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Relationship between lipophilicity and antitumor activity of molecule library of Mannich ketones determined by high-performance liquid chromatography, clogP calculation and cytotoxicity test

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Abstract

A series of Mannich ketones were synthesized in order to study the relative importance of structure and specific substitutions in relation to their lipophilicity and antitumor activity. Substitutions were carried out with morpholinyl, pirrolidinyl, piperidyl and tetrahydro-isoquinolyl groups in various positions on three different skeletons. Lipophilicity of Mannich ketones was characterised by chromatography data (log k') and by software calculated parameters (clogP). Compounds were tested on their ability to inhibit the proliferation of the A431 human adenocarcinoma cell line evaluated by MTT and apoptosis assays. The results suggest that the higher the lipophilicity values (log k' and clogP), the higher the antitumor and apoptotic activity of Mannich ketones. Determination of lipophilicity by measuring the log k' or by calculating the clogP values of the compounds may help to predict their biological activities.

Keywords: Hydrophobicity; Mannich ketones

1. Introduction

Appearance of new methods (e.g. combinatorial molecular libraries in pharmaceutical research) arouse two tremendous problems: one is the analysis demanding separation of thousands of synthesized molecules (often having very similar chemical structure and character), while the other is the physico-chemical/biological/biochemical characterisation of this huge number of molecules [1]. The number of molecules to be tested by expensive, labour- and sample consuming biological/biochemical methods has extremely increased in recent years. This situation enhanced the need for invention of rational methods in drug design [2]. One of the current approaches in rational drug design is to estimate hydrophobic/lipophilic nature of the molecules on

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the basis of certain experimentally determined physico-chemical parameters, because hydrophobic/lipophilic properties of the molecules play an important role in the mechanism of their biological action, as well as in the structure-biological activity relationships [3–5]. Another possibility is the use of different softwares to calculate hydrophobicity/lipophilicity on the basis of the structural moieties building up the molecule investigated [6].

The expressions ''hydrophobicity'' and ''lipophilicity'' are often used interchangeably, and their frequency of usage is different in various scientific fields. While medicinal chemists prefer lipophilicity term, chromatographers tend to use hydrophobicity to describe partition processes in RP-HPLC [30]. According to valid IUPAC recommendation, hydrophobicity is the association of non-polar groups or molecules in an aqueous environment, which arises from the tendency of water to exclude non-polar molecules [3]. Hydrophobicity was recognised as one of the most important parameters influencing the fate of a molecule within the body [7] and it has a pivotal role in QSAR studies [6].

According to the definition suggested by IUPAC, lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment and it is commonly measured by its distribution behaviour in a biphasic system such as liquid/liquid or solid/liquid system. Since the proposal of Hansch, the lipophilicity of molecules has been typically characterised by the log 1-octanol/water partition coefficient (log Pow) [8,9]. In the last decades, different reasons promoted the displacement of the shake flask method conventionally used to determine log Pow. One of them is the experimental difficulty of this method, other drawbacks aroused by the invention of the combinatorial molecular libraries in the rational drug design [10].

These demands contribute to the development and application of those separation methods of high-performance, which beyond the fast separation of the analyzed components are simultaneously able to provide data characterising physico-chemical properties (e.g. distribution coefficient, retention factor, lipophilicity) of the compounds analysed. High-performance liquid chromatography in reversed-phase separation mode has long been recognized as a potential method for lipophilicity determination [11,12]. Various approaches have been described which employ octanol in the chromatographic system or just use conventional octadecyl silica column and hydroorganic mobile phase [3,11,12]. The properties of the compounds are characterised directly from the chromatographic retention determined by the interaction of solutes with the stationary and mobile phases. When highly efficient reversed-phase stationary phases were used with hydroorganic mobile phases, the correlation between the chromatographic partition data and the octanol/water partition data was strong when structurally related compounds were investigated [13].

The aim in this respect is to estimate lipophilic character of the molecules on the basis of their retention factors (k') determined in various separation processes. Expected biological activity of the constituents of a molecule library can be evaluated on the basis of their retention factors [3]. This may prove to be especially useful for pre-screening of thousands of molecules synthesized by combinatorial chemistry methods. The number of molecules to be biologically tested can be considerably reduced if biological activity of the compounds can be reliably predicted on the basis of those data. In that case, there is no need to perform biological characterisation of every single member of the library [7].

Among the various biological parameters investigated, the knowledge of cytotoxicity of the compounds is one of the most important data. Many different methods are available to assess cytotoxicity in culture including the microculture tetrazolium assay (e.g. MTT) [14,15]. Since measurements of in vitro growth in microculture wells by cell mediated reduction of tetrazolium salt to water insoluble formazan crystals showed excellent correlation with measurements of cellular protein in adherent cell line, as well as viable cell count in suspension cell cultures [16]. This assay provides sensitive and reproducible indices of growth as well as drug sensitivity in individual cell lines. Thus, this colorimetric assay based on enzyme activity of various dehydrogenases of the living cells is suitable for cytotoxicity testing of antitumor drugs in vitro [16,17].

However, as it was observed in many cases, cytotoxic agents like antitumor compounds can reduce not only the number of tumor cells as a

measure of decreased MTT reduction, but they can induce various types of cell death including apoptosis [18]. Apoptosis is a physiological phenomenon, which means apoptotic or programmed cell death where the dying cell plays an active part in its own destruction relying on the new protein synthesis, and among others, morphologic hallmarks [19]. Furthermore, it is becoming increasingly clear that apoptosis is a significant mode of cell death in the response of tumor cells to various anticancer treatment. Therefore, new apoptosis-inducer molecules provide new targets for drug design [20].

In our earlier work, we reported on the antifungal effect of *E*-2-arylidene-1-tetralones, *E*-3-arylidenechroman-4-ones and *E*-3-arylidene-1-thiochroman-4-ones-homoisoflavones [21]. These compounds were screened against human pathogenic yeasts showing marked antifungal activity [22]. Furthermore, several Mannich ketones are described having antibacterial, antifungal and cytotoxic activity [23–28]. To search for new types of antiproliferative compounds, possessing apoptosis-inducing activity a library of fused Mannich ketones was designed. A total of 16 Mannich ketones with different base structures and substituents were synthesized.

The aim of the present work was to separate the members of the Mannich ketone library by a suitable RP-HPLC method, to characterise their lipophilicity by experimental parameter obtained from the separation process (retention factor, k') and by a parameter obtained by computer prediction method (clogP calculation). Furthermore, antitumor activity of Mannich ketones was determined by MTT test and cytology. The relationship between the lipophilicity parameters (k' and clogP) and the antitumor activity was also studied.

2. Materials and methods

2.1. Chemicals

Mannich ketones were synthesized in our laboratory. Purity of the compounds was better than 95% in each case as shown by HPLC analysis. Triethylamin and phosphoric acid were purchased from Fluka (Buchs, Switzerland); acetonitrile (ACN) from Chemolab (Budapest, Hungary). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and bovine serum albumin were obtained from Sigma. DMSO was purchased from Merck (Darmstadt, Germany). Foetal calf serum and RPMI-1640 medium were obtained from GIBCO (Grand Island, NY, USA). Formic acid was extra pure grade (27001) from Riedel-deHaën.

2.2. Synthesis of Mannich ketones

Mannich ketones have been prepared from the corresponding fused ketones as tetralones, indanones etc. The method was the classical Mannich reaction applying ethanol as a solvent and HCl as a catalist [22]. The products were purified through recrystallization and Mannich bases were liberated and purified using very mild conditions. Then they were precipitated with methanolic HCl. The NMR measurements were done on the Mannich bases. All structures were validated by a Waters LC/MS system equipped with a Waters 996 DAD UV detector and a Micromass ZMD MS detector. Mannich ketones with different base structures and substituents (R2) are summarized in Table 1. R1 substituents may be H- or OMe-, and their name and position are given in the Table 2.

Table 1
Basic structures of Mannich ketones

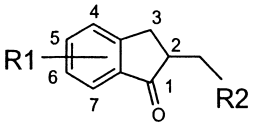
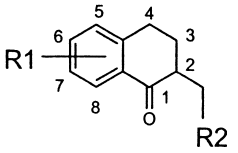
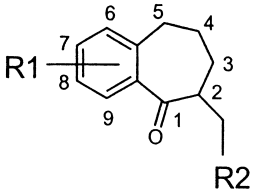
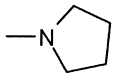
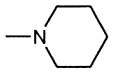
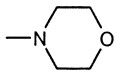
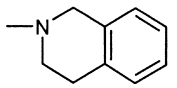
Type of structure	Structure
A	
B	
C	

Table 2
R2 substituents of the basic structures

Substituents	R2
1-pyrrolidinyl	
1-piperidyl	
4-morpholinyl	
2-(1,2,3,4-tetrahydro)-isoquinolinyl	

2.3. HPLC measurements

For chromatographic analysis stock solutions of 1.0 mg/ml of the samples in acetonitril:water (1:1) were prepared and filtered through a 0.2-μm Millipore filter unit. These solutions were kept in Eppendorf tubes at 4 °C.

HPLC analysis of the samples were performed with Varian (Basel, Switzerland) 9012 Solvent Delivery System, Varian 9065 Polychrom Diode Array Detector; column: Hypersil 5 MOS 5 μm, 300×4.6 mm (BST, Hungary); injector: Rheodyne; eluents: (A) 0.25 N triethyl ammonium phosphate (TEAP), pH 2.25; (B) 80% ACN+20% A. Isocratic runs were performed in an eluent of 24 v/v% ACN in A eluent. Flow-rate: 1 ml/min. Temperature: 20 °C. 20 μl portion from the stock solution was injected into loop of 50 μl volume and four parallel injections were analyzed. Retention factors (k') of the samples were calculated from the experimentally determined retention data:

$$k' = (t_R - t_o)/t_o.$$

2.4. Calculation of clogP data

Software-predicted lipophilicity of the compounds was calculated with the computer program clogP which predicts this parameter with the so-called ''fragment constant'' method on the basis of the chemical structure of the compound processed. Briefly, the clogP program is based on Hansch-Leo's logP calculation method. It divides molecules into fragments and uses the constants of these fragments and correction factors taken from its database for logP calculation [9,29]. A clogP database was prepared with the computer program accessible via internet (www.daylight.com/daycgi/clogp). Lipophilicity values were computed for the 16 Mannich ketones and are designed in the followings as clogP values (see Table 3).

2.5. MTT assay

To evaluate the antiproliferative effect of Mannich ketones on A431 cells (American Type Culture Collection No. CRL-1555), the MTT colorimetric assay was performed as described in Refs. [16,17]. The amount of formazan could be determined by photometer at 570 nm. Cells were plated into 96-well flat bottomed culture plates (Greiner, Germany) at a concentration of 10^4 cells per well in complete RPMI 1640 culture medium. A period of 24 h after plating, foetal calf serum containing medium was removed and test solution of Mannich ketones was given to cells in various final concentration such as 200, 100, 50, 25 and 10 μg/ml. After incubation with drugs for 24 h, MTT solution was added to the wells and plates and were incubated at 37 °C for 4 h. Then sodium dodecyl sulphate (10 w/v% in 0.01 *M* HCl) was added and the amount of formazan formed was measured. Six wells per dose and time points were counted in three different experiments. The MTT assay have been performed only on selected members of the library. Compounds with highly hydrophobic nature and those with low lipophilicity were selected. Percent of inhibition was calculated from the values of triplicate experiments and the results are expressed as percent of controls.

2.6. Apoptosis assay

To evaluate the apoptosis-inducing effect of Mannich ketones, 7×10^4/ml of A431 cells were cultured over glass coverslips in 6-well flasks. Treatment was performed 24 h after plating with the following compounds: No. 1, 2, 11 and 13. Apoptosis-inducing activity was investigated at the final concentration of

Table 3
Groups in the library of Mannich ketones

Group	Type of structure	Number of compound	R1	R2	k'[a]	clogP	MTT[b]
I	B	1	H	4-morpholinyl	0.46	2.08	5.2±3
	B	2	H	1-pirrolidinyl	0.73	2.59	–
	B	3	H	1-piperidyl	0.97	2.74	–
	B	4	H	2-(1,2,3,4-tetrahydro)-isoquinolyl	2.46	3.96	35.5±8
II	B	5	6-OMe	4-morpholinyl	0.68	2.30	31.3±5
	B	6	6-OMe	1-pirrolidinyl	1.01	2.81	–
	B	7	6-OMe	1-piperidyl	1.33	2.96	–
	B	8	6-OMe	2-(1,2,3,4-tetrahydro)-isoquinolyl	3.48	5.46	37.8±4
III	B	9	5-OMe	1-piperidyl	1.24	2.96	36.4±7
	B	7	6-OMe	1-piperidyl	1.33	2.96	–
	B	10	7-OMe	1-piperidyl	1.31	2.96	37.1±5
IV	B	11	H	1-piperidyl	0.97	2.74	27.7±3
	B	7	6-OMe	1-piperidyl	1.33	2.96	–
	B	10	7-OMe	1-piperidyl	1.31	2.96	37.6±8
V	B	1	H	4-morpholinyl	0.46	2.08	5.3±7
	B	5	6-OMe	4-morpholinyl	0.68	2.30	–
	B	12	7-OMe	4-morpholinyl	0.78	2.30	31.4±5
VI	A	13	H	4-morpholinyl	0.25	1.67	26.6±3
	B	1	H	4-morpholinyl	0.46	2.08	–
	C	14	H	4-morpholinyl	0.68	2.64	28.4±7
VII	A	13	H	4-morpholinyl	0.25	1.67	26.3±5
	A	15	H	1-pirrolidinyl	2.99	2.14	–
	A	16	H	1-piperidyl	0.46	2.33	39.6±8

[a] Values are means of three parallel measurements where the standard error was less than 0.1%.
[b] Values are means of three different experiments with six wells per dose and time point in each cases and ±SD.

100 μg/ml. Three cover slips per time point of both control and treated cells were fixed in ethanol/acetic acid (3:1) and stained with Haematoxylin and Eosin. At least 2000 cells per coverslip were investigated and counted. The cell pellets from control and treated cultures were resuspended at 1×10^7 cells/ml in fresh medium, and 25 μl of a solution of acridin orange and ethidium bromide was added. The mixture was immediately placed on the stage of an Olympus Fluorescence microscope (Germany) and the image was analyzed and photographed. Apoptotic cells were characterised by morphological changes including shrinkage of the cell and the nucleus; condensation and fragmentation of the nuclear chromatin; membran blebbing; and the segmentation of the cell into apoptotic bodies. Apoptotic, necrotic and live cells were compared, scored and counted under microscope according to our earlier work [17].

3. Results and discussion

3.1. The results of the HPLC measurements

A suitable isocratic ACN/TEAP eluent system has been developed for the separation of structurally relative compounds of the Mannich ketone library containing 16 compounds (Table 1) bearing different substituents on the same skeleton and compounds heaving the same substituents on different skeletons

(rings with different carbon number). Compounds were successfully separated within 25 min (Fig. 1).

The retention factor for each compound was calculated as indicated in Table 3. In all cases, tetrahydro-isoquinolyl group as substituent R2 caused the strongest impact on the retention data. Compounds with tetrahydro-isoquinolyl group showed the highest retention factor (Number 4 and 8), while Mannich ketones containing morpholinyl group had the lowest retention factor (Number 1, 5, 12 and 13). When morpholinyl group was replaced by pirrolidinyl or piperidyl group, the retention factor became greater (Group I, II and VII).

Considering the phenomena of isomerism library of Mannich ketones contains compounds which are structural isomers (Group III, IV, V). During the course of HPLC investigation, the stationer phase applied (Hypersil 5 MOS) was sensitive enough to separate the isomers, however, significant discrepancies in the retention factors of the isomers we could not achieved.

3.2. The results of the clogP calculation

Lipophilicity values predictable on the base of chemical structure (clogP) were calculated for Mannich ketones. These calculated clogP values were compared with the experimentally determined retention factors of the same compounds (Tables 3).

The tendency of retention factors (log k') and calculated lipophilicity (clogP) values were in good agreement. Comparison of the retention factors and clogP values for 14 compounds revealed that good linear correlation exists between the lipophilicity parameters determined experimentally (log k') and the calculated (clogP) ones on the basis of the chemical structure: $R=0.9476$, SD $=0.1787$ (Fig. 2). Similarly to the measured retention factor values tetrahydro-isoquinolyl group caused the strongest impact on clogP values. Compounds with tetrahydro-isoquinolyl group showed the highest clogP values (Group I, II). In contrary, Mannich ketones with morpholinyl substituent had the lowest retention factor (Number 1, 5, 12 and 13). The clogP values became greater when the morpholinyl group was replaced by pyrrolidinyl or piperidyl moieties (Group I, II and VII).

When the influence of carbon number of the

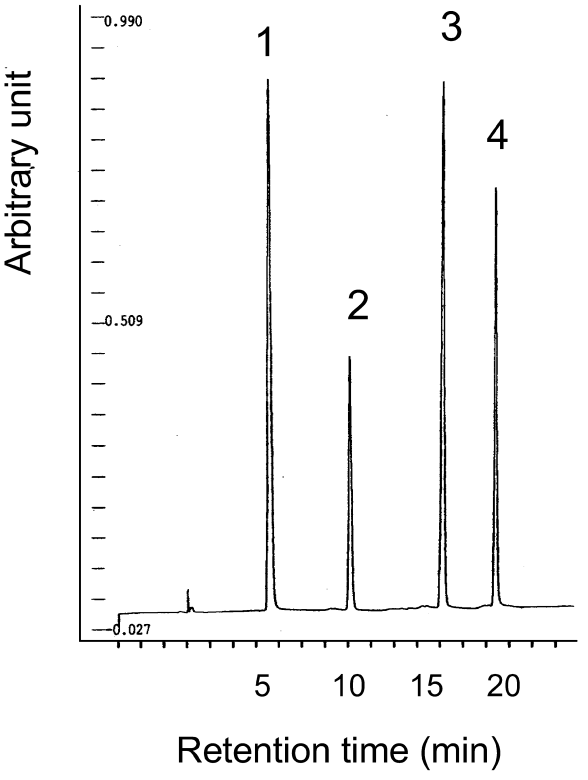


Fig. 1. HPLC chromatograms of fused Mannich ketones. Isocratic run was performed in an eluent of 24 v/v% ACN in eluent A.

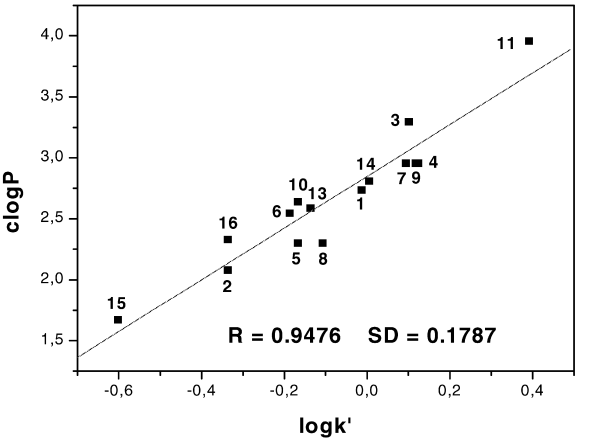


Fig. 2. Relationship between log k' and calculated lipophylicity values (clogP) of 14 Mannich ketones.

skeletons on the retention factor and clogP values of the ketones having the same substituent on different rings was investigated, we found that the retention factor and the clogP values increased with the carbon number (e.g. in the case of Group VI, where in each member of the group R1 and R2 had H- and morpholinyl moiety, respectively), the retention factor and clogP grew according to the increasing carbon number of the non-aromatic saturated rings in the order of A→B→C. Retention factors and clogP values were also increased as H- was substituted for Me- or MeO- groups (Group IV, V).

3.3. The results of the MTT assay

Molecules with slightly or highly lipophilic properties were chosen from all groups of the library to analyse the biological significance of lipophilicity and the influence of the substituents on antitumor activity. Antiproliferative activity of the selected compounds was determined at 100 μg/ml. Comparison of the inhibitory effect of Mannich ketones on A431 cell proliferation (MTT activity) and lipophilicity of the selected compounds revealed that the higher the measured or calculated lipophilicity of the molecule, the higher its MTT activity (Table 3). Antiproliferative activity of the compounds was within a relatively narrow range (from 5 to 39% of inhibition). However, we could observe the same trend with the MTT values as we found with the log k' and clogP data. The highly lipophilic compounds were more active in all groups than their less lipophilic member.

3.4. The results of the apoptosis experiments

The first four compounds of the library were selected for the apoptosis assay (Table 4). In this assay, apoptosis-inducing ability of Mannich ketones was evaluated on the basis of cytomorphological criteria at the same concentration as was used in the MTT test (100 μg/ml). Our findings showed that all of the compounds investigated were active apoptosis-inducers at that concentration. According to Table 4, compounds having tetrahydro-isoquinolyl substituent were nearly twice more active than the compound having morpholinyl group. Furthermore, apoptosis-inducing ability of the compounds was compared to their measured/predicted lipophilicity values and to their MTT activities. These comparisons showed that the higher the lipophilicity (either the experimentally measured values or the predicted/calculated ones) and the MTT activity (cell proliferation inhibition), the greater the apoptosis-inducing capability of the molecules investigated. This suggests that lipophilicity of the molecules has influence on the apoptosis-inducing activity of Mannich ketones.

Table 4
Apoptosis-inducing effect (% of inhibition in 100 μg/ml) of selected compounds. Values are means of three parallel experiments and ±SD

Group	Type of structure	Number of compounds	Induction of apoptosis
I	B	1	23.4±2.6
	B	2	30.5±3.5
	B	3	35.4±4.4
	B	4	39.1±6.2

4. Conclusions

Reversed phase HPLC method applied in this work proved to be applicable for fast analysis of Mannich ketone library. Separation of 16 Mannich ketones possessing similar chemical structure could be obtained within 25 min. Advantage of the method applied is that beyond the check of the chromatographic purity of the compounds synthesized, an experimental physico-chemical parameter can be obtained during the course of the analysis which characterises the lipophilicity of the compounds (retention factor, k'). Furthermore, good correlation was found between the experimentally determined lipophilicity parameter (retention factor) and the computer predicted parameter (clogP). In the case of Mannich ketone library, this good correlation confirms that both parameters can be well used for the characterisation of lipophilicity.

The role of the lipophilicity in the antiproliferative/antitumor activity and in apoptotic activity of the fused Mannich ketones was proved. The higher the lipophilicity, the higher the antitumor- and apoptotic activity of Mannich ketones. The log k' and clogP values of Mannich ketones showed similar

behaviour to the results of the cell proliferation (MTT) and apoptosis assays, clearly indicating that lipophilicity of the compounds investigated plays a role in their anti-tumour activity. In other words, the calculated clogP values along with the fast determined physico-chemical parameter (log k'), characterising the lipophilicity may offer useful data for the prediction or pre-screening the biological activity. Pre-selection of the molecules can easily be performed on this basis. There is no need to perform sample-, time-, chemicals- and labour-demanding biological tests on each member of the library, only the molecules pre-selected on this basis must be processed. It makes possible the utilisation of this method in cases where fast characterisation of lipophilicity of great number of molecules with similar chemical structure (e.g. members of a combinatorial library) is needed.

Taken together all these findings, we can say that characterisation of lipophilicity on this basis may help to predict the biological activity of the element of a molecule library and it offers a help to compose a more rational library. Such a system may be suitable for further characterisation in the relationship between the lipophilicity parameters and antitumor activity of the drugs.

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