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Evaluation of lipophilicity and antitumour activity of parallel carboxamide libraries

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Abstract

Searching for molecules possessing antitumour activity, a parallel molecule library of aromatic carboxamides has been designed and synthesised. This work resulted in a ''thiophene'' sub-library containing a thiophene core and of a ''furoyl'' sub-library with a furoyl core, respectively. In both sub-libraries substitutions were carried out with six different groups resulting in six pairs of compounds differing in only the heteroatom of aromatic ring of the cores. To study the importance of the type of cores and the specific substitutions in relation to their lipophilicity and antitumour activity, lipophilicity of carboxamides was determined by chromatographical data (log k') and by software calculated parameters (CLOGP). Pairs of compounds were tested for their ability to inhibit the proliferation of the A431 cells by MTT assay. The isosteric molecule pairs were successfully separated. Our results showed that the experimentally determined (log k') and the calculated (CLOGP) lipophilicity parameters correlated well with each other. Furthermore, lipophilicity values of the thiophene sub-library were always higher than those in the furoyl sub-library. Moreover, compounds of the thiophene sub-library were more active than their respective furoyl pairs in our MTT antiproliferative assay. From these observations we can conclude that the higher the lipophilicity values the higher the antitumour activity of the carboxamides synthesised. Therefore, determination of lipophilicity by measuring the log k' or by calculating the CLOGP values of the carboxamide sub-libraries may help to predict their biological activities.
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Keywords: Lipophilicity; Carboxamide parallel library

1. Introduction

Combinatorial chemistry provides drug discovery facilities with a growing number of biologically active compounds [1]. Construction of analogues around these potential drug candidates is required for the hit-to-lead avenue, further increasing the necessity of high throughput (HT) pre-screening. It is because the in vitro nature of HT screening techniques generates leads with often unfavourably poor bioavailability [2]. For initial chemical screening of activity of newly synthesised compounds it is recommended first to determine their lipophilicity since the hydrophobic character of a molecule often seems to be the most important physico-chemical parameter in accounting for the variations of biological activity. The importance of hydrophobicity/lipophilicity of

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molecules has long been recognised in drug action including absorption, blood–brain distribution, drug–receptor interaction etc. [3,4]. It is usually measured by determining the equilibrium concentration of the compound in two immiscible liquids and expressed as the logarithm of the partition coefficient [5] first demonstrated the relationship between bioactivity and logarithm of the *n*-octanol–water partition coefficient (log P_{ow}). Since then, log P_{ow} has become the most widely used scale for solute hydrophobicity [5], because it has been shown that this partition system is a good model for many biological partition processes [6]. Early octanol–water partition data were generated using the shake flask technique. This method suffers from long analysis times, interference from solvent/solute impurities, the inherent problems of trace analysis at polarity extremes since solute concentration in both phases must be analysed, and has a limited dynamic range [7,8]. Different techniques have been used to estimate log P_{ow} values through its linear relationship with chromatographic retention factor, k' [9,10], as $\log P_{ow} = a \log k' + b$.

Much work has been done for the ''best'' chromatographic conditions in order to establish this equation for a wide range of compounds. Various chromatographic techniques such as centrifugal partition chromatography [11], micellar liquid chromatography [12,13], and gas chromatography [14] have been used for estimation of log P_{ow}. Among liquid chromatography methods high-performance liquid chromatography in reversed-phase separation mode (RP–HPLC) is an alternative technique that can correlate the hydrophobicity of compounds with retention parameters [15,16]. Chromatographic experiments have a lot of practical advantages over the direct determination of partition coefficients i.e. small amounts of material are required, impurities can be separated during the measurements, there is no need for concentration determination, and the process can be easily automated. These demands contribute to the development and application of those separation methods of high-performance, which beyond the fast separation of the analysed components are simultaneously able to provide data for drug development characterising physico-chemical properties (e.g. distribution coefficient, retention factor, lipophilicity) of the compounds analysed. The properties of the compounds are characterised directly from the chromatographic retention determined by the interaction of solutes with the stationary and mobile phases. It is widely accepted that the logarithm of retention factor in RP–HPLC at a purely aqueous mobile phase, log k'_w, provides the best correlation with log P_{ow} [17,18]. When highly efficient reversed-phase stationary phases were used with hydro-organic mobile phases, the correlation between the chromatographic partition data and the octanol–water partition data was strong when structurally related compounds were investigated [19]. Therefore, the measurement of log k' in the RP–HPLC system provides a useful tool for the determination of hydrophobicity parameters in molecule libraries containing structurally close relative compounds. 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 [20]. This may prove to be especially useful for pre-screening of thousands of molecules synthesised by combinational chemistry methods.

Another possibility of the current approaches in rational drug design is to estimate lipophilic nature of the molecules on the basis of computer-assisted prediction, models or expert systems to calculate physico-chemical parameters on the basis of the structural moieties building up the molecule investigated [21]. Methods, which allow physico-chemical predictions, are ''badly needed in both early discovery and pharmaceutical development setting'' [22]. This statement can be supplemented with the necessity of toxicity assessment as well as with the early prediction of the first pass effect (metabolic clearance) [23]. There are multiple advantages of computer-assisted prediction, models and expert systems [24]. They are not only capable of screening vast numbers of molecules with relatively small investment in space and equipment but can also be used in a network environment or via the Internet. On the other hand, the users should be aware that computer-calculated data and software-generated models have accuracy with certain limitations and can not reflect the true complexity of the chemical and biological systems [25]. An example for this comes from the

field of chromatography where discrepancies between the calculated physico-chemical parameters and the measured retention factors of isomers (ortho, meta and para) of the same compound can regularly be observed. In many cases isomers can be separated well by various chromatographical methods but the calculation of lipophilicity data (CLOGP) gives the same value for the different isomers [30a,b]. In this case structure-based calculation seems to be ''blind'' for the characterisation of lipophilicity of the isomers. These systems are mainly rule- and structure-based approaches, represented by quantitative structure–activity relationship.

The knowledge of cytotoxicity of the compounds is one of the most important data among the various biological parameters investigated. Many different methods are available to assess cytotoxicity in culture including the microculture tetrazolium assay which has an excellent correlation with the cell number [26,27]. 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 elements of the synthetic combinatorial molecule libraries in vitro [28,29].

In summary, prediction of hydophobicity using the computer-assisted log P calculation data (CLOGP) along with the experimentally (RP–HPLC) determined log k' parameters and with biological (cytotoxicity) data can offer further opportunities of evaluating and of screening synthetic molecule libraries to get a more focussed target library. In rational drug design, one of the major approaches generating highly diverse libraries is to construct a small central core (scaffold or centroid) which is simultaneously or consecutively ''decorated'' around with substituents [34,35]. The central core is frequently considered as a biologically inert skeleton which links together substituents oriented into different spatial regions. These substituents hold the recognition elements for protein binding [36].

In our earlier works we reported on the relationship between lipophilicity and antitumour activity of molecule libraries of Mannich ketones [30a,b]. A series of fused Mannich ketones as well as a library of Mannich ketones of cycloalkanones were synthesised in order to study the relative importance of structure and specific substitutions in relation to their lipophilicity and antitumour activity. In the case of both libraries we found strong correlation between hydrophobicity and antitumour activity of the compounds.

Searching for new types of molecules possessing antitumour activity in various cell lines, a parallel molecule library of aromatic carboxamides has been designed and synthesised. We have synthesised five-membered aromatic molecules in pairs in which the heteroatom in the aromatic ring was either oxygen or sulphur (isosteric compounds). This work resulted in thiophene carboxamide derivatives (designated as ''thiophene'' sub-library containing a thiophene core) and of furan carboxamide derivatives (designated as ''furoyl'' sub-library containing a furoyl core). In both sub-libraries substitution of the cores with the same substituents have resulted in six pairs of compounds.

The aim of the present work was to separate the members of the parallel carboxamide library by a suitable RP–HPLC method, to characterise their lipophilicity by an experimentally-determined parameter obtained from the separation process (retention factor, k') and by a parameter originated from the computer calculation method (CLOGP). Another aim of the present study was to determine the antitumour activity of the carboxamide compounds by MTT test and to investigate the relationship between their lipophilicity parameters (log k' and CLOGP) and their antitumour activity (LD_{50}).

2. Materials and methods

2.1. Chemicals

Furoyl and thiophene derivatives were synthesised in our laboratory. 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 parallel carboxamide libraries

Members of the parallel sub-libraries of carboxamides were synthesised by the acid chloride method as described [31]. Six pairs of compounds have been prepared from the corresponding furan and thiophene basic compounds. For the preparation of each member of sub-libraries, 11 m*M* 2-thiophenecarbonyl chloride (Aldrich) or 11 m*M* 2-furoyl chloride (Aldrich) was added to 10 m*M* amines (anilin derivatives purchased from Aldrich) dissolved in 1 ml dimethylformamide. The reaction mixture was stirred vigorously and heated up to 60 °C for 1 h. Ten milliliters of water was added to the reaction mixture and the precipitation was filtered on filter paper. The crude materials were dried in a desiccator over natrium-hydroxide and then recrystallised from 40–70% ethanol–water. During the preparation process the purity of the materials were tested by silica TLC in acetone–toluol 2:1; ethylacetate–pyridine–acetic acid–water 48:20:6:1 solvent systems. All structures were validated by a Waters LC/MS system equipped with a Waters 996 DAD UV detector and a Micromass ZMD MS detector. Furoyl and thiophene core structures for parallel sub-libraries and their substituents (R) are summarised in Fig. 1.

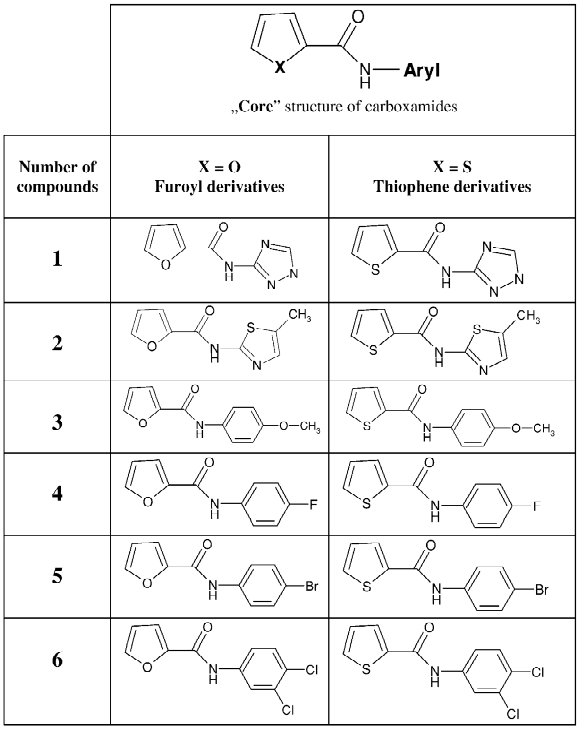

Fig. 1. ‘‘Core’’ structures and substituents in the library of carboxamide compounds. These structures resulted in two parallel sub-libraries: a furoyl and a thiophene sub-library.

2.3. Separation of the members of parallel libraries and determination of lipophilicity by a chromatographic method

For chromatographic analysis stock solutions of 1.0 mg/ml of the samples in acetonitrile: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 a 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 at 24 v/v% ACN. Flow-rate: 1 ml/min. Temperature: 20 °C. A 20 μl portion from the stock solution was injected into a loop of 50 μl volume and four parallel injections were analysed. 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 log *P* calculation method. It divides molecules into fragments and uses the constants of these fragments and correction factors taken from its database for log *P* calculation [32,33]. A CLOGP database was prepared with the computer program accessible via the Internet (www.daylight.com/day-cgi/clogp). Lipophilicity values were computed for the 12 carboxamide compounds and are designed in the following as CLOGP values.

2.5. Evaluation of antitumour activity by MTT assay

To evaluate the antiproliferative effect of carboxamides on A431 cells (American Type Culture Collection No. CRL-1555), the MTT colorimetric assay was performed as described earlier [26,29]. 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. Twenty-four hours after plating, the medium containing foetal calf serum was removed and test solutions were given to cells in various final concentrations 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 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. Percent of inhibition was calculated from the values of triplicate experiments and the results are expressed as percent of controls.

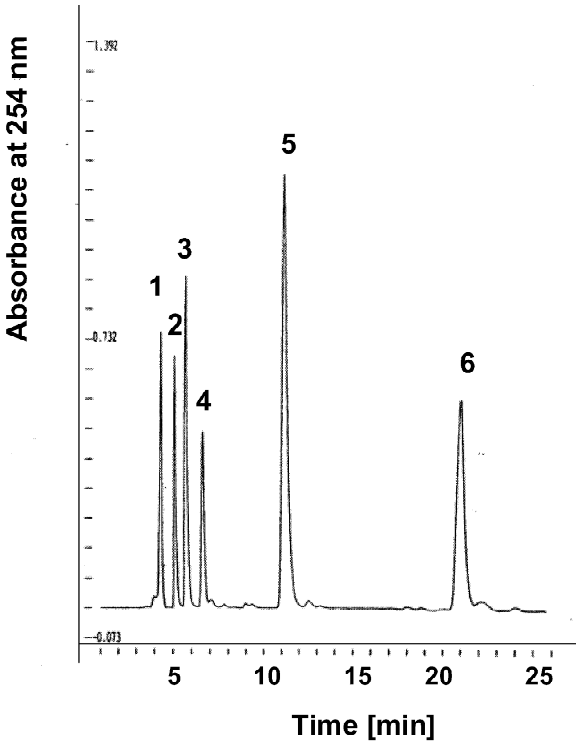

Fig. 2. HPLC chromatograms of the members of the furoyl sub-library. Isocratic run was performed at 24 v/v% ACN using a Hypersyl 5 MOS column.

3. Results and discussion

In our work parallel libraries of carboxamides were designed and synthesised. This work resulted in two sub-libraries: a ''thiophene'' and a ''furoyl'' sub-library (Fig. 1). To study the importance of the type of cores and the specific substitutions in relation to their lipophilicity and antitumour activity, lipophilicity of carboxamides was determined by HPLC.

3.1. The results of the HPLC measurements

A suitable isocratic ACN/TEAP eluent system has been developed for the separation of 12 carboxamide compounds of the parallel libraries. Using the same isocratic system (24 v/v% ACN in eluent A) for the separation of furoyl and thiophene derivatives, compounds were successfully separated within 25 and 45 min, respectively (Figs. 2 and 3.). Analysing the impact of the substitution pattern of the aryl group on the retention factors, we found that substitution of the methoxy group with halogen atoms (flouro, bromo and dichloro substituents) resulted in large increase in their retention. Furthermore, by the application of the stationary phase of Hypersil 5 MOS, our system was sensitive enough to separate isosteric pairs of compounds having the same substituents as exemplified by the chromatogram of a mixture of 5 pairs of compounds (Fig. 4.). Comparing the retention factors of the molecule pairs bearing the same substituents revealed that the retention factor of the thiophene derivative was always greater than that of the respective furoyl derivative. This means that replacement of the oxygen atom for sulphur heteroatom in the core structure significantly increased the retention factors and the direction of this change was always the same.

3.2. The results of the CLOGP calculation

Lipophilicity values predictable on the basis of

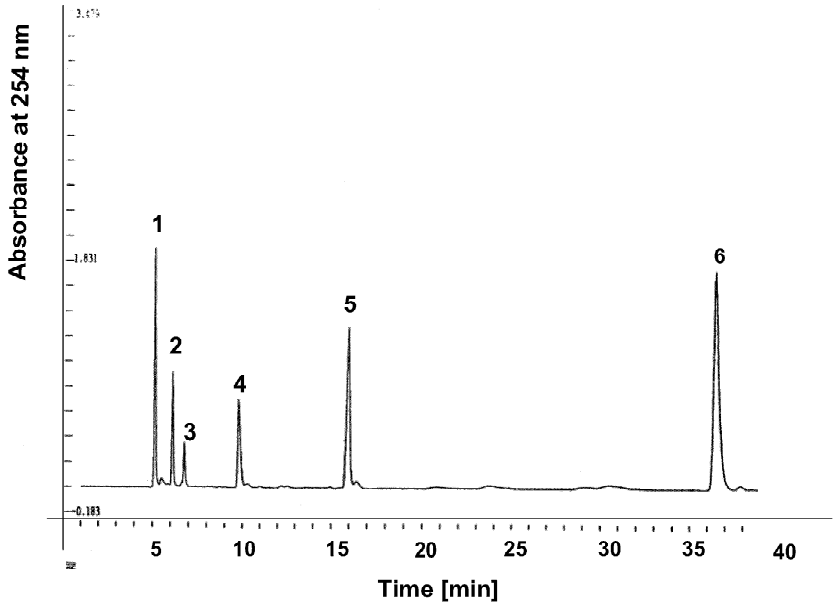

Fig. 3. HPLC chromatograms of the members of the thiophene sub-library. Isocratic run was performed under the same conditions as indicated in Fig. 2.

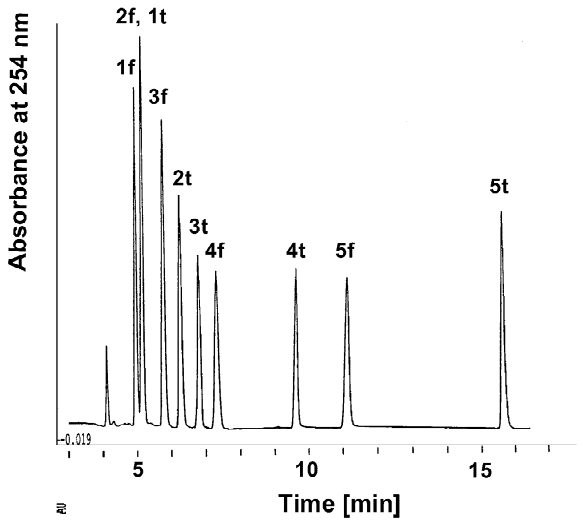

Fig. 4. HPLC chromatograms of the co-injected pairs of compounds where abbreviation ''f'' means the furoyl compounds while ''t'' represents the corresponding thiophene derivatives of the same core structures. Isocratic conditions applied were the same as described in Fig. 2.

chemical structure (CLOGP) were calculated for both sub-libraries. These calculated CLOGP values were compared with the experimentally determined retention factors of the same compounds (See Table 1 and 2). Comparison of the retention factors (log k') and CLOGP values for the compounds of both sub-libraries revealed that good linear correlation exists between the experimentally determined and the calculated lipophilicity parameters.

Table 1
Experimentally determined and calculated lipophilicity values of thiophene sub-library

No. of compound	log k'	CLOGP	LD_{50} [μg/ml]	
			Mean	SD
1.	0.169	1.901	93	30
2.	0.177	1.741	91	27
3.	0.335	2.097	85	11
4.	0.398	2.227	87	25
5.	0.682	2.947	55	12
6.	1.001	3.480	50	19

Cytotoxic activities of thiophene derivatives are also indicated as means and standard deviations.

Table 2
Experimentally determined and calculated lipophilicity values of furoyl sub-library

No. of compound	log k'	CLOGP	LD_{50} [μg/ml]	
			Mean	SD
1.	−0.115	1.211	190	30
2.	0.098	1.547	170	14
3.	0.401	2.470	138	35
4.	0.607	2.975	150	19
5.	0.872	3.477	139	16
6.	1.275	4.015	82	9

Cytotoxic activities of furoyl derivatives are also indicated as means and standard deviations.

For the furoyls: $R=0.9891$; SD=0.1797; $P<0.000177$ and for the thiophene series $R=0.9935$; SD=0.0854; $P<0.0001$ (Fig. 5).

Similarly to the measured retention factor CLOGP data of the thiophene derivatives were always higher than that of the respective furoyl derivatives. In the case of both sub-libraries halogen-containing aryl groups caused the strongest impact on CLOGP values. Compounds with bromo- and dichlorophenol moieties had the highest CLOGP values. In contrary, in both sub-libraries compounds having N-containing five-membered ring as substituents showed the lowest CLOGP data and retention factors, respectively.

3.3. The results of the MTT assay

To study the biological significance of lipophilicity and the influence of the substituents on anti-

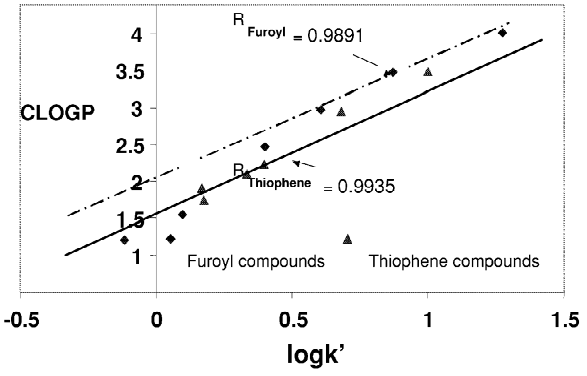

Fig. 5. Relationship between measured (log k') values and calculated lipophilicity data (CLOGP) of the members of parallel carboxamide libraries.

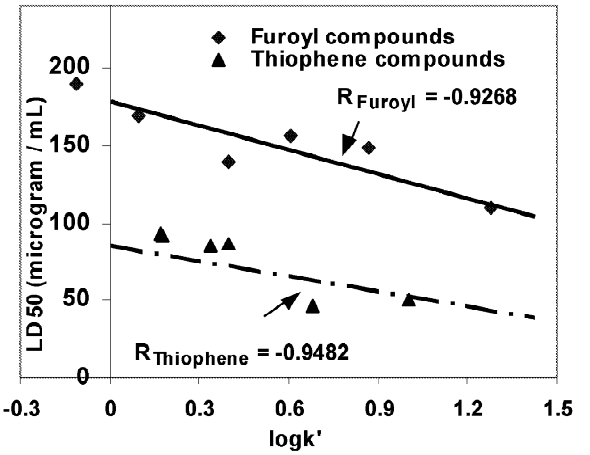

Fig. 6. Relationship between log k' and cytotoxicity values (LD_{50}) in the sub-libraries.

tumour activity, compounds of parallel sub-libraries were tested on A431 cell proliferation by MTT assay. Antitumour activity of the carboxamide compounds was expressed as LD_{50} values. Comparison of the experimentally determined lipophilicity data (log k') and the LD_{50} values of the compounds from both sub-libraries revealed that the higher the measured (or calculated) lipophilicity of the molecules the higher their antitumour activity as shown by lower MTT values. Considering the MTT values in the thiophene sub-library, compounds were nearly twice more active than their respective pair in the furoyl sub-library as exemplified by Fig. 6 and Table 1 and 2. We could observe the same trend with the

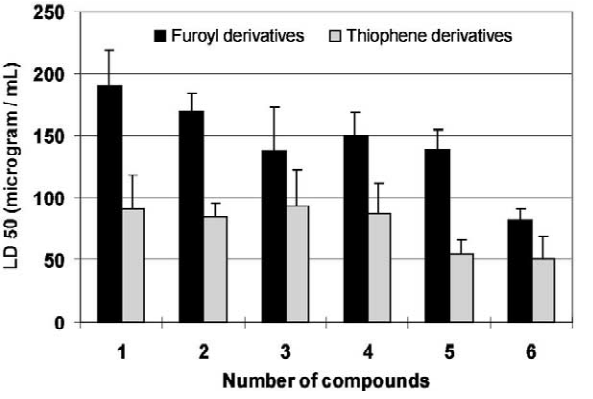

Fig. 7. Cytotoxic activity of carboxamide compounds expressed as LD_{50} values.

MTT values as we found with the log k' and CLOGP data.

For the furoyls: $R=-0.9268$; SD=15.4353; $P<0.0078$ and for the thiophene series $R=-0.9482$; SD=6.8043; $P<0.0039$ (Fig. 6).

Comparing the LD_{50} values of the isosters we found that carboxamides having a furoyl core structure showed higher MTT value (lower activity) than that of compounds having a thiophene core (Fig. 7). The highly lipophilic compounds were more active in all groups than their less lipophilic members.

4. Conclusions

The RP–HPLC method applied in this work proved to be applicable for fast analysis of parallel libraries containing thiophene and furoyl derivatives. Separation of six pairs of carboxamide compounds having the same substituents could be obtained within 45 min. Beyond the fast separation and the check of the chromatographic purity of the compounds synthesised, the advantage of this method is that experimental physico-chemical parameters can be obtained during the course of the analysis which characterises the lipophilicity of the compounds. Furthermore, good correlation was found between the experimentally determined lipophilicity parameter (retention factor) and the computer predicted one (CLOGP). This good correlation confirms that in our cases both parameters can be well used for the characterisation of lipophilicity.

Our experiments proved the role of lipophilicity in the antiproliferative activity of carboxamide compounds. The present results showed that the higher the lipophilicity the higher the antitumour activity in both sub-libraries. Furthermore, from our data it is also clear that the highest values for both biological activity and lipophilicity were achieved when halogen-containing aromatic ''R'' substituents were applied. The most active material was found to be the dichlorophenyl-substituted furoyl-type carboxamide compound. In order to obtain new lead chemicals our results suggest that highly halogenated carboxamides might be more active antitumour compounds. The good correlation between the measured and the calculated lipophilicity (log k' and CLOGP) and the MTT activity proved clearly that lipophilicity of the compounds investigated played a role in their antiproliferative 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 the biological activity. Pre-selection of the molecules can easily be performed on this basis. This method is especially useful when fast characterisation of lipophilicity of a number of molecules with a similar structure (e.g. combinatorial libraries) is needed.

Taking all these findings together we can say that preliminary 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 more rational libraries. Such a system may be suitable for further characterisation the relationship between the lipophilicity parameters and antitumour activity of the drugs.

Acknowledgements

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