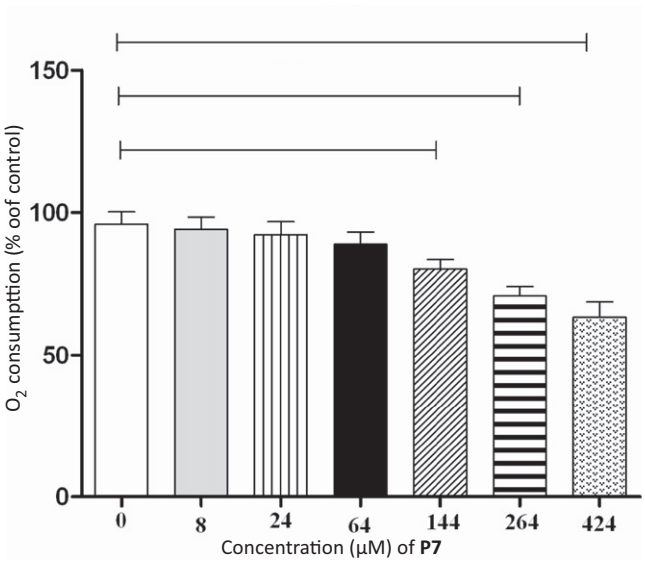


Figure 1. *In vitro* effects of increasing concentrations of **P7** on rat liver mitochondrial respiratory chain. $p < 0.05$ (one-way ANOVA followed by Tukey).

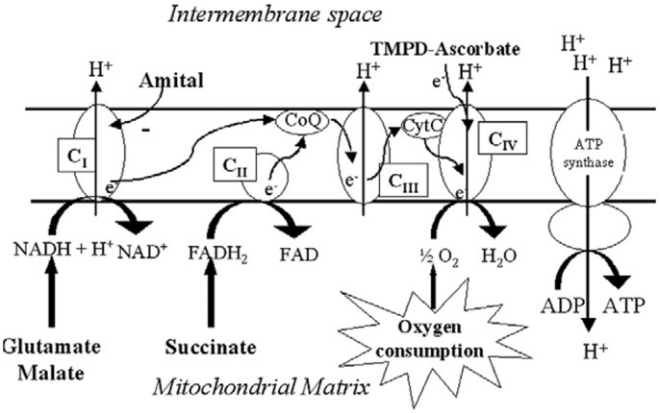


Figure 2. Schematic representation of the mitochondrial respiratory chain with specific substrates and inhibitors (modified from a previous study[53]). CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride; H, proton.

Discussion

In this study, hydrazone compounds were designed as acid-sensitive compounds targeting cancer cells that have lower pH value than corresponding normal tissues. There are three suggested mechanisms of action for the cytotoxicity of designed chemical structures[51,52]: firstly, hydrazone compounds may undergo deamination to produce N,N′-bis(1-aryl-propylidene)hydrazine, then interact with cellular thiol to form cytotoxicity. Secondly, hydrazone compound undergoes hydrolysis at the acidic pH of cancer cells to produce 2 mols of mono Mannich bases (**Pm**-Series) from 1 mol of hydrazone (**P**-Series) first, then they may undergo deamination to produce biologically active species α,β-unsaturated ketones, which alkylate cellular thiols to produce cytotoxicity. Thirdly, inhibition of mitochondrial respiration by the compounds.

The chemical structures of newly synthesized compounds were assigned on the basis of their spectroscopic data such as UV, ^{1}H NMR and ^{13}C NMR and HRMS. Spectral data are presented in the experimental section. Spectral data of the compounds were in accordance with the chemical structures of the compounds synthesized. As an example, compound **P1**'s spectral data can be summarized. When examined, the ^{1}H NMR spectrum of **P1**, the protons on the phenyl ring in the molecule were observed as 2-hydrogen-multiplet and 8-hydrogen-multiplet in δ 8.03–8.06 ppm and δ 7.51–7.56 ppm, respectively. The protons belonged to ethylene chain between nitrogen atom of piperidine ring and carbon atom of imine group gave 8-hydrogen multiplet in δ 3.54–3.63 ppm. The protons of methylene group next to the nitrogen atom in piperidine ring were observed in δ 2.97 ppm as 8-hydrogen multiplet. The other protons of piperidine ring gave 12-hydrogen multiplet in δ 1.80–1.95 ppm. In ^{13}C NMR spectrum, the carbon signal belonged to the imine group, which has shown that expected structure had synthesized, was observed in δ 163.27 ppm and it was the most important signal of the spectrum. The signal observed in δ 136.05 ppm belonged to carbons of phenyl ring next to the imine group. The signals of other protons of phenyl ring was in δ 131.09 ppm, δ 128.92 ppm and δ 127.39 ppm. The carbon atoms of methylene group next to nitrogen atom in piperidine ring gave signals of δ 53.50 ppm. The carbon atoms of ethylene chain next to nitrogen atom of piperidine ring was observed at δ 53.15 ppm, while the signal of carbon atoms in the same ring next to the imine group was in δ 23.74 ppm. The carbon atoms on the position 4 according to the nitrogen atom of piperidine ring gave signal in δ 23.05 ppm. In the spectrum, the signal observed in δ 21.39 ppm belonged to the carbon atoms which were on positions 3 and 5 according to the nitrogen atom of piperidine ring. In HRMS spectrum of compound **P1**, molecular ion signal which confirmed the structure of **P1** was observed in m/z 431.32. M + 1 signal was in m/z 432.32. The signal that had the highest rate of relative abundance and confirmed that the fragmentation of the molecule occured between the imine groups was in m/z 216.16. In UV spectrum of compound **P1**, the signal which was observed in 303 nm belonged to the chromophor imine group-conjugated phenyl ring. This signal was due to $\pi \rightarrow \pi^*$ electronic transition of imine group.

When the relationships between the cytotoxicity of the compounds and physicochemical parameters tested (Hammett σ, Hansch π and log P, data not shown) were investigated, there were no correlation. Data were analysed by using the bivariate, Pearson correlation method in SPSS for Windows program (IBM, New York, NY). Observed cytotoxicities with **P**-Series of compounds against cell lines used, may be explained with the optimum deamination ratio of the compounds in cancer cells tested to produce biologically active alkylation centre or interaction with some steps at the electron transport chain in mitochondria. On the other hand, absence of cytotoxicity with some compounds of **P**-Series may be explained by faster deamination of the compounds than the optimal level in the tested cells, which can cause faster elimination of the active metabolite before reaching their target. Second explanation for the absence of cytotoxicity may be the lack of interaction with the proper receptors for the bioactivity or the production of 1-aryl-3-(3-aryl-4,5-dihydropyrazol-1-yl)propan-1-one, which does not have suitable centre for thiol alkylation. Third, the compounds which dont have cytotoxicity may not have any interaction with electron transport chain in mitochondria.

Previously, it was shown that cytotoxic Mannich bases derived from styryl ketones inhibit mitochondrial respiration in rat liver cells[21,22]. Accordingly, inhibition of mitochondrial respiratory chain by the compound **P7** in this study suggests that inhibition of mitochondrial respiration may be one of the contributing mechanisms to the cytotoxic activity of **P7** and the other cytotoxic compounds.

Conclusions

In conclusion, new hydrazones synthesized having a piperidine moiety as potential anti-cancer candidate deserved further structural modification and pharmacological evaluation. We reported here the compounds **P2**–**P8** for the first time with their synthesis, spectral analysis and cytotoxicities of the **P**-series of compounds against Huh7 and T47D cell lines. Compounds **P1** (4.86 times), **P2** (10.08 times), **P7** (5.26 times) and **P8** (1.07 times, almost equal to 5-FU), against Huh7 cell line, compound **P2** (1.45 times) against T47D cell line may serve as model candidate compounds for further synthetic designs and studies. Inhibition of the mitochondrial respiration by the representative compound **P7** suggests that it may be one of the contributing mechanisms to the cytotoxic activity of the hydrozones.

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Declaration of interest

The authors report no conflict of interest.

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