Determination of optimal lipophilicity and phospholipophilicity range by different potential anti-tumour molecules and NOX inhibitors

PhD thesis

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1. Introduction

Drug research is a very diverse procedure which has several different steps. A potential drug candidate should be completely characterised in order to produce a real drug molecule.

The first step of the drug development is the pharmacokinetic description. This characterisation procedure was not widely accepted till the last decade of the past century. Its importance has only been admitted by the drug research companies by the end of 1990. In a clear relation to it, in 1993 the 40% of the drug molecules failed because of their ineligible ADME(T) properties, due to the changing attitude, by the end of 1990, only 11% of them proved to be inacceptable pharmacokinetically.

Among many advantages, the ADME(T) characterisation has great importance because it can reduce the cost needed during the selection of active drug molecules, it is reasonably fast and can be used to predict and estimate the biological effectiveness of a molecule. Compounds having improper pharmacokinetic or pharmaco-chemical characteristics cannot be considered as drug candidate molecules. On the basis of that applying correlation analysis between the physico-chemical and biological properties, the active molecules can be separated from the inactive ones and the most effective members can be characterised further using pharmacodinamic studies.

These quick, cost-effective, highly essential methods which are performed at the early stage of the drug research generally called „early ADME(T) characterisation".

1.1 Physico-chemical parameters

1.1.1 Lipophilicity

The lipophilicity of a molecule describes the absorption behaviour of a molecule and characterises the hydrophobic interaction between the compound and the biological membrane.

1.1.2 Phospholipophilicity

Phospholipophilicity also describes the absorption, especially the intestinal drug absorption ability of a molecule. This parameter can be measured by immobilized artificial membrane column and may involve the combination of hydrophobic, H-bonding and ion-pair interactions between the stationary phase and the molecule.

1.1.3 Permeability

Permeability is also frequently used ADME(T) parameter. This can be applied in order to describe the penetration ability of a drug molecule through the cell membrane and also the gastrointestinal oral-absorption of the compound.

1.2 Biological investigation

At the pharmacokinetic state of the drug research mainly cell based, in vitro biological assays are carried out. The aim is to determine the biological activity of the molecules on animal or human cell line.

MTT and MB assays are widely applied techniques for the pre-screening of mitochondrial activity of potential anti-tumour molecules. Using the results gained from these studies correlation analysis can be performed where the physico-chemically and biologically most ideal molecules are selected. After this pre-screening procedure, the most active compounds are tested for their apoptotic effect. Apoptotic properties could be measured by different methods, for instance by light microscopic morphological studies, by immunoblotting where the fragmentation of PARP enzyme is investigated or by flow-cytometric assay.

Our studies concentrated mainly on flavonoid derivatives. These molecules are well known and characterised compounds. Besides their antiproliferative activity, flavonoids are also good against ROS (reactive oxygene species) molecules as they act as NADPH oxydase inhibitors. On the basis of that correlation analysis was also performed between the NOX-4 inhibitory effect and the physico-chemical properties of different flavonoids.

2. Aims

The main goal is the physico-chemical characterization of four different molecule libraries including flavonoid derivatives and the comparison of these data with the determined biological parameters.

Out of the four libraries three are analysed as potential antiproliferative compounds and the fourth as potential NOX inhibitors. All of these biological parameters will be compared to the physico-chemical data and will be examined according to the structural properties.

It is also our aim to develop HPLC based methods which can be successfully applied for the simultaneous analysis of geometrical isomers and for the selective and fast detection of the compounds. Using these methods the lipo- and phospholipophilicity values will also be determined and compared to the calculated lipophilicity.
The two applied columns, with the RP and the IAM stationary phase, will be characterised with the four different molecule libraries.
Permeability assay will be used to characterise bioavailability and drug absorption.
On the basis of the collected data, "early ADME(T) characterisation" can be developed, which should be able to provide a fast and cost-effective method for the determination of desirable structural properties and optimal lipo- and phospholipophilicity range. As an outcome we should be able to select the most effective drug candidates as the real drug-like molecules proposed for clinical studies.

3. Materials and Methods

3.1 Studied molecular libraries

The investigated compounds were all flavonoid derivatives:

- isochromanones (IC libr.)
- reduced Mannich-ketones (red MK libr.)
- aurones, tioaurones, sulphones (Aurones)
- chromenones

3.1.1 Physico-chemical methods

3.1.1.1 Lipophilicity and phospholipophilicty measurements

Both assays are HPLC-based.
The differences between the methods can be seen in Table 1. and 2..
Samples: 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.

Applied buffer: 0.083 M triethyl-ammonium phosphate, made by weighing the calculated quantities of triethyl amine and phosphoric acid.

Table 1.: Applied chromatographic parameters

Parameters	Lipophilicity measurement (RP column)		Phospholipophilicity measurement (IAM column)	
	IC libr., red MK libr., aurones	**NOX inh.**	**IC libr., red MK libr., NOX inh.**	**Aurones**
HPLC	Varian 9012 solvent delivery system, 9065 Polikróm Diode Array detector, Rheodyne injector	JASCO 2089 solvent delivery system, 2077 multiwavelength detektor, Rheodyne injector	JASCO 2089 solvent delivery system, 2077 multiwavelength detektor, Rheodyne injector	Varian 9012 solvent delivery system, 9065 Polikróm Diode Array detector, Rheodyne injector
Column	Hypersil 5 MOS 5µm, 250x4.6 mm	Gemini C18, 5µm, 150X4.6mm	IAM.PC.DD2, 12µm, 100X4.6mm	IAM.PC.DD, 12 µm, 150X4.6mm
Eluents	*A"* eluent: 0.083M TEAP, pH 2.25		*A"* eluent: 0.083M TEAP, pH 7.4	
	„B" eluent: 95% AcN + 5% *„A"* eluent		*„B"* eluent: 95% AcN + 5% *„A"* eluent	
Flow rate	1 ml/min		1 ml/min	
Injected volume	20 µl		20 µl	
$\lambda_{det.}$	254 nm		254 nm	
T_{column}	20 ºC		20 ºC	

Table 2.: Eluent composition of the applied isocratic methods

Studied library	Eluent composition	
	RP column	**IAM column**
IC libr.	40% AcN / 60% eluent *„A"*	24% AcN / 76% eluent *„A"*
Red MK libr.	24% AcN / 76% eluent *„A"*	24% AcN / 76% eluent *„A"*
Aurones	52% AcN / 48% eluent *„A"*	33% AcN / 67% eluent *„A"*
NOX inhibitors	40% AcN / 60% eluent *„A"*	33% AcN / 67% eluent *„A"*

3.1.1.2 CLOGP-determination

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, *2002*) based on the number and the position of the fragment.

3.1.1.3 Permeability measurement

- Samples: 10 mM stock solution prepared in DMSO was diluted to 200 µM using PBS (therefore the individual compound solutions contained 2% DMSO).

- PAMPA plate: BD Gentest pre-coated PAMPA plate system (Cat.No.: 353015-G)
- Detector: BioTek, Synergy2 Multi-Mode Microplate Reader (UV/VIS detection)

- *Protocol*:

- PAMPA plates stored at -20ºC were warmed up to room temperature.
- 300 µl, 200 µM compound solution was added into the donor plate.
- 200 µl PBS was pipetted into the acceptor plate.
- Incubation time: 5 hours.
- Detection of the solutions on the acceptor and donor plates were performed by UV/VIS detector.

3.1.2 Biological methods

3.1.2.1 Antiproliferative assay (MTT and MB test)

- *Protocol*
- 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% CO2.
- Cells were seeded into 96-well plates and incubated for 16 hours.
- Incubation time after treating the cells with the compound solutions was 48 hours.
- Cells were fixed by 10% buffered paraformaldehyde in 0.9% NaCl.
- Wells were then stained by 1% MB or MTT.
- Following a thorough washing by PBS, both apoptotic and necrotic cells previously detached from the surface of wells are 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.

3.1.2.2 Light microscopic assay

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.

3.1.2.3 Immunoblotting studies

- *Sample preparation:*

- A431 cells were treated for 24 hours with the compounds 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 1200 g/min 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 and boiled for 5 min followed by Western blot analysis.

- *Western blot:*

- The lysate was run on 8% SDS-PAGE, and blotted onto nitrocellulose sheets.
- Blots were processed using rabbit anticleaved PARP Ab (Cell Signaling Technology Danvers, MA, USA; Cat. no: 9546) followed by a peroxidase-labeled goat 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).

3.1.2.4 Flow cytometric assay

- *Protocol:*
  - 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.
  - A431 cells were treated with the compounds applying the doses of 1 and 10 µM.
  - The cells were washed and fixed with 70% ethanol for one day.
  - The fixed cells were spin down at 1000 g/min, at 4 ºC-on, for 10 min.
  - After addition of apoptotic buffer (200 mM Na_2HPO_4 (pH = 7.8)) and 100 µg/ml RNase, staining was performed with 10 µg/ml propidium-iodide.

- The stained cells were subjected to flow cytometry (FACSa Calibur, BD Biosciences) to detect and quantify apoptosis (subG1 fraction).
- The analysis was performed with CellQuest software.

3.1.2.5 H_2O_2/Tyr/LPO celullar assay

- *Protocol:*
  - Transfected free-style 293F HEKcells were incubated at 37 °C for 24 h in DMEM supplemented with 5% FCS, 1% penicillin-streptomycin.
  - After the treatment with the compounds applying in 10 µM, the incubation time was half an hour at 37 ºC.
  - The inhibition reaction was stopped by the addition of 50 µl extracellular medium containing 2 mM L-tyrosine and 160 mU/ml lactoperoxidase.
  - The analysis was performed by fluorescent detection (λ_{ex} = 330 ± 40 nm, λ_{em} = 405 ± 10 nm).

4. Results and discussion

4.1 Isochromanone molecular library

4.1.1 Chromatographic characterisation

Our chromatographic method was able to perceive the small structural differences resulting in fine alteration of lipophilicity (in contrast of calculation method).
Baseline separation was achieved in the case of different *E* and *Z*-isomers and simultaneous analysis was carried out for 6-6 *E*-isomers both on RP and on IAM column.
Linear correlation was found between the *CLOGP* and *logk* values for the whole library (Fig.1) and individually for the *E*- and Z-isomers. Therefore biological activity indirectly can also be predicted from the *CLOGP* parameter.

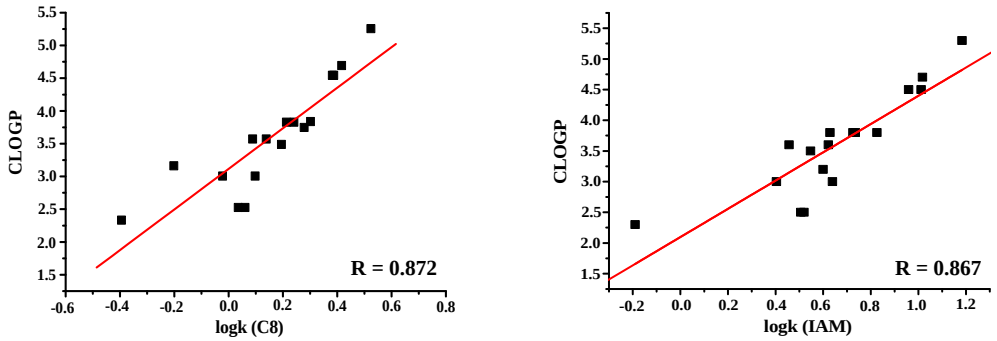

Fig. 1: Calculated (*CLOGP*) vs. measured (*logk*) lipo/phospholipophilicity values of the whole molecular library

4.1.2 Permeability study

Every compound showed high ability to penetrate the artificial lipid membrane according to their $logP_e$ > -5.82 values.

4.1.3 Study of antiproliferative activity

Three of the 17 isochromanones investigated showed good antiproliferative activity on A431 cells with low IC_{50} (antiproliferative effect) values in the µM range and one proved to be outstanding with as low IC_{50} value as 0.3 µM.

Generally, the *E*-isomers showed higher antiproliferative activity than their *Z*-counterparts.

Among these isomers**,** compounds having phenyl-ring in the R_1-substituent showed the greatest biological activity.

Using parabolic regression a minimum range of IC_{50} data was determined by the set of optimal lipophilicity (k_{C8}) and phospholipophilicity (k_{IAM}) values (Fig. 2).

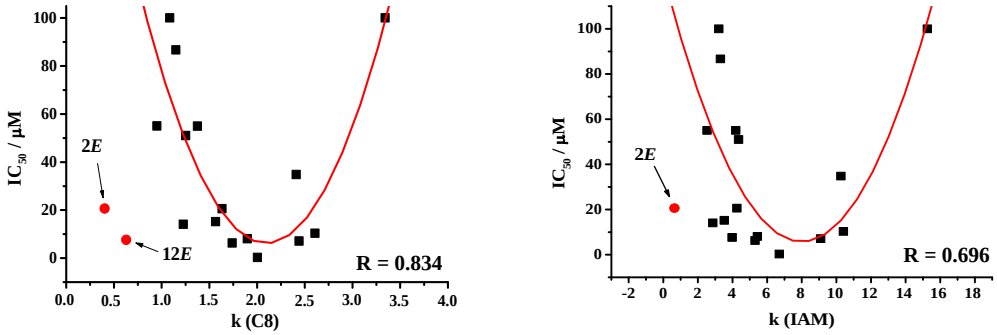

Fig. 2: Antiproliferative activity (IC_{50}) vs. measured lipo/phospholipophilicity (*k*) of the whole molecular.

According to the parabolic relationship defined above, molecules with medium lipophilicity (k = 2.1) and phospholipophilicity (k = 8) values could be the best drug candidates.

4.1.4 Light microscopic study

In further biological investigations the focus was on the most effective compound **8*E*** (4E-((2',4'-dimethoxy-phenyl-4-methylene)-3-isochromanon) with IC_{50} of 0.3 µM and with the optimal k value of 2.0 (Fig. 3).

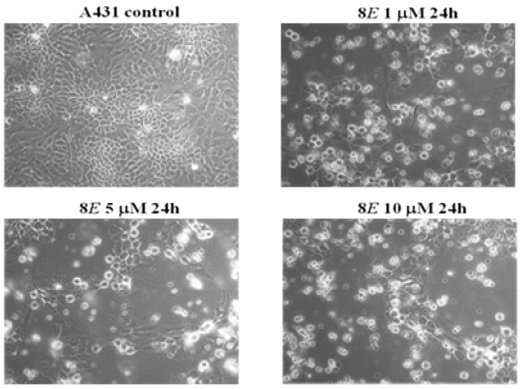

Fig. 3: Light microscopical study using A431 cells after compound **8*E*** treatment in 1, 5 and 10µM concentrations after 24 hours.

As shown in Fig. 3, 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.

4.1.4.1 Immunoblotting study by PARP fragmentation

The study was performed both using cleaved and normal PARP antibody. In both cases the appearance of the main PARP fragment, which is a hallmark of apoptosis, was clearly detected at the lowest concentration (1 µM) we applied (Fig. 4).

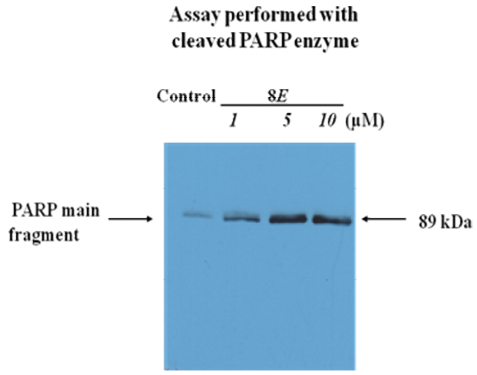

Fig. 4: Immunoblotting analysis of PARP fragmentation using the molecule **8*E*** in 1, 5 and 10 μM concentrations. Spontaneous apoptosis is approximately 5%.

4.1.4.2 Flow cytometric study

The flow cytometric assay proved that compound **1*E*** (phenyl derivative) is an almost inactive isomer – just like a negative control. and **5*E*** (2'-methoxyphenyl derivative) is a significantly active isomer but still far behind the most active **8*E*** isomer. This outstanding compound gave very significant percentage of the apoptotic fraction, namely 68.28 ± 1.67% after 24 hour treatment using 1 μM **8*E*** (Fig. 5).

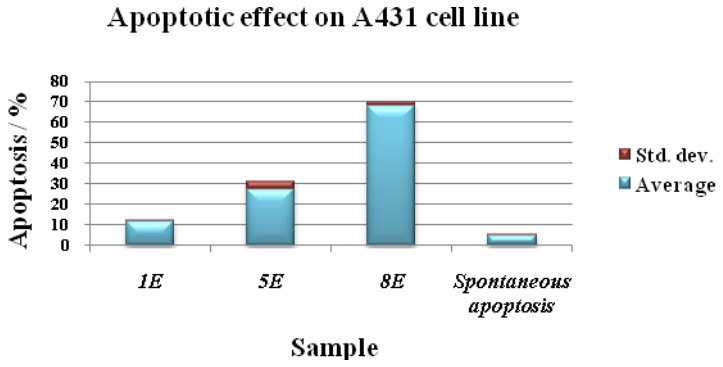

Fig. 5: The results of the flow cytometric analysis

In summary, all of the assays carried out on the molecules support our idea that 4E-(2',4' dimethoxyphenyl-4-methylene)-3-izokromanon can be an outstanding apoptosis inducer. These results suggest **8*E*** may have potential anticancer effect against epidermal carcinoma cells by inducing strong apoptosis.

4.2 Reduced Mannich ketones

4.2.1 Chromatographic parameters

Compounds having equal calculated lipophilicity (*CLOGP*) could be separated both on IAM and on RP column. Good separation was achieved both for the *E*/*Z* isomers and also for molecules with same basic structure, same substituents, but different ring size.
Linear correlation was found between the *CLOGP* and *logk* values (Fig. 6).

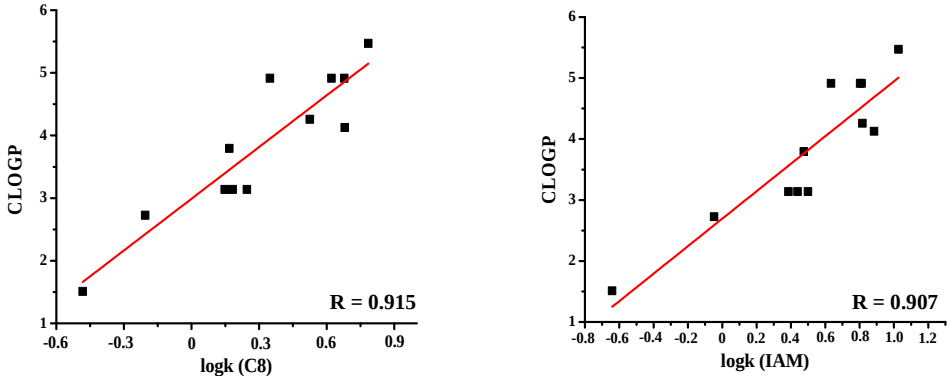

Fig. 6: Calculated (*CLOGP*) vs. measured (*logk*) lipo/phospholipophilicity values of the red MK library

4.2.2 Permeability study

According to the applied PAMPA method, all of the molecules are able to penetrate the lipid layer of the artificial membrane by passive transport.

4.2.3 Study of antiproliferative activity

The antiproliferative activity of Red MK compound library was also measured on A431 cell line and was compared to the activity (IC_{50}) of the MK compounds, which are the starting materials of the reduced molecules.
The reduced and oxidized molecules showed significant difference in their activity: the reduction decreased the antiproliferative activity.
Red MK molecules in 10 µM were also tested on six other cell lines. None of the investigated compounds proved to be effective in these assays. It seems, on the basis of these results, that the keto-group might play an important role in biological activity through its reaction with the thiol group of the enzyme.

4.3 Aurones

Characterisation of auron, thioauron and sulphon molecular libraries were also carried out. Generally, these three groups of compounds are called “aurones”.
The characterization of the molecules by their elution pattern on RP column has been done previously by B. Hallgas et al.

4.3.1 Chromatographic characterisation

Sulphones and sulphoxide molecule showed the lowest phospholipophilicity on the IAM column which indicates that the higher the number of the oxygen atoms on the sulphur atom in the heterocycle, the lower is the phospholipophilicity. As it was the case at the previously described molecular libraries, molecules with small structural differences could also be analysed with the method applied.
Linear correlation was found between the measured and calculated phospholipophilicity both by the complete auron library and by the individual sub-libraries (Fig. 7).

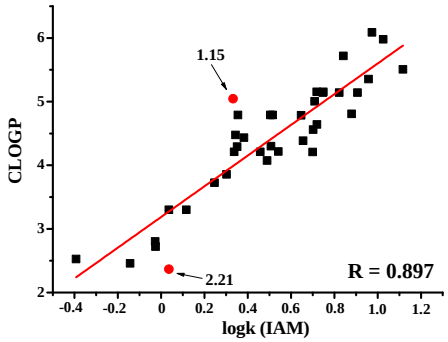

Fig. 7: Calculated (*CLOGP*) vs. measured (*logk*) phospholipophilicity values of the whole auron library

4.3.2 Permeability study

All of the compounds showed high permeability. Therefore the passive transportation of the molecules through the cell membrane is not incapacitated.

4.3.3 Study of antiproliferative activity

The study proved that the very low lipo- and phospholipophilicity of the sulphones are in correlation with their very low antiproliferative activity. Thioaurones showed higher

activity than aurones, therefore the conclusion is that the O/S exchange increase, the SO/SO2 exchange decrease the “drug-likeness” behaviour.

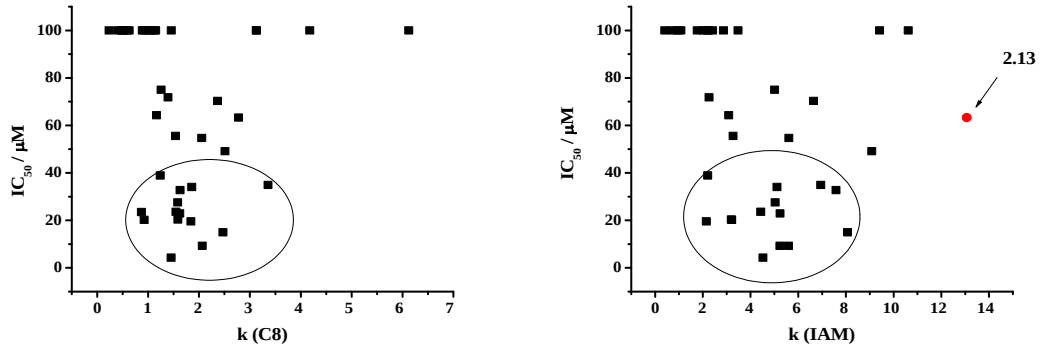

Fig. 8: Antiproliferative activity (IC_{50}) vs. measured lipo/phospholipophilicity (k) of the whole library.

As Figure 8 shows, the optimal lipophilicity range (k_{C8}) was between 1 and 3, the optimal phospholipophilicity (k_{IAM}) was between 2 and 8 for the whole auron library.

4.4 Chromenones

As an extension of the study of early ADME(T) characterisation and optimalisation method, NOX-4 inhibitors have also been tested and analysed to find the optimal values which are needed to produce promising drug candidates.

4.4.1 Chromatographic characterisation

The characterization of NOX-4 inhibitors by IAM chromatography has arisen some problems. The most potent NOX-4 inhibitors could not be analysed by IAM chromatography. This probably related to the simultaneous occurrence of the ionic groups on the column and the four or even more hydroxyl groups on the most active chromenones. The position of the substituents had also great importance. Molecules having substituents at 3,5 or 3,5,7 positions could not be or not so easily be detected as molecules with substituents attached at 5,7 position.

Linear correlation was found between the *CLOGP* and *logk* values in the case of chromenones (Fig. 9), too when measured on C18 column.

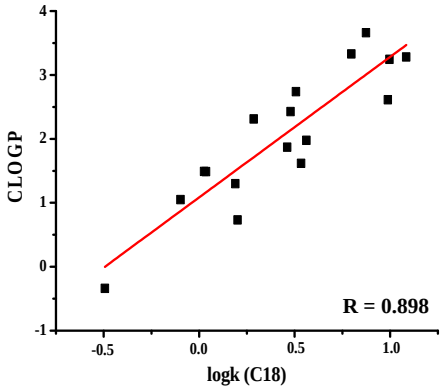

Fig. 9: Calculated (*CLOGP*) vs. measured (*logk*) lipophilicity values of the chromenone library

4.4.2 Permeability study

Except 5 compounds all of the molecules showed low or extremely low permeability. These data were in good correlation with the data gained by HPLC.

4.4.3 Study of the NOX inhibitory effect

Molecules with low or extremely low lipophilicity or more than three hydroxyl-groups showed the highest activity (Fig. 10). Substitution at the position 3,5 or 3,5,7 proved to be the most advantageous. Therefore the optimal lipophilicity range (k = 0.3-4) was much lower than in case of the studied potential antiproliferative moleculelibraries.

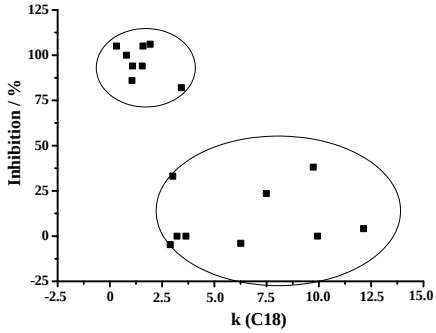

Fig. 10: *NOX-4 inhibition* vs. k_{C18} by the chromenone library

5. Conclusion

Following the general theories described in more details in the introduction, we were able to highlight the importance of the role of early stage drug research ("early ADME(T)") based on our chromatographic characterisation and basic but efficient and cost effective biological studies. Optimalisation of lipo- and phospholipophilicity values can be really successfully applied by the selection of drug candidates which are promising to be subjected to clinical studies. According to our antiproliferative studies we found that molecules with medium lipo- and phospholipophilicity values should have the most ideal hydrophobic properties. Parabolic correlation of the isochromanone (IC) molecules affirms this theory and the IC_{50}-*k* relationship analysis of the aurones also proves that compounds with medium lipo/phospholipophilicity values are the most potent anti-tumour candidates. Reduced Mannich ketones (red MK) have also been studied but from a different aspect. The main interest was on the study of the biological role of the keto-group (which is present in Mannich ketones but absent in the reduced form).As the red MK compounds proved to be inactive on 7 different cell lines, this structure-activity relationship study pointed out the significance of the functional group in a molecule.

Structure-activity relationship was also investigated by the IC library. *E*-isomers proved to be more appropriate both biologically and physico-chemically as the *Z*-ones. By the aurone compounds the effect of the heteroatom in the ring was also investigated and found to be a significant modifying factor of the pharmacokinetic behaviour. While the oxygen/sulphur exchange in the ring increased, the replacement of SO group to SO_2 decreased the biological activity.

Linear correlation was found by all of the libraries investigated between the calculated and the measured lipo- and phospholipophilicity values which means, that the biological activity can also be predicted indirectly from the *CLOGP* parameter. Moreover according to the good correlations we are able to describe the optimal lipophilicity range not only with optimal *k* values but optimal *CLOGP* data as well. This can also be useful at the molecules (mainly geometrical isomers) with same *CLOGP* values.

The compound libraries have been characterised on two different columns. As our main interest was on the imitation of the membrane bilayer and the investigation of the penetration properties of the drug candidates, besides the generally applied RP column,

IAM (immobilized artificial membrane) column was also used. Therefore not only the optimal lipophilicity range could be determined but also the ideal phospholipophilicity values. The applied methods proved to be selective and the resolution was also sufficient by both types of the columns. However the kinetic efficiency (half-width at half maximum) of the RP column was higher than of the IAM column, which may be due to the complex interactions existing between the molecules and the groups of the IAM stationary phase.

Difficulties have been experienced only by the NOX-4 inhibitors (chromenones). IAM chromatography was not an appropriate method to analyse compounds with extreme low lipophilicity values. The study of these compounds was really valuable as we found significant discrepancies from the potential anti-apoptotic molecules. By the chromenones the optimal lipo- and phospholipophilicity range was defined not at medium level but at low or extreme low values. This suggests that the penetration ability of the compounds is very small which was also affirmed by the PAMPA assay. It could mean, on one hand, that it is not necessarily for the compounds to penetrate completely through the cell membrane or, on the other hand, the penetration is accomplished by active transport mechanism.

Our data proved that selecting the effective and sorting out the ineffective molecules at the early stage of the drug research can be perfectly performed by the determination of lipo- and phospholipophilicity and permeability values as well as the biological activity measured by basic antiproliferative assays.

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. As a proof of concept, determining and applying this optimal lipophilicity range, using isochromanone molecular library, we successfully developed a novel, potential isochromanone drug candidate (4E-((2’,4’-dimethoxyphenyl-4-methylene)-3-isochromanon) with high antitumour effect.

Publications

List of own publications related to the dissertation:

- **Huszar M.**, Varga A., Horvath A., Lorand T., Agocs A., Idei M., Mandl J., Vantus T., Keri, G.. (2010) Comparative Characterization of Experimental and Calculated Lipophilicity and Anti-Tumour Activity of Isochromanone Derivatives. Curr. Med. Chem., 17: 321-333. **IF: 4.94.**
- **Huszár M.**, Hallgas B., Idei M., Kiss-Szikszai A., Horváth A., Patonay T.. (2008) Lipophilicity of Substituted Aurones and Related Compounds Measured on Immobilized Artificial Membrane (IAM) and Conventional C8 (MOS) Columns. J. of Liq. Chrom. and Rel. Technologies, 31: 3143–3158. **IF: 0.98.**
- **Huszár M.**, Idei M., Vántus T., Varga A., Agócs A., Kéri G., Lóránd T.. Characterisation of the promising members from the isochromanone molecule library. *ISC 2008 - 27th International Symposium on Chromatography*, 2008, Münster, Germany.
- Lóránd T., **Huszár M.**, Vántus T., Idei M., Horváth A., Kéri G.. Lipophilicity and antiproliferative activity profiling of 4-(arylmethylene)-3-isochromanones. *4th Summer School "Medicinal Chemistry"*, 2008, Regensburg, Germany.
- **Huszár M.**, Hallgas B., Idei M., Erős D., Szabó E. Z., Bökönyi G., Vántus T., Kéri G., Lóránd T.. Measuring of the lipophilicity and biological properties of the isochromanone molecular library. *31st International Symposium on High Performance Liquid Phase Separations and Related Techniques,* 2007, Ghent, Belgium.
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