Biological Activity of 1-Aryl-3-phenethylamino-1-propanone Hydrochlorides and 3-Aroyl-4-aryl-1-phenethyl-4-piperidinols on PC-3 Cells and DNA Topoisomerase I Enzyme

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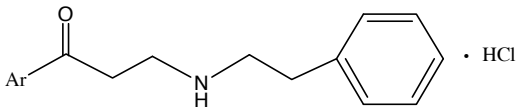
A number of studies reported Mannich bases to manifest antimicrobial, cytotoxic, anticancer, anti-inflammatory, and anticonvulsant activities. A considerable number of therapeutically important cytotoxic compounds are active on DNA topoisomerases that regulate the DNA topology. In the present study we evaluated the biological activity of mono-Mannich bases, 1-aryl-3-phenethylamino-1-propanone hydrochlorides (**1a**–**10a**), and semicyclic mono-Mannich bases, 3-aroyl-4-aryl-1-phenethyl-4-piperidinols (**1b**–**9b**), synthesized in our laboratory. We employed androgen-independent human prostate cancer cells (PC-3) to assess the cytotoxicity of the compounds and extended the biological activity evaluation to cover supercoil relaxation assays of mammalian type I topoisomerases. Our results showed that the compounds had cytotoxicity within the 8.2–32.1 μM range, while two compounds gave rise to a comparable average value in topo I interference of 42% and 40% for **10a** (with a hydroxy substituent on the phenyl ring from mono-Mannich bases) and **5b** (with a fluoro substituent on the phenyl ring from the semicyclic mono-Mannich base series, piperidinols), respectively.

Key words: Mono-Mannich Bases, Cytotoxic Activity, PC-3 Cell, DNA Topoisomerase I

Introduction

Mannich bases are formed by the reaction between a compound containing a reactive hydrogen atom, formaldehyde, and generally a secondary amine (Dimmock and Kumar, 1997). Mannich bases have several biological activities such as antimicrobial (Gul M. *et al*., 2005), cytotoxic (Gul H. I. *et al*., 2003, 2006, 2007, 2008; Gul M. *et al*., 2005a, 2009), anti-inflammatory (Suleyman *et al*., 2007; Gul H. I. *et al*., 2009), and anticonvulsant activities (Gul H. I. *et al*., 2004). Considerable anticancer activity was also attributed to Mannich bases (Dimmock and Kumar, 1997).

Cancer is the second leading cause of deaths in the world following cardiovascular diseases (Menotti *et al*., 2004). The available anticancer drugs on the markets need improvements to overcome the toxicity problems, side effects, and drug resistance. Therefore, new anticancer agents need to be investigated. Several researchers reported that a considerable number of chemotherapeutical agents target DNA (Martin-Cordero *et al*., 2003; Ishar *et al*., 2006; Alpan *et al*., 2007; Topcu *et al*., 2008) and/or interfere with DNA-protein interactions (Topcu and Borden, 2000) in particular the topoisomerase-DNA catalytic cycle. Topoisomerases are essential enzymes that break and rejoin DNA strands through a covalent protein-DNA intermediate (Wang, 1996). They are divided into two classes, which vary in their mechanisms of action: Type I topoisomerases produce transient single-strand breaks in DNA, while type II enzymes make transient double-strand breaks



a

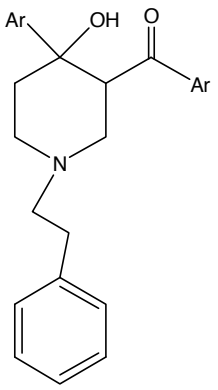


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Fig. 1. Chemical structures of the compounds tested: (a) Mono-Mannich bases; (b) semicyclic mono-Mannich bases, *i.e.* piperidinols. Ar = (C_6H_5) for **1a**, **1b**; (4-$CH_3C_6H_4$) for **2a**, **2b**; (4-$CH_3OC_6H_4$) for **3a**, **3b**; (4-ClC_6H_4) for **4a**, **4b**; (4-FC_6H_4) for **5a**, **5b**; (4-BrC_6H_4) for **6a**, **6b**; (2,4-$(Cl)_2C_6H_3$) for **7a**, **7b**; (4-$NO_2C_6H_4$) for **8a**, **8b**; (C_4H_3S(2-yl)) for **9a**, **9b**; (4-HOC_6H_4) for **10a**.

(Wang, 1996; Bjornsti and Osheroff, 1999). As a result, the former enzymes remove supercoils from DNA, one at a time, whereas the latter ones also remove supercoils, but two at a time.

To the best of our knowledge, there is limited information about the effects of Mannich bases on DNA topoisomerase I (Canturk *et al.*, 2008). In this study, we assessed the cytotoxicity of 1-aryl-3-phenethylamino-1-propanone hydrochlorides and 3-aroyl-4-aryl-1-phenethyl-4-piperidinols (Fig. 1), synthesized in our laboratory (Mete *et al.*, 2010), using androgen-independent prostate cancer cells (PC-3) in relation to supercoil relaxation activity DNA topoisomerase I.

Results and Discussion

Cytotoxic activity results of the compounds against PC-3 cells are shown in Table I. Among the mono-Mannich bases, compound **7a** with two chloro substituents on the phenyl ring, **8a** with a nitro substituent on the phenyl ring, and **10a** with a hydroxy substituent on the phenyl ring were found to be effective against PC-3 cells while the others were not effective. On the other hand, all semicyclic mono-Mannich bases (piperidinols) were effective against the PC-3 cell line and had higher cytotoxicity than their corresponding mono-Mannich base analogues (Table I). Although, all of the compounds had lower cytotoxicity than the reference compound 5-fluorouracil, their cytotoxicity was within the 8.2–32.1 μM concentration range.

Among the piperidinol compounds on which the halogen atom is located at *p*-position of the phenyl ring, the most effective one in terms of cytotoxic activity was **4b** having a chloro substituent, the second one was **6b** having a bromo substituent, and the third one was **5b** having a fluoro substituent (Table I). When the number of chlorine atoms increased in compound **7b** by addition of a second chlorine atom at 2-position of the phenyl ring, cytotoxicity increased about 1.77 times compared with **4b**. Replacement of the phenyl group in **1b** by the thiophene in **9b** had no effect on the cytotoxicity.

When the chemical structures of the compounds were considered, cytotoxicity increased in all semicyclic mono-Mannich bases, *i.e.* piperidinols, compared with the corresponding mono-Mannich base analogues. It is possible to quantify the increase in cytotoxicity by comparing the cytotoxic activities of compounds **7a** and **8a** with the analogue compounds **7b** and **8b** as examples. Cytotoxicity increased 2.85 times in **7b** and 1.67 times in **8b**, which are piperidinol compounds (Table I). This suggests that the piperidinol structure may produce more alkylation centres than the corresponding mono analogues leading to higher cytotoxicity. It is known that Mannich bases can undergo deamination to generate an α,β-unsaturated ketone under physiological conditions *in vivo* or simulated physiological conditions *in vitro*. Cytotoxicity can be attributed to thiol alkylation of the generated α,β-unsaturated ketone (Gul M., 2005; Gul M. *et al.*, 2005b; Pati *et al.*, 2007). In the case of piperidinols mentioned in this study, they should have produced a mono-Mannich base, 1-aryl-3-phenethylamino-1-propanone and 1-aryl-2-propene-1-one, under *in vivo* or *in vitro* conditions by degradation, while mono-Mannich bases might have undergone deamination to produce 1-aryl-2-propene-1-one. It is also important that the optimum drug or compound concentration

Table I. Cytotoxic activities of the compounds **1a**–**10a** and **1b**–**9b** against PC-3 cells.

Compound	Ar	Cytotoxicity [μM]	Log P	σ_p
1a	C_6H_5	-	4.83	0.00
1b	C_6H_5	8.5 ± 0.9	4.48	0.00
2a	4-$CH_3C_6H_4$	-	5.10	-0.17
2b	4-$CH_3C_6H_4$	21.4 ± 0.9	5.18	-0.17
3a	4-$CH_3OC_6H_4$	-	4.89	-0.27
3b	4-$CH_3OC_6H_4$	10.3 ± 1.8	4.09	-0.27
4a	4-ClC_6H_4	-	5.32	0.23
4b	4-ClC_6H_4	14.5 ± 3.5	5.65	0.23
5a	4-FC_6H_4	-	4.82	0.06
5b	4-FC_6H_4	32.1 ± 1.1	4.41	0.06
6a	4-BrC_6H_4	-	*	0.23
6b	4-BrC_6H_4	22.2 ± 1.5	*	0.23
7a	2,4-$(Cl)_2C_6H_3$	23.4 ± 3.5	5.49	&
7b	2,4-$(Cl)_2C_6H_3$	8.2 ± 1.4	6.16	&
8a	4-$NO_2C_6H_4$	15.2 ± 1.8	4.95	0.79
8b	4-$NO_2C_6H_4$	9.1 ± 1.9	5.54	0.79
9a	C_4H_3S(2-yl)	-	4.84	&
9b	C_4H_3S(2-yl)	8.5 ± 2.1	4.16	&
10a	4-HOC_6H_4	17.2 ± 1.3	4.12	-0.37

Cytotoxic activity of 5-fluorouracil was (2.8 ± 0.8) μM.
* Could not be calculated; - not active; & not considered.

is needed for optimum bioactivity at the target side. Piperidinol compounds might have provided optimum compound concentration at the target side, while mono-Mannich bases could not have provided this because of several reasons, such as faster deamination or faster extraction.

There was no correlation between IC_{50} values and log P values of the compounds, which reflect the hydrophobicity of the compounds, and IC_{50} values of the compounds and Hammett values of the substituents (σ_p), which reflect electronic nature of the substituent. This suggests that cytotoxicity of the compounds did not result from the hydrophobicity of the compounds or electronic nature of the substituents.

We, next, employed the supercoil relaxation assay that uses plasmid DNA substrate with mammalian DNA topoisomerase I. The method relies on the ability of the enzyme to relax supercoiled DNA (*sc*DNA) to its relaxed form (*rlx*DNA). The result of a representative supercoil relaxation assay monitored on 1% agarose gel using 1 μg/μL test compound is given in Fig. 2. The *sc*DNA (Fig. 2A, upper lane 1) was fully relaxed (Fig. 2A, upper lane 2) in the presence of 1 unit of the enzyme. Relaxation of *sc*DNA substrate was inhibited upon incubation with **10a** (Fig. 2A, upper lane 7) and **5b** (Fig. 2A, lower lane 6) while the other compounds did not exert a detectable interference on the enzyme (Fig. 2A, upper lanes 3–6, 8–12 and lower lanes 1–5, 7–9). Presence of the organic solvent DMSO (10%) did not interfere with the topoisomerase I activity (Canturk *et al*., 2008). As the interference decreased upon serial dilution (data not shown), our data shows that the effects obtained were due to the presence of the test compounds **10a** and **5b**. We then quantified the *sc*DNA and *rlx*DNA band intensities for these two compounds, and the average values are summarized in Fig. 2B. We estimated that the mono-Mannich bases **10a**, which has a hydroxy substituent on the phenyl ring, and **5b**, which has a fluoro substituent on the phenyl ring from the semicyclic mono-Mannich base series, had an interference of 42% and 40%, respectively (Fig. 2B). This suggests that the inhibition of DNA topoisomerase I may be one of the possible mechanisms for the cytotoxicity of compounds **10a** and **5b**. The hydroxy substituent has the ability of hydrogen bonding with receptors, while fluorine is the most electronegative element in the periodic table. These properties of the substituents could also contribute to their effects on DNA topoisomerase I.

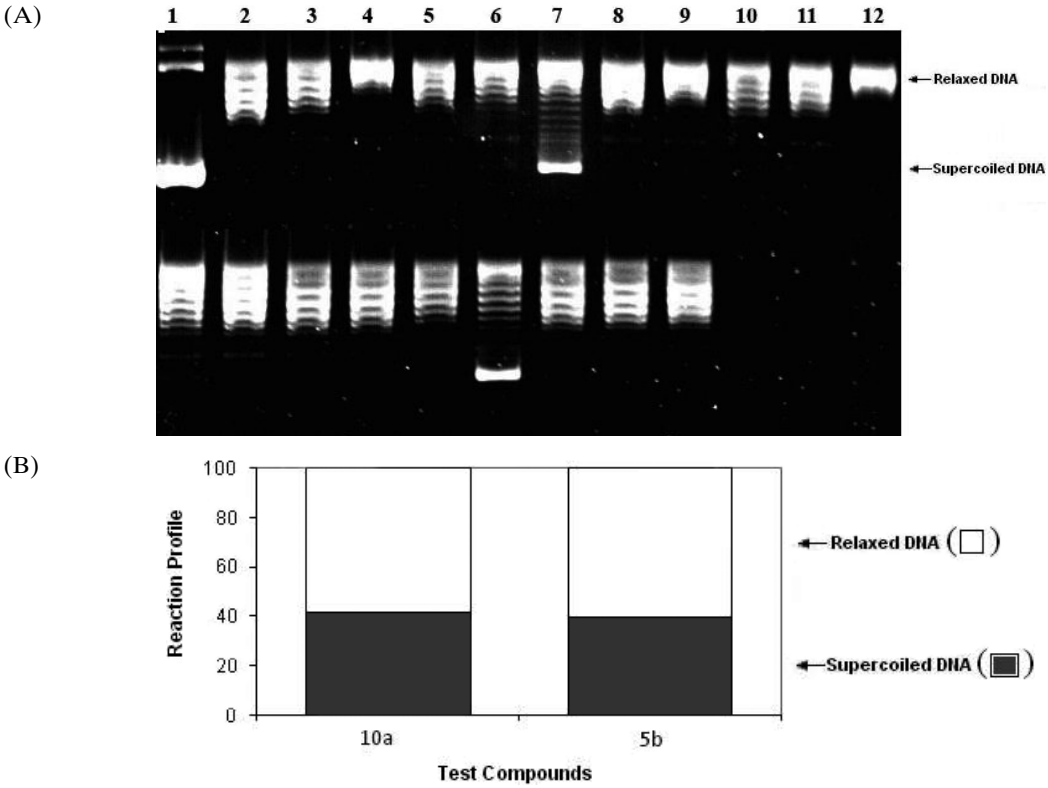

Fig. 2. Inhibitory activity of the test compounds on mammalian DNA topoisomerase I. (A) A representative assay using 1 μg/μL of the test compounds in topo I reactions. Upper lane 1, pBR322 DNA without enzyme; upper lane 2, supercoil relaxation with 1 unit of DNA topoisomerase I; upper lanes 3–12 correspond to the compounds **2a**, **3a**, **6a**, **7a**, **10a**, **8a**, **5a**, **9a**, **1a**, and **4a**, respectively. Lower lanes 1–9 correspond to **2b**, **3b**, **6b**, **7b**, **8b**, **5b**, **9b**, **1b**, and **4b**, respectively. (B) Quantitative assessment of the inhibition obtained with compounds **10a** and **5b**. Two of the test compounds, **10a** and **5b**, were selected for quantitative evaluation. Supercoiled DNA population, representing the inhibition, and the relaxed DNA population, representing the enzyme activity, are shown with dark and blank areas, respectively (see "Materials and Methods" for details).

In conclusion, piperidinol-type semicyclic mono-Mannich bases are more promising derivatives in developing new anticancer agents compared to their corresponding mono-Mannich base analogues. In further studies, new compounds having the same substituents at different position/positions of the phenyl ring will be synthesized, since duplication of the substituent improved the cytotoxicity in the present study.

Material and Methods

Materials

PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 medium, PBS, FBS, glutamine, and gentamicin reagent solution were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA solution (10x) and trypan blue solution were purchased from Sigma Chemical (St. Louis, MO, USA). Cell Titre 96 Aqueous Non-Radioactive Cell Proliferation kit was purchased from Promega Corporation (Madison, WI, USA). Cell culture flasks and plates were purchased from Corning Inc. (Union City, CA, USA). A Marathon 3200 centrifuge was purchased from Fisher Scientific (Pittsburg, PA, USA). A HERA cell incubator and HERA safe cell culture safety hood were purchased from Kendro Laboratory Products (Newton, CT, USA). An IV900 series inverted microscope was purchased from Microscoptics Inc. (Holy, MI, USA). A SpectraMax 384 Plus spectrophotometer was purchased from Molecular Devices (Sunnyvale, CA, USA). Compounds

tested were synthesized in our laboratory (Mete *et al.*, 2010).

Cell maintenance

PC-3 cells were maintained in T-75 flasks at 37 °C and 5% CO_2 with RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, and 0.02% gentamicin solution (10 mg/mL).

Determination of the cytotoxic activity of the compounds

Cells were plated into 96-well plates at a cell density of 1000 cells per well in 100 μL of medium. The cells were incubated at 37 °C and 5% CO_2 for 24 h to allow the cells to attach to the plate surface. Compounds were dissolved in DMSO to 10 mM. Dilutions were made in medium to a final concentration of 50 and 10 μM in final volume of 200 μL. A lower starting concentration of 25 μM was used for the compounds having solubility problems at higher concentrations. The content of DMSO did not exceed 0.2% of the total volume in each well. Triplicates per compound per plate were performed as well as a no-drug control, which received 100 μL of medium with DMSO only. The compounds were incubated with the cells for 72 h at 37 °C and 5% CO_2. Promega Cell Titre 96 Aqueous Non-Radioactive Cell Proliferation kit was used to determine the viability of cells. For four plates 8 mL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) solution were combined with 400 μL of phenazine methosulfate (PMS) solution. MTS/PMS solution was added to each well and the 96-well plates were incubated for 3–5 h at 37 °C and 5% CO_2. Absorbance was determined at 490 nM using a Molecular Devices SpectraMax 384 Plus spectrophotometer with SOFTmax Pro software. The absorbance data were copied into Microsoft Excel, and the replicates of each compound were averaged, as the values for the control of each plate. A background value of 0.41 representing the absorbance of media and MTS/PMS solution alone was subtracted from each average. Percent inhibition of cell proliferation was calculated as described (Cory *et al.*, 1991; Gul H. I. *et al.*, 2006, 2007, 2008). Compounds that inhibited cell viability by 50% or more at 50 μM concentration were then used for IC_{50} determinations with the starting concentration of the compounds of 150 μM. Three-fold serial dilutions were performed and the concentration of the last well was 8 nM. Compounds with percent inhibition between 30 and 50% had a starting concentration of 200 μM. Following incubation for 72 h, the MTS assay was performed using the Cell Titre 96 Aqueous kit. IC_{50} values were calculated using the SOFTmax Pro plate reader program. The control used was 5-fluorouracil. Each compound was tested at least three times.

Plasmid supercoil relaxation assays

Plasmid supercoil relaxation assays were carried out as described (Alpan *et al.*, 2007; Topcu *et al.*, 2008). 20 μL of reaction mixture contained one unit of calf thymus topoisomerase I, 0.5 mg of supercoiled (sc) pBR322 DNA (Takara, Otsu-Shiga, Japan), in the presence or absence of the test compounds in 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, and 0.1% bovine serum albumin. The relaxation products were analysed on 1% agarose gels in TBE buffer (45 mM Tris borate and 1 mM EDTA, pH 8.0) in a horizontal electrophoresis apparatus (5 V/cm) (Thermo EC250) and photographed under UV light after staining in ethidium bromide (EtdBr) solution (0.5 μg/mL). The relationship between the binding of EtdBr and the amount of fluorescence given by sc and relaxed DNA under UV light was determined as described (Alpan *et al.*, 2007). DNA bands were quantified from gel photographs using BioRad Multianalyst (ver. 1.1). One unit of the enzyme activity was defined as the activity removing the supercoils from 500 ng of *sc*DNA plasmid substrate at 37 °C within 30 min.

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