Comparative Characterization of Experimental and Calculated Lipophilicity and Anti-Tumour Activity of Isochromanone Derivatives

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Abstract: Compound lipophilicity connected to ADME(T)[a] has great importance in drug development and it has to be evaluated by the generally used drug developmental process. In addition to the importance of lipophilicity in ADMET, recently it has been reported that lipophilicity of small molecules correlates with their antiproliferative activity because of certain specific hydrophobic and lipophilic interactions. Due to the complexity of ADME(T) parameters an efficient and fast method is needed to characterize the many promising candidate lead molecules as a preselection in order not to be rejected from the latter phase of drug development. In the present paper we provide an overview of the importance of lipophilicity of drug candidates for biological action and for ADME(T) and describe a novel approach for drug-likeness characterization of a molecular library using correlation study between lipophilicity and biological activity. Lipophilicity and molecular characteristics have been measured, predicted and optimized for a diverse library from which the best members have been selected to describe their biological, chemical and drug-likeness properties. Molecules were selected from the family of α,β-unsaturated ketones and thorough HPLC characterization for lipophilicity and morphological, antiproliferative and flow cytometric studies were carried out on them. Based on the results 17 member isochromanone library including E and Z geometric isomers were selected for further characterization. In this focused library linear correlation has been found between the calculated and measured lipophilicity and significant parabolic correlation was found between the antiproliferative effect and lipophilicity. Using our efficient and fast method, from a diverse library, we identified an outstandingly effective inhibitor of A431 tumour cell growth *via* a PARP[a] cleavage dependent apoptosis. In summary the optimized HPLC analyses of lipophilicity combined with the cell-culture assay, introduced above, resulted in the determination of an optimal lipophilicity range. This optimized lipophilicity range should be used in designing novel antiproliferative compounds.

Keywords: Lipophilicity, apoptosis, isochromanone derivative, antiproliferative effect.

INTRODUCTION

Physico-chemical parameters are among the most important properties to be evaluated in the early drug developmental phase [1-4]. Compounds having improper pharmacokinetic or pharmaco-chemical characteristics cannot be considered as drug candidate molecules [5]. One of the most important physico-chemical parameters, in which case early prescreening is available, is lipophilicity [6]. In the last few years it has been realized that the correlation analysis of lipophilicity and biological activity, such as cytotoxicity, antiproliferative effect or apoptosis, has a major impact on drug development. If a precisely defined relationship can be determined it can reduce the cost needed during the selection of active drug candidates and can be used for prediction and estimation of biological effectiveness [7-12].

Lipophilicity of a non-ionic compound whose partition is independent of the pH is commonly characterized by the n-octanol/water (biphasic) partition coefficient ($P_{o/w}$[a], and $\log P_{o/w}$). It has long been recognised that the retention of a compound in different separation methods (RP-HPLC[a], micellar electrokinetic chromatography (MEKC[a]), micellar liquid chromatography) is governed by its lipophilicity and thus it correlates with the $\log P_{o/w}$ measured in n-octanol-water system.

It is a plausible alternative to use quick, automatizable separation methods of high performance and low sample demand (e.g. RP-HPLC, MEKC) as a substitute for the classical slow and uncomfortable shake-flask method to characterize lipophilicity of a compound [13-17]. A great number of publications on the efforts made to adjust HPLC methods and to improve stationary phases to substitute $P_{o/w}$ measurements are well reviewed by Testa [18] or Valkó [6].

Another choice to characterize lipophilicity of a molecule is the computerized calculation frequently based on the fragment approach [13, 19, 20] or on atomic-based (e.g. Crippen's, Viswanadhan's, Broto's method) [21] or molecular lipop-hilicity potential (MLP[a]), developed by Testa *et al*. methods [22].

Lipophilicity strongly depends on structural properties. Adrien Albert was one of the first researchers who studied the relationship between the structure and the activity of different drug molecules using QSAR[a] studies. That was later extended to carry out relationship analyses for different physico-chemical parameters, between these parameters and the biological activity [23-26].

Lipophilicity can be modified and easily determined by characterizing molecules prepared with different moieties. On the base of that correlation studies using lipophilicity parameter as a basic factor could be used for prediction of biological activity.

The relationship between the antiproliferative activity and lipophilic property has been established for different

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molecular libraries such as platinum complexes [10], dehydroartemisinin derivatives [11], calcium antagonist drug molecules [12] or isoquinoline-4,6-dione analogues where the assumption was that the correlation between the lipophilicity and the antitumour activity on A549 non-small-cell drug resistant lung carcinoma cells was caused by a change in the receptor structure termed allosteric reaction [27-28]. These studies established linear correlation mainly but parabolic relationship could also be found in some cases.

In this paper, with the collection of data from several publications and on the basis of our own data, we would like to provide a full insight how the relationship can be described between physico-chemical parameters, especially lipophilicity, and biological parameters, how the molecule-structure can affect the biological activity or how this structural property is in connection with the presence of activation or hydrophobic pocket.

Investigating inhibitors of choline kinase, a novel validated target kinase, Campos *et al.* proved the critical importance of the relationship between lipophilicity, structural properties and antitumour activity resulting in novel achievements in anticancer drug design [29].

Villar *et al.* and Encío *et al.* studied the effect of structural properties on the biological activity in case of benzo[b]thiophene 1,1-dioxide and its derivatives. It was found that these molecules have high cytotoxic and inhibitory activity on tumour-associated NADH oxidase [8, 9] and on human leukemic CCRF-CEM cells [7] confirming that cytotoxic effect is in some cases in a clear correlation with the lipophilicity (logP) due to structural phenomenon. Based on that, the authors synthesised a new benzo[b]thiophene 1,1-dioxide derivative with relatively higher lipophilicity, predicted that it has strong inhibitory effect on six human tumour cell lines. They also found quantitative structure-activity relationship when this drug candidate was tested under reductive and oxidative conditions. In reductive condition they found high inhibitory activity on the tNADH oxidase while in oxidative condition the compound was absolutely inactive [8, 9].

The relationship between lipophilicity and biological activity was also shown by Toth *et al.* [30] In their effort to find an inhibitor to the human neutrophil elastase, which has a significant role in several disorders such as rheumatoid arthritis or periodontitis, they tested several peptides with increasing number of lipophilic amino acids coupled to them. They found an increase in biological activity with the growing number of the lipophilic amino acid in the conjugate. It was confirmed that the presence of a hydrophobic binding site, a hydrophobic pocket, which was located near to the active centre of the human neutrophil elastase, most likely has an impact on the increased biological activity. This finding also shows a clear correlation between lipophilicity and biological activity [30-33].

Interaction between a hydrophobic pocket and an inhibitor is strongly depending on the lipophilicity of the drug candidate. Quartare *et al.* studied cyclic pseudopeptide NK-2 antagonists and determined that the potency of the antagonists increased with the lipophilic side chains. They also found linear correlation between the biological activity and the lipophilicity which generated the conclusion that the lipophilic pocket interacted with the hydrophobic moiety of the cyclic pseudopeptide [34]. Regan *et al.* [35-36] and Kim *et al.* [37] in their structure-activity studies also emphasized the connection between lipophilicity and the role of a lipophilic pocket. In the latter study influenza neuraminidase inhibitors have been investigated and found that the inhibitory activity depends on the presence of a hydrophobic domain. Regan *et al.* studied a specific compound, called 1-(5-*tert*-butyl-2-*p*-tolyl-2*H*-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl]urea against p38α MAP kinase. Their aim was to find a method for the treatment of autoimmune diseases. According to their observation a lipophilic domain of the kinase has major role in the inhibition, as the tert. butyl group of the drug candidate fit into the activation loop of the kinase completely.

On the basis of the described studies, it seems that lipophilicity indeed has a great importance in pre-screening and drug development.

In different biological processes the limitation of the lipophilicity value is indispensable. Several studies have been made to estimate the upper and lower level of this parameter and to estimate the optimal value of it [38-43].

The ideal range can be dependent on several key factors, such as the cell or tissue types (where the molecule should penetrate through) [5].

Coats *et al.* were among the first researchers who pointed clearly out the need of an optimal lipophilicity range in drug discovery. They used parabolic correlation analysis, plotting the cytotoxic activity against the lipophilicity of copper chelates, to determine an optimal range [44].

Michael J. Waring recently published a paper where this limitation was studied. For a very long time, only the upper level of compound lipophilicity has been used as criteria in drug design. Nowadays it is already determined that the too polar molecules can also fail in clinical studies [45].

Upper limitation is required due to several expected drug properties such as water solubility (which is very important especially by orally applied drugs), easier and quicker excretion and clearance through the kidney, faster transport by the plasma and bigger concentration in it [3, 46]. It has recently been stated that the risk for adverse toxicological behaviour increases by drugs having high logP value [38-42, 45]. The determination of a lower limit is also essential. Estimating the optimal lipophilicity range it has to be concerned that while too low lipophilicity reduces the ability of a drug candidate to penetrate into the hydrophobic membrane then too high logP value increases the possibility of the drug molecule sticking in the membrane [47-48]. Veldman *et al.* showed an optimal lipophilicity range for the drug transport through the cell membrane using parabolic correlation analysis [49].

The drugs penetration through the blood-brain barrier has also great importance for molecules which are expected to have an effect in the brain. Their interaction with the blood brain barrier should be proper and the brain uptake of drug molecules highly depends on the lipophilic property [50-53]. Too low lipophilicity prohibits the penetration through the blood-brain barrier, high lipophilicity causes a limitless

fluxuation through it and too high may cause trapping of the compounds inside the membrane [54].

Very often a parabolic relationship has been observed between the logP and the brain uptake. Timmermans found that optimal lipophilicity value for the ideal concentration of the drug in the blood is logP value of 2.16 [55].

According to all of these, several predictions can be made beware of the lipophilicity value of a drug candidate but one of the most essential is to estimate biological effectiveness such as cytotoxicity, antiproliferative activity or apoptotic effect.

In the course of drug development we investigated a wide spectrum of molecules with different core structures. To our lipophilicity studies we selected tetralones, chromanones, benzylidencycloalkanones and isochromanones.

The naturally occurring isochromanone derivatives display various biological effects. Some α,β-unsaturated ketones are also known as synthetic homoisoflavones. 3-Isochromanones exhibited antibacterial, antifungal, insecticide and phytotoxic effects (oosponol, fusarubin and canescin) [56-60] and also play an important role in the utilization of all fluorene carbons in fluorene metabolism by *Arthrobacter sp*. strain F101 [61]. Some homoisoflavones as tetralone, chromanone or thiochromanone derivatives [62-63] have also been screened against human pathogenic yeasts (*Candida spp*). Some of them showed in these *in vitro* tests as high activity (MIC values[a]) as 1.5-6 μg/mL [64].

Chromanone derivatives are also well known of their strong antiproliferative activity. [65-66]. In our previous studies we have already described molecular libraries, which belong to the same molecular family as the isochromanones, such as *E*-2-arylmethylene-1-tetralones, *E*-3-arylmethylene-chroman-4-ones, *E*-3-arylmethylene-1-thiochroman- 4-ones and aurones and their derivatives [67-68]. These compounds have also been tested for their antiproliferative effect which showed good correlation plotted against the measured lipophilicity. Moreover by the tetralone derivatives we were able to determine an optimal lipophilicity value which belongs to the best molecules with antiproliferative effect [67].

To establish a system specific although in basics, generally applicable interrelationship between lipophilicity and certain cellular functions, we further extended our studies where we closely investigated the isomeric 4-arylmethylene-3-isochromanones [69-70] as a new type of antiproliferative compound library. The type of the substituent on the 3-isochromanone molecule was varied in order to investigate the structure- lipophilicity - biological activity relationship. We clearly showed how this correlation analysis can be applied to predict the efficiency of inhibitory activity on our cancer cell line system of selected molecular libraries with the same core-structure.

In harmony with the literature data and as an extension of our previous studies [69-71], we created a molecular library where the lipophilicity and the biological activity of the molecules are in parabolic relationship. This established correlation enabled us to determine an optimal lipophilicity range for biological studies. This optimal range comprises inhibitor molecules with the greatest biological activity and using this area further prediction could be made for the structural properties of potentially active drug candidate molecules. A promising hit-lead molecule with drug-like character and strong antitumour effect on A431 cancer cells was selected during the determination of lipophilicity optimal range.

MATERIALS AND METHODS

Triethylamine, ACN[a], orthophosphoric acid, methanol, ethanol, piperidine, Methylene Blue dye and the chemicals utilised in the synthesis (aldehydes) were purchased from Fluka (Buchs, Switzerland). Solutions were prepared of deionised, bacteria-free water made by Elgastat UHP system (Elga Ltd. Bucks, England). A431 cells (ECACC No.85090402, European Collection of Cell Cultures, Salisbury, Wiltshire, UK), PARP antibody (Cell Signalling Technology, Inc. Danvers, MA, USA), PI[a] (Sigma Aldrich Inc , St. Louis, MO, USA), DMEM[a] cell culture medium (Sigma Aldrich Inc , St. Louis, MO, USA), Trypsine (Sigma Aldrich Inc., St. Louis, MO, USA), FCS[a], cell culture flask (Corning Incorporated, Corning, NY, USA), 24-well plate (Sarstedt Inc, Newton, NC, USA), MB[a] stain (Sigma Aldrich Inc , St. Louis, MO, USA), anti-mouse secondary Ab (Amersham, Buckinghamshire, U.K), X-ray film (KODAK X-OMAT AR FILM, Cedex, France).

Synthesis

A series of 4-(arylmethylene)-3-isochromanones (Fig. (**1**)) has been prepared by base catalysed Knoevenagel condensation as it has been published in [70]. The synthetic method involves a solvent free condensation of 3-isochromanone and the appropriate aldehyde in equimolar amounts in the presence of catalytic amount of piperidine at 140 ^{o}C under argon atmosphere. The separation of the *E*-*Z*-isomers and the purification of the reaction mixture were performed by column chromatography and the title compounds were recrystallized from ethanol.

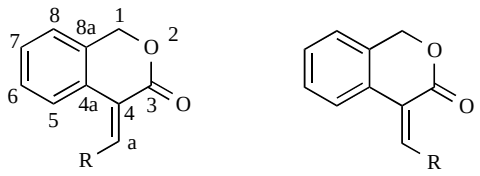


Fig. (1). 4-(arylmethylene)-3-isochromanones (*E*- and *Z*-isomers).

Stereochemical and conformational analysis were performed by ^{1}H and ^{13}C NMR methods, and X-ray crystallography has been used to complement the configurational assignment for a representative product [62]. The ^{13}C NMR spectroscopy was applied for the assignment of the configuration of these ene-lactones.

HPLC Measurements

For high performance liquid chromatographic analysis stock solutions (0.5 mg/ml) of the samples in ACN: water (3:1) were prepared freshly before the analysis and filtered through a 0.2 μm Millipore filter unit. HPLC analysis of the samples was performed with Varian (Basel, Switzerland) 9012 Solvent Delivery System, Varian 9065 Polychrom Di-

ode Array Detector; column: Hypersil 5 MOS 5 μm , 250 x 4.6 mm (BST, Hungary); injector: Rheodyne. In many cases different alkyl-ammonium phosphates were used in the eluent for buffering when the determination of the interaction between the analyte and the hydrophobic alkyl chains of the stationary phase is intended [16, 72-75]. Based on the former practice triethyl-ammonium phosphate (TEAP[a]) was chosen as mobile phase additive. Eluents: A: 0.083 M TEAP (made by weighing the calculated quantities of triethyl amine and phosphoric acid), pH 2.25; B: 95 % ACN + 5 % A (TEAP).

Isocratic runs were performed in an eluent of 40 v/v% ACN in A eluent; flow-rate: 1 ml/min; temperature: 20 °C. Injected volume 20 μl, the compounds investigated here were injected individually, number of intra-day repetitions was 3 (n=3) for each of the compounds. The k^a values of the samples were calculated from the experimentally determined retention data: ($k = (t_R^a - t_o)/t_o$, where t_o has been determined by injection of water [76-77]). Correlation between the k and CLOGP[a] has been investigated.

Calculation of CLOGP Data

The software-predicted lipophilicity of the compounds was calculated with the program called 3DNET4W (Vichem Ltd., 1022 Budapest, Hermann O. u. 15., istvan.kovesdi@ eqnet.hu <mailto:istvan.kovesdi@eqnet.hu>, *2002*) based on the number and the position of the fragment. Thus the calculations set out from the base structure of the molecule and the number, the type and the position of the substituent, but they cannot distinguish between the different geometric isomers.

Antiproliferative Assay

A431 human epidermoid carcinoma cell line was used utilizing the MB test [78]. This colorimetric assay based on the enzyme activity of various dehydrogenases of the living cells, is suitable for testing the cytotoxic activity of antitumour candidates *in vitro*. Human A431 epidermoid carcinoma cells were cultured in DMEM supplemented with 10% FCS (200 mM *L*-glutamine, 10000 U/ml penicillin and 10 mg/ml streptomycin (Gibco Life Sci) at 37°C and 5% CO_2. Cells were seeded into 96-well plates and incubated for 16 hours before serial dilutions of compounds were added (3 replicates have been made at every concentration for each of the compounds investigated). Antiproliferative efficacy was assessed after 48 hours: cells were fixed by 10% buffered paraformaldehyde in 0.9% NaCl. Wells were then stained by 1% MB, followed by thorough washing. Both apoptotic and necrotic cells previously detached from the surface of wells are thus removed. MB stain from cells entrapped on the plate surface was dissolved by ethanol (100%): 0.1 M HCl 1:1 and optical densities measured by a microtiter plate photometric reader at 650 nm.

Microscopic Studies

A431 human epidermoid carcinoma cells maintained in culture flasks or 24-well plates were studied and photographed using Zeiss Axiovert 200 microscope equipped with photo camera at 200x magnification.

Flow Cytometric Studies

A431 cells were incubated at 37 °C for 24 h in DMEM supplemented with 10 % FCS using 24-well plates, seeding 50 000 cells per well. After treatment, the cells were washed, fixed with 70 % ethanol, and were stored at -20 °C or used for PI staining. The stained cells were subjected to flow cytometry (FACS[a] Calibur, BD Biosciences) to detect and quantify apoptosis. Cells with their DNA content less than that of G1 phase cells (sub-G1) were assumed to be apoptotic [79]. The analysis was performed with CellQuest software.

Immunoblotting Studies

For western blot experiments A431 cells in 25 cm^2 culture flasks were treated for 24 hours with **8*E*** applying the doses of 1, 5 and 10 μM. Samples were lysed by adding a Triton-based lysis buffer with protease and phosphatase inhibitors followed by a 10-min incubation on ice and spinning down at 12 000 g for 10 min at 4°C. The Triton-soluble supernatant was used in further immunoblot determinations. The Triton-soluble lysate was mixed with sample buffer, boiled for 5 min, run on 8 % SDS-PAGE, and blotted onto nitrocellulose sheets. Blots were processed using mouse anti-cleaved PARP Ab (Cell Signaling Technology Danvers, MA, USA; Cat. no:9546) followed by a peroxidase-labeled anti-mouse secondary Ab (Cell Signalling Technology Danvers, MA, USA; Cat. no:7076). Blots were developed using Amersham's enhanced chemiluminescence system (ECL Western Blotting Detection Reagents) and exposed to X-ray film (KODAK X-OMAT AR FILM).

RESULTS AND DISCUSSION

A reliable, fast and accurate HPLC method was developed to investigate the 17-member molecular library. The lipophilicity of the compounds was evaluated not only experimentally (HPLC) but by in silico calculation based on their chemical structure, too. Antiproliferative effect of the molecules was determined on A431 cells by the MB method [78]. Both the relationship between the measured (k) and calculated (CLOGP) lipophilicity data and that of the biological activity and the lipophilicity data obtained by HPLC or by calculation have been investigated. Possibility of cell death after treatment with the isochromanones has been determined by light microscopic analysis based on the cell morphology. Furthermore, more specific biological studies, like PARP fragmentation by western blotting and flow cytometry were applied to investigate the possibility of apoptosis.

Chromatographic Separation

The RP-HPLC method applied in this work proved to be applicable for fast analysis of the isochromanone molecular library.

Isocratic separation was achieved within 14 minutes, k values are shown in Table **1**. Our chromatographic method was able to perceive the small structural differences resulting in fine alteration of lipophilicity (in contrast of calculation method). Ability of the method to separate members of the

library is demonstrated on a representative chromatogram: baseline separation has been achieved for the samples **2*E***, **3*E***, **5*E***, **7*E***, **11*E*** and **12*E*** (Fig. (**2**)).

The chromatographic method worked well for the separation of the structural isomers, too. Impact of the spherical factor on the k value was shown by the structural isomers, where for example two compounds, a monomethoxy and a dimethoxy derivative (**5*E*** and **7*E***, respectively) eluted with a 0.3 difference in their retention coefficient (Table **1**). Not only the monomethoxy and dimethoxy compounds (**5*E***, **7*E***), but the isomeric dimethoxy compounds (**7*E***, **8*E***) have been separated well with a difference of 0.4 in their k values. These results confirm the importance of the spherical factor: not only the chemical nature of the substituent, but its position is also able to influence lipophilicity of the molecule as it has been found earlier for the aurones, thioaurones, 2-aryliden-tetralones and Mannich ketones, too [67-68, 80-81].

The synthesis by Knoevenagel condensation may result in both the *E*- and *Z*- isomers. The separation of isomers can be performed only by chromatographic method. That is also the reason why in correlation study the k value is more suitable than the CLOGP. The method reported here was able to

Table 1. Calculated CLOGP and Measured k Values and Antiproliferative Activity of 17 Compounds

Compound	Substituent (R)	k*	CLOGP	IC_{50}** / μM
1*E*	Ph	1.7	3.8	6.3
1*Z*	Ph	1.6	3.8	20.6
2*E*	3'-pyridyl	0.4	2.3	20.6
3*E*	1-methyl-2'-pyrrolyl	1.2	2.5	86.7
3*Z*	1-methyl-2'-pyrrolyl	1.1	2.5	100.0
4*Z*	2',6'-Cl_2-C_6H_3	3.3	5.3	100.0
5*E*	2'-CH_3O-C_6H_4	1.9	3.8	8.1
6*Z*	2'-Cl-C_6H_4	2.4	4.5	34.8
6*E*	2'-Cl-C_6H_4	2.4	4.5	7.1
7*E*	2',3'-$(CH_3O)_2$-C_6H_3	1.6	3.5	15.2
8*E*	2',4'-$(CH_3O)_2$-C_6H_3	2.0	3.8	0.3
9*Z*	2'-O_2N-C_6H_4	1.4	3.6	55.0
9*E*	2'-O_2N-C_6H_4	1.2	3.6	14.1
10*Z*	2'-furyl	1.3	3.0	51.0
10*E*	2'-furyl	1.0	3.0	55.0
11*E*	2'-Br-C_6H_4	2.6	4.7	10.3
12*E*	3'-OH-C_6H_4	0.6	3.2	7.6

*R.S.D. of the k values was less, than 2%, the compounds investigated here were injected individually, number of intra-day repetitions was 3 (n=3) for each of the compounds.
** R.S.D. of the IC_{50} values were less, than 10 %, three replicates have been made for each of the compounds.

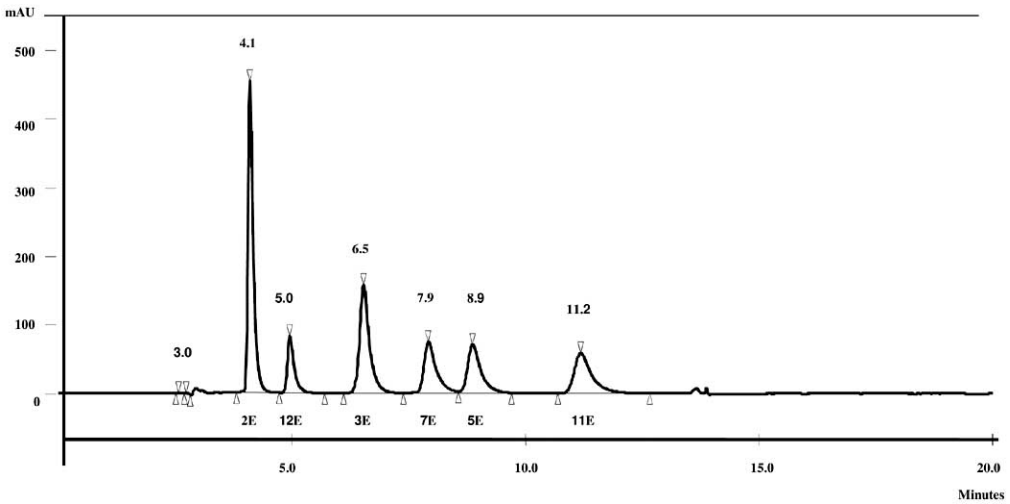


Fig. (2). Representative chromatogram of the separation of the compounds **2*E***, **3*E***, **5*E***, **7*E***, **11*E***, **12*E***.

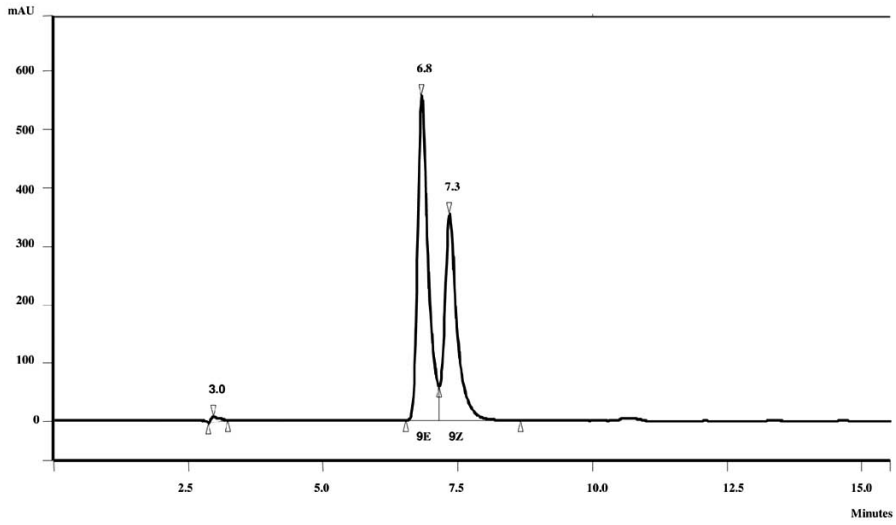


Fig. (3). Separation of the **9*E*/*Z*** isomer pair.

distinguish between the *E*- and *Z*- isomers, it was able to separate the isomers **1*E*** – **1*Z***, **9*E*** – **9*Z*** and **10*E*** – **10*Z*** (Table **1**). As a representative, Fig. (**3**) shows the elution profile of the **9*E*** and **9*Z*** isomers.

CLOGP Values

Investigating the relationship between the experimental and predicted lipophilicity values a good linear correlation has been found:

$$CLOGP = A \cdot \log k + B$$

Correlation was determined for the whole set of compounds investigated (Fig. (**4**) and Table **2**).

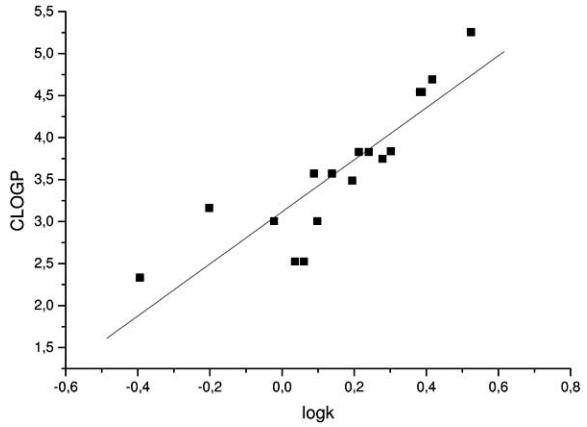


Fig. (4). Calculated (CLOGP) vs. measured (logk) lipophilicity values of the whole molecular library (Table **1**). Abscissa: logk; ordinate: CLOGP.

However calculation method is mainly insensitive for little structural differences and thus the chromatographic method is more suitable for the characterisation of the lipophilicity, CLOGP values proved to be also informative and useful. As we found linear correlation between the measured and calculated lipophilicity, biological activity indirectly can also be predicted from the CLOGP parameter. Moreover according to the good correlation we are able to describe the optimal lipophilicity range not only with optimal k values but optimal CLOGP data as well.

Results of the Antiproliferative Assay

The A431 human epidermoid carcinoma cell line is well known as epidermal growth factor overexpressing system and indicates potential EGFR[a]-related apoptotic effects. Small molecules as inhibitors of protein kinases constitute one of the most major class of the target-selective agents and this system is a straightforward screening setup to test compounds with EGFR inhibitory potential. As several protein kinases comprise the above mentioned hydrophobic pocket their inhibition highly depends on the compound lipophilicity [35-36]. Thus determination of optimal lipophilicity range can be used with higher impact for the prediction of biological activity. The investigated compounds were evaluated in this screening system to determine their cytotoxicity features, to examine the impact of the various substituents on the bioactivity regarding to their ability to alter lipophilicity and to determine an optimal lipophilicity range where the inhibitory activity is on maximum level.

IC_{50} -k Relationship

As lipophilicity can be a great predictor for biological activity, it is a generally used method to compare the inhibitory efficiency to the lipophilicity (logP or k value). For instance Fish *et al.* studied the relationship between the selective inhibition of noradrenalin reuptake (NRI) and the CLOGP. While they could not find an optimal lipophilicity range they discovered that the inhibitory activity of NRI increases with the lipophilicity [82].

Four of the 17 isochromanones investigated showed good antiproliferative activity with low IC_{50} (antiproliferative effect) values in the μM range (Table **1** compounds: **5*E***, **6*E***, **8*E***, **12*E***). The *E*-isomers having phenyl-ring in the R – substituent showed the greatest biological activity. The highest activity was shown by the compound **8*E*** (R:2',4'-$(OCH_3)_2$-C_6H_3) with as low IC_{50} values as 0.3 (exactly 0.266 μM). Generally, the *E*-isomers showed higher antiproliferative activity than their *Z*-counterparts.

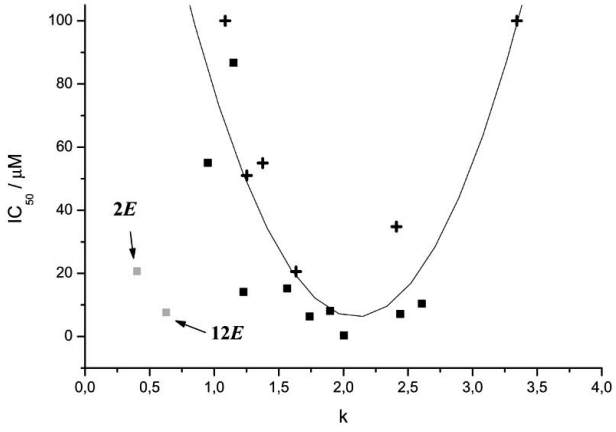
Table 2. Correlation Parameters of the CLOGP– logk Relationship for the Whole Set of Molecular Library and Separately for the *E*- and *Z*-Isomers

Parameters of the linear*	CLOGP vs. logk	CLOGP vs. logk by the *E*-isomers	CLOGP vs. logk by the *Z*-isomers
A	3.11560	3.20941	-0.46580
B	3.09785	2.54915	0.18444
R	0.8724	0.86425	0.98340
SD	0.41319	0.39200	0.03797
P	<0.0001	$6.0005*10^{-4}$	$4.1128*10^{-4}$
N	17	11	6

**A*, *B*, *R*, *SD*, *P* and *N* are the parameters of the linear.
A: intercept value and its standard error, *B:* slope value and its standard error, *R:* correlation coefficient, *SD:* standard deviation of the fit, *P:* probability (that R is zero), N: number of data points.

In our laboratory Hallgas *et al*. has already succeeded to determine an optimal lipophilicity range, plotted IC_{50} versus k, on the structurally similar tetralone library. Using parabolic regression a minimum range of IC_{50} data was determined by the set of optimal lipophilicity values [67].

Here we also found good parabolic correlation (with a regression coefficient (R) of 0.8343) between the IC_{50} and k value for 15 out of 17 molecules excluding two outliers (**2*E***, **12*E***) found by the "leave one out" method of Allen *et al.* [83] (Fig. (**5**)), (Table **3**). This lipophilicity range has the optimal k value of 2.1.



*where ■ symbols belong to the E-isomers, + to the Z-isomers and, → symbols show the two outliers.

Fig. (5). Antiproliferative activity (IC_{50}) vs. measured lipophilicity (k) of the whole molecular library. Abscissa: k, ordinate: IC_{50} (µmol/l).

Parabolic correlation and therefore optimal k value could also be determined by the separately studied *E*- (Fig. (**6**)), (Table **3**) and *Z*-isomers.

With this new criterion, according to our successful studies both on the tetralones and on the isochromanones, we are able to estimate biological activity, such as apoptotic or antiproliferative effect, for any other new library member. After the determination of this optimal lipophilicity the most promising member was studied in further biological assays to get a more complete characterization of its antiproliferative effect.

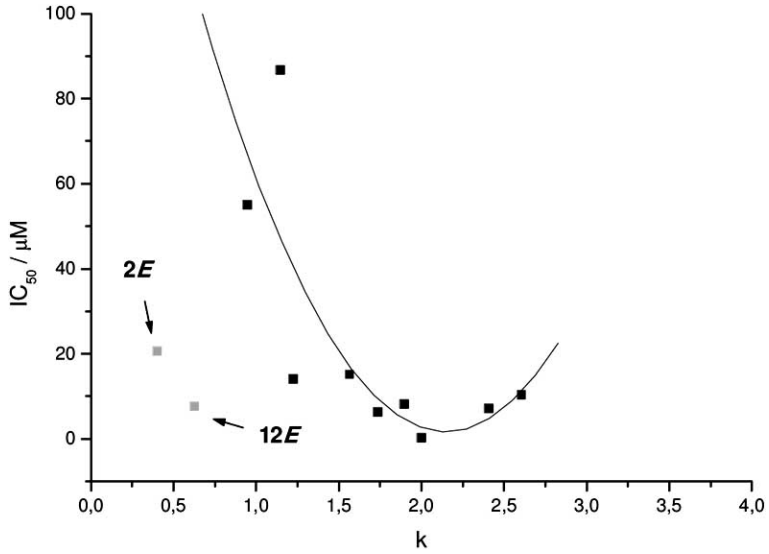
Light Microscopic Morphological Studies

It is well known that A431 epidermal carcinoma cell line is a fast-growing and a well-characterized cell target for signal transduction studies. Besides cell proliferation assays it is also applicable for other biological observations, as well. Therefore, morphological evaluation was continued in A431 cells. In the further biological investigations the focus was on the most effective compound **8*E*** with IC_{50} of 0.3 µM and with the optimal k value of 2.0. Considering the very low IC_{50} value of compound **8*E*** to show significant differences when compared to control, in further experiments at least 1 µM concentration of compound **8*E*** was applied. As shown in Fig. (**7**), even the simplest phase-contrast light microscopic study could clearly show that the minimal concentration (1 µM) of compound **8*E*** was perfectly enough to induce robust cell (shape) morphology changes –looks like apoptosis- of A431 cells in culture after 24 hours comparing to the normal-growing control cells.

Table 3. Correlation Parameters of the IC_{50} – k Relationship

Parameters of the linear	IC_{50}** vs. k without outliers	IC_{50} vs. k by the *E*-isomers without outliers
A	268.01564	210.37100
B_1	-249.72270	-194.23249
B_2	59.53753	45.19956
R^2	0.69606	0.63287
SD	20.90323	20.17173
P	$7.88376*10^{-4}$	0.04948
N	15	9

*A, B_1, B_2, R^2, SD, P and N are the parameters of the polynomial.
A, B_1, B_1: coefficient and the standard error for each order, R^2: square of the correlation coefficient, SD: standard deviation of the fit, P: probability (that R is zero), N: number of data points.
**R.S.D. of the IC_{50} values were less, than 10 %, three replicates has been made for each of the compounds.



*where → symbols show the two outliers.

Fig. (6). Antiproliferative activity (IC_{50}) vs. measured lipophilicity (k) of the *E*-isomers. Abscissa: k, ordinate: IC_{50} (µmol/l).

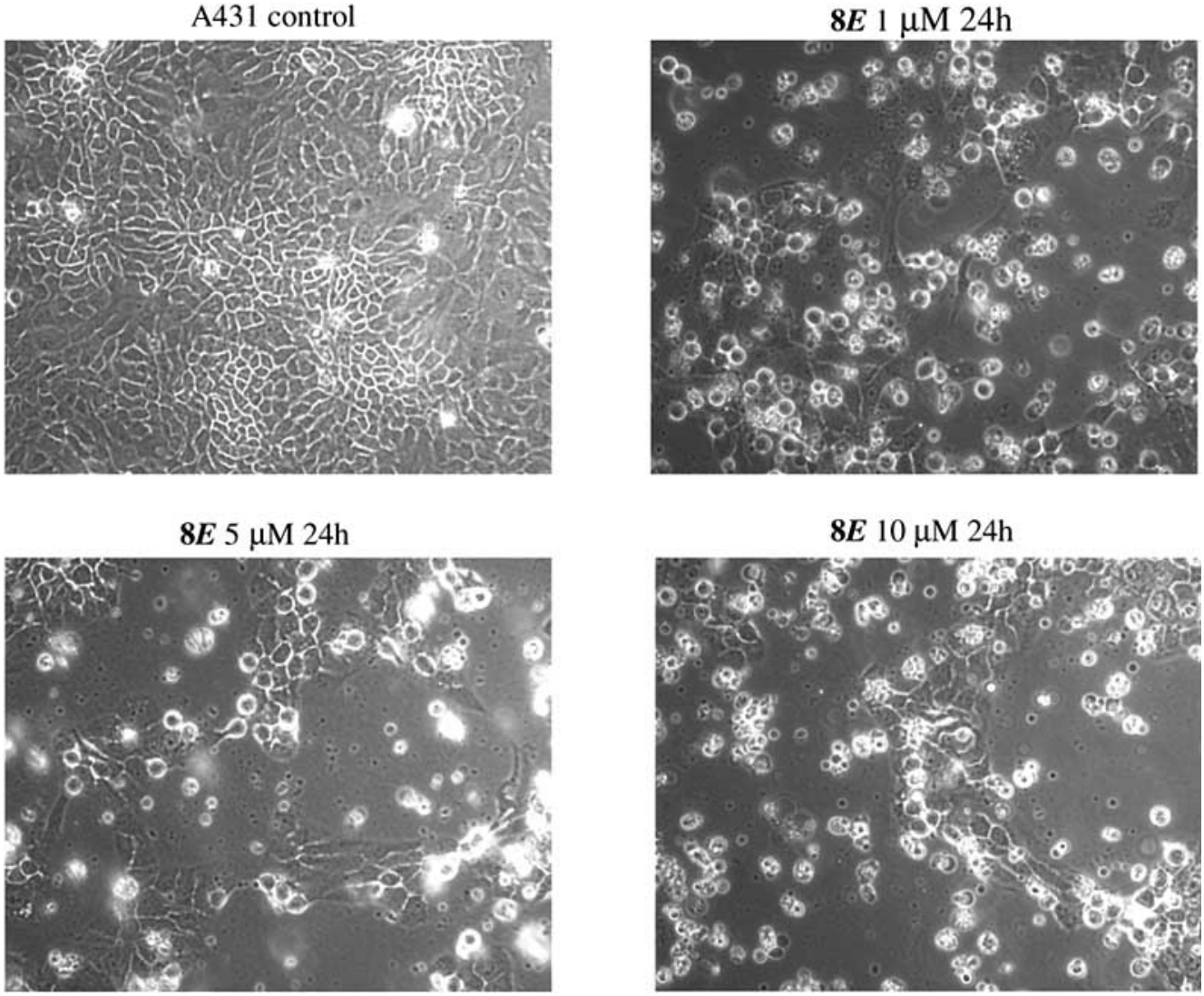


Fig. (7). Light microscopical study using A431 cells after compound **8*E*** treatment in different concentrations.

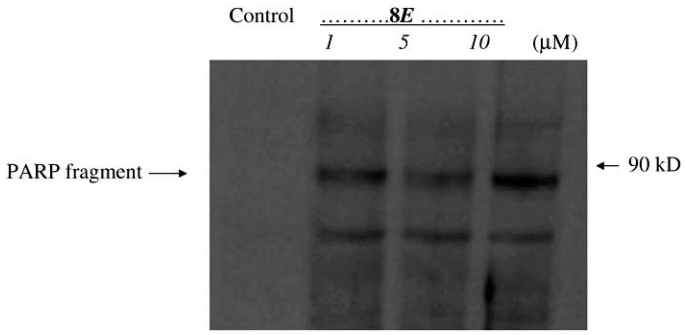


Fig. (8). Immunoblotting analysis of PARP fragmentation using the molecule **8*E***. The results shown are representative of three independent experiments.

The majority of compound **8*E*** treated cells were rounded up, shrunk and retracted from their neighbouring cells, and then eventually floated into the media which might be already an indicative of cell death, most probably apoptosis as well.

These data (pictures) - in agreement with the antiproliferative experiments - suggest that apoptosis induction might be a major the mechanism of **8*E***-caused death of epidermal carcinoma cells. Using other isochromanones, weaker or no cell death induction has been observed compared to control in A431 cells.

Immunoblotting and Flow Cytometric Studies Investigating the 8*E* Compound Induced Cell Death

To determine whether the **8*E***-induced loss of the proliferation capacity and cell viability of the epidermal carcinoma cancer cells was associated with the induction of apoptosis, A431 cells were treated with **8*E*** for further apoptosis assays. To examine whether cells with shrunken abnormal phenotype are apoptotic, immunoblot staining of PARP and FACS analysis (both known as appropriate methods to check certain criteria of apoptosis) has been performed on cells.

PARP is a protein that detects DNA strand breaks and functions in their repair. Once PARP is cleaved, this repair function does not exist longer. Although PARP is not essential for apoptosis, the cleavage of PARP may contribute to the commitment to apoptosis [84]. To investigate the effect of compound **8*E*** on the cleavage of PARP, SDS polyacrylamide gel electrophoresis was applied using 8 % gel and specific antibody staining against the cleaved, approximately 85 kD main fragment of PARP. Treating A431 cells with solvent (control), 1 μM, 5 μM and 10μM doses of compound **8*E*** for 24h, the appearance of the main PARP fragment, which is a hallmark of apoptosis, was clearly detected at the lowest concentration (1μM) we applied (Fig. (**8**)).

Besides, as a further proof of apoptosis, the ratio of apoptotic cells were assessed and quantified using FACS analysis, where the apoptotic fraction was measured applying flow cytometric analysis with the PI staining (Fig. (**9**)).

Another event associated with the process of apoptosis is DNA fragmentation, as a result of endonuclease activation. During the fragmentation, DNA molecules become smaller (fragmented) with the appearance of more free DNA ends in

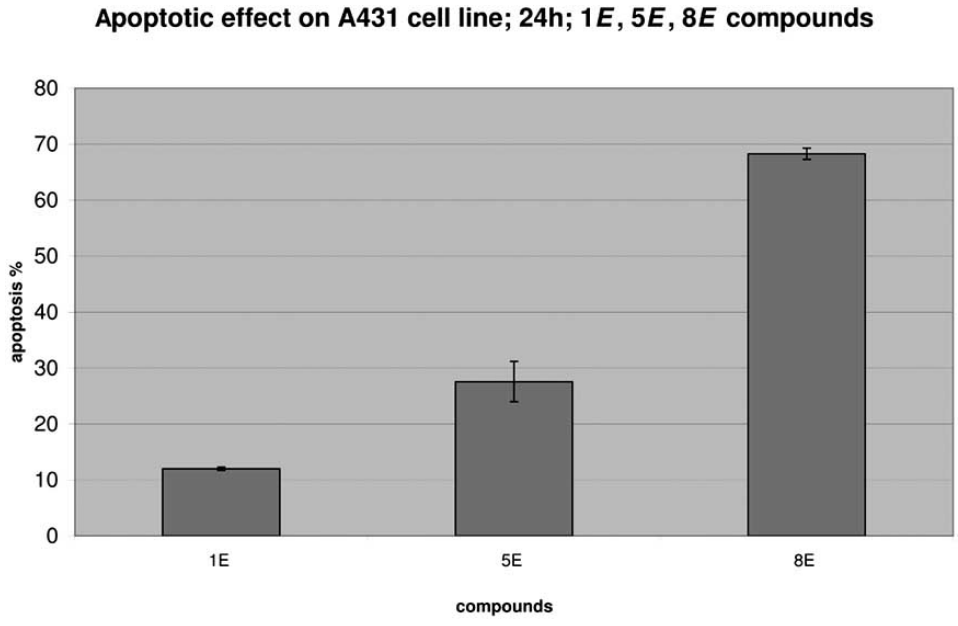


Fig. (9). The results of the flow cytometric analysis of the compounds **1*E***, **5*E*** and the **8*E*** (10 μM). The columns represent the percentage of the apoptotic fraction.

the cell. PI can be used to detect the later phase of apoptotic cell death (data not shown, ref.: [79]).

Since all our earlier measurements suggested that there was no need to apply higher dose than 1 μM concentration of compound **8*E*** for a robust effect, the same amount was used accompanying **8*E*** with two more isomers of different behaviour, **1*E*** and **5*E*** showing the big variety in biological effect of isochromanone family. Compound **1*E*** is an almost inactive isomer according to the microscopic studies – just like a negative control, and **5*E*** is a significantly active isomer but still far behind the most active **8*E*** isomer. Considering the expectation based on the IC_{50} and other data of **8*E***, it was not surprising to observe a very significant percentage of apoptotic fraction, namely approximately 68 % after 24 hour treatment using either 1 or 10 μM **8*E***. In this cellular system the spontaneous apoptosis was around 5 % (data not shown). As shown in Fig. (**9**), no significant apoptosis was observed after treatment with **1*E***, even the more effective isomer **5*E*** could show much less increase of DNA fragmentation comparing to the effect of highly effective **8*E***, in full agreement with our earlier results.

Both assay types with completely different approaches support our idea that **8*E*** is an outstanding apoptosis inducer even at low doses *via* PARP and DNA fragmentation. These results suggest **8*E*** may have potential anticancer effects against epidermal carcinoma cells by inducing strong apoptosis.

DISCUSSION

Since the elucidation of the critical role for the lipophilicity parameter in drug development, it has been established that prescreening for this value is essential early step in drug development. In the present paper we provided an overview of the importance of lipophilicity of drug candidates both for biological action related efficacy and for ADME(T) and described a novel approach for drug-likeness characterization of a molecular library using correlation study between lipophilicity and biological activity. Our research has led to the conclusion that a better knowledge of the relationship between lipophilicity and the compounds biological effect could provide an easy but powerful screening platform to select drug candidate molecules without expensive biological studies.

In line with other recent publications where other different types of molecular libraries have been investigated [67-68, 10-12, 27-29], our study provides additional evidence on the clear relationship between biological activity and lipophilicity characterizing an isochromanone molecular library for logP, k and cell growth on A431 human tumour cells.

An applicable RP-HPLC method for fast analysis and characterization of lipophilicity of the members 4-(arylmethylene)-3-isochromanones was also developed. Separation of 17 4-(arylmethylene)-3-isochromanones possessing similar chemical structure could be obtained.

The chromatographic method developed here proved to be able to distinguish between the small lipophilicity differences of the members of the library. While the prediction gave the same calculated lipophilicity value for the *E*/*Z* pairs, the chromatographic method developed here was able to distinguish the *E* and *Z* members of the isomer pairs having the same calculated lipophilicity but different antiproliferative activity. On the basis of that we plotted the inhibitory activity (IC_{50}) rather against the more informative HPLC determined lipophilicity (k) values to the computer calculated ones (CLOGP).

In the current paper we also summarized the most important literature data about the impact of hydrophobic pocket on biological activity [30-37] or about the optimalisation of lipophilicity value and range connected to biological effectiveness [38-43, 45]. We collected data how this optimal range could be defined by parabolic correlation where this range was determined between lipophilicity and some other, very useful drug developmental parameter such as drug transport through cell membrane [49] or brain uptake [55]. Following our previous observations by other molecular libraries [67-68] we succeeded to determine parabolic correlation between lipophilicity (k) and cytotoxic activity (IC_{50}).

The molecular family investigated here interferes with cell proliferation mostly by apoptosis. Magnitude and type of the effects depended on the nature of the substituents of the core structure. Studying the antiproliferative activity (IC_{50}) and experimental lipophilicity (k) the defined parabolic correlation enabled us to determine an optimal lipophilicity value (k = 2.1) and to predict a range where the biological activity is the most effective. Also the biological activity can be compared now not only to the k value but to the CLOGP value as well, as good linear correlation was found between the experimentally determined (logk) and the computer calculated (CLOGP) lipophilicity parameters. With the determination of this criterion pre-selection of the biologically effective molecules can be made within molecular libraries containing chemically similar core structure as the isochromanones.

Taking into account the determined optimal lipophilicity range, the most promising members of the molecular library has been selected. As generally the activity of the *E* isomers was greater than that of the *Z*-ones, these molecules also belonged to the E-isomers. The E-isomer of the 2'-methoxy-phenil (**5*E***), 2'-chloro-phenil (**6*E***) and 3'-hydroxo-phenil (**12*E***) derivatives showed good, while the molecule included 2',4'-dimethoxy-phenil substituent (**8*E***) showed outstanding antiproliferative activity. Compound **8*E*** proved to be prominently efficient in the proliferation and apoptosis assays, showed a very robust and outstandingly efficient antiproliferative effect in A431 cell culture model system with lower than 1 μM IC_{50} value. The mechanism of action of **8*E*** proved to be apoptosis induction, supported by the morphology and other more specific biological studies, adding extra value to its antiproliferative effect since besides the blocking of the tumour cell proliferation, it can eliminate cancer cells with false cell proliferation signalling, too. Based on its unique structure isomer **8*E*** has very promising anti-tumour characteristics for developing pre-clinical lead molecules.

In summary, we found that analysing the interrelationship between the optimal lipophilicity range and biological effect would be crucial for further drug development in a cost effective but still efficient way. Determining and applying this optimal lipophilicity range, using isochromanone

molecular library, we could successfully develop a novel potential isochromanone drug candidate (**8*E***) with high anti-tumour effect as a proof of concept considering our previous results in this experimental system.

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ABBREVIATIONS:

ADMET	=	Absorption, Distribution, Metabolism, Excretion, and Toxicity
QSAR	=	Quantitative structure-activity relationship
PARP	=	Poly(ADP-ribose) polymerase
$P_{o/w}$	=	n-octanol/water (biphasic) partition coefficient
RP-HPLC	=	Reversed phase-high performance liquid chromatography
MEKC	=	Micellar electrokinetic chromatography
MLP	=	Molecular lipophilicity potential
MIC values	=	Minimum inhibitory concentration
CLOGP	=	Calculated logP
k	=	Retention coefficient
ACN	=	acetonitrile
PI	=	Propidium iodide
DMEM	=	Dulbecco's Mod Eagle Medium
FCS	=	foetal calf serum
MB	=	Methylene blue
EGFR	=	Epidermal growth factor receptor
t_R	=	Retention time
FACS	=	Fluorescence-activated cell-sorting

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