Quantitative Structure – Activity Relationship (QSAR) of N-Arylsubstituted Hydroxamic Acids as Inhibitors of Human Adenocarinoma Cells A431

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Abstract: Hydroxamic acids the multifunctional molecules with general formula R'–C(=O)NROH have interesting medicinal and biological potentiality. The antiproliferative activity of 12 hydroxamic acids has been tested *in vitro* towards human adenocarcinama cell line by MTT assay. The IC_{50} values were found to be in the range from 12 to 152.8μM. The most potent product identified is N-*p*-chlorophenyl-4-nitrobenzohydroxamic acid with IC_{50} value 12μM. The RP-HPLC experiment of these molecules was performed with 50:50 $^{V}/_{V}$ % methanol - water mixture as mobile phase. A QSAR is developed for the human adenocarcinoma cells inhibitory activity of a series of hydroxamic acids (n=1-12) that are structurally related to hydroxyurea. Multivariate analytical tool, projection to latent structures was used to develop a suitably predictive model for the purpose of optimizing and identifying members with more potent inhibitory activity. The cross-validated Q^2cum values for two optimal PLS models of hydroxamic acids are above 0.690 (remarkably higher 0.500), indicating good predictive abilities for log1/IC_{50} values of HAs. By partial least squares regression, two QSAR models revealed that, besides the essential pharmacophore – NOH·C=O, retention capacity factor, logk', polar surface area, PSA, Dipole moment, Dm, total no. of hydrogen bond donor and acceptor atoms, H, and chlorine atoms attached in upper or/and lower phenyl rings, I_{Cl}, are important determinants for the inhibitory potency against A431 cells.

Key Words: QSAR, PLS, hydroxamic acids, A431 cell.

INTRODUCTION

Quantitative structure-activity relationship (QSAR) studies have received widespread attention as a powerful drug design tool for the optimization of promising drug candidates [1-8]. Using QSAR approach, molecular descriptors such as hydrophobic, steric, electronic, and topological can be generated for molecule with similar structural features, in order to quantify their molecular characteristics.

Multiple linear regression (MLR) is one of the mostly used modeling methods in QSAR [9]. The co-linearity problem of the MLR method has been overcome through the development of the partial least square projection to latent structure (PLS) method, which has been shown to be an efficient approach in monitoring many complex processes, reducing the high dimensional strongly cross-correlated data to smaller and interpretable set of principal components or latest variables. PLS and PCA have been widely used with these kinds of processes because they can be used with redundant data sets [10].

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 [11,12]. This assay provides sensitive and reproducible indices of growth as well as drug sensitivity in individual's cell lines. Thus this colorimetric assay based on enzyme activity of various dehydrogenase of the living cells is suitable for cytotoxicity testing of elements of the synthetic combinational molecule libraries *in vitro* [13,14].

The lipophilicity, logP, of products of therapeutic interest is an important parameter to understand the transport process across the biological barriers. The traditional shake flask method for determination of $logP_{O/W}$ is time consuming, tedious, prone to experimental difficulties and required relatively large amount of pure compound [15, 16]. The reversed-phase HPLC (RP-HPLC) method is a promising alternative to the shake flask method, having advantages such as, higher throughput, insensitivity to impurities or degradation products and broader lipophilicity range.

Hydroxamic acids, a group of very weak organic acids of general formula, R_2 –C(=O) –NR_1OH (where R_1 and R_2 are phenyl or substituted phenyl groups), fulfill a variety of role in biology and medicine, for example, as siderophores for iron (III) [17], as potent and selective inhibitors of enzymes such as peroxidase [18, 19], ureases [20,21], and matrix metalloproteinase [22-24], as hypotensive [25], antitumor [26-28], antituberculous and antifungal [29], agents.

The aim of the present work was to separate the members of N-arylsubstituted hydroxamic acids by suitable RP-HPLC method, to characterize their lipophilicity by experimental parameters obtained from the separation process (retention factor, k'). Furthermore, *in vitro* antitumor activity against the A431 cells of these molecules was determined by MTT test. The QSAR analysis of 12 N-arylsubstituted hydroxamic acids as inhibitors of A431 cells has been performed using logk', calculated molar refraction (CM_R), Dipole moment (Dm), Polar surface area (PSA), Ovality (O), Wiener index

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(WI), Balaban index (J) and suitable indicator variable using PLS method.

EXPERIMENTAL SECTION

Materials and Methods

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from GIBCO, NY, USA and fetal bovine serum (FBS) was obtained from Biomedia, France. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from Sigma, USA. DMSO and HPLC grade methanol were purchased from Merck (Darmstadt, Germany). Hydroxamic acids were synthesized in the laboratory following the procedure reported in literature [30, 31]. These were purified by recrystallisation from benzene thrice and dried in vacuum over phosphorous pentaoxide for 24 hrs prior to use. The purity of these compounds was ascertained by determining the melting point, U.V. and I.R. spectra, which tallied with literature data [32]. Whereever analyses are only indicated with element symbols, analytical results obtained for those elements were within 0.4% of the theoretical values.

N-phenylbenzohydroxamic acid (1). Anal. ($C_{13}H_{11}NO_2$): C, H, N.

N-phenyl-4-methyl-3-nitrobenzo hydroxamic acid (2). Anal. ($C_{14}H_{12}N_2O_4$): Calcd C, 61.76; H, 4.44; N, 10.29. Found: C, 61.74; H, 4.04; N, 10.24.

N-phenyl-2-iodobenzohydroxamic acid (3). Anal. ($C_{13}H_{10}INO_2$): Calcd C, 46.04; H, 2.97; N, 4.13. Found: C, 46.09; H, 2.89; N, 4.20.

N-*m*-chlorophenyl-2-methoxybenzo hydroxamic acid (4). Anal. ($C_{14}H_{12}NO_3Cl$) : C, H, N.

N-*p*-chlorophenyl-4-chlorobenzo hydroxamic acid (5). Anal. ($C_{13}H_9NO_2Cl_2$): Calcd C, 55.34; H, 3.22; N, 4.96. Found: C, 55.29; H, 3.13; N, 5.03.

N-*p*-chlorophenyl-4-bromobenzo hydroxamic acid (6). Anal. ($C_{13}H_9NO_2ClBr$): C, H, N.

N-*p*-chlorophenyl-4-nitrobenzo hydroxamic acid (7). Anal. ($C_{13}H_9N_2O_4Cl$): C, H, N.

N-*o*-tolyl-2-chlorobenzohydroxamic acid (8). Anal. ($C_{14}H_{12}NO_2Cl$): C, H, N.

N-*o*-tolyl-4-chlorobenzohydroxamic acid (9). Anal. ($C_{14}H_{12}NO_2Cl$): C, H, N.

N-*o*-tolyl-4-ethoxybenzohydroxamic acid (10). Anal. ($C_{16}H_{17}NO_3$): C, H, N.

N-*p*-tolyl-4-ethoxybenzohydroxamic acid (11). Anal. ($C_{16}H_{17}NO_3$): Calcd C, 70.83; H, 6.32; N, 5.16. Found: C, 70.79; H, 6.24; N, 5.08.

N-*o*-tolyl-3-nitrobenzohydroxamic acid (12). Anal. ($C_{14}H_{12}N_2O_4$): C, H, N.

Biological Activity *In Vitro*

The Human Adenocarcinoma cell was obtained from national centre for cell science, Pune, India. The cells were maintained in a 5% CO_2 humidified atmosphere at 37^0C in DMEM supplemented with FBS (10%) and antibiotics (streptomycin 200µg /m) and penicillium 100 units /ml). Cells were harvested from 80% to 90% confluent culture by trypinization. The antiproliferative effect of N-arylsubstituted hydroxamic acid derivatives against A431 cells were determined using MTT assay in microliter plates. For each experiment, the cells were suspended in DMEM with 10% FBS at $1x10^5$ / ml and seeded in 96 well plates at 100µl/well and allowed to grow for 24hrs. Each compound initially dissolved in 100% DMSO immediately before use and further into DMEM to obtained ten gradual concentrations ranging from $1.25x10^{-5}$ to $4x10^{-4}$M. Each dilution was added to the wells as 100µl/well in quadruplicate. The final concentration of DMSO in the cell culture was 0.5% for the highest drug concentration and in corresponding controls, all others had < 0.25% to avoid the toxicity of DMSO. Hydroxyurea (Aldrich chemical St. Louis, Mo.) was used as positive controls. Negative control containing only DMSO at identical dilutions was run with each experiment. The plate was incubated in 5%CO_2 humidified in atmosphere at 37^0C for 48 hrs. MTT (0.1 mg/well) was added and plate was incubated for 3 more hrs. The formazon crystals formed were solubilized by incubating the cells with 10% sodium dodecyl sulphate (10%w/v in 0.01MHCl) overnight. The absorbance of the solution was measured at 550nm, using a microplate reader (Bio-Tek instruments, USA). All experiments were repeated at least thrice. IC_{50} values of hydroxamic acids are presented in Table **1**.

HPLC Measurement

For chromatographic analysis stock solutions of 1.0mg/ml of the sample in methanol was prepared and filtered through a 0.2µm Millipore filter unit. These solutions were kept in eppendorf tubes at 4^0C. HPLC analysis of the sample performed with an 880-PU-HPLC pump and an 875-UV-Vis detector (both from Jasco, Tokyo, Japan). The column was Hypersil Gold (250 x 4.6 mm I.D.5 µm) from Thermo electron cooperation, North America. Isocratic runs were performed at 50:50 v/v% methanol/water. Flow time: 1ml/min. A 20 µl portion from the stock solution was injected into loop of 50 µl volume and detection was done by measuring UV absorption at 254nm and four parallel injections were analyzed.

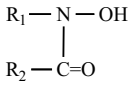
Molecular Descriptors Generated

The Bio-Loom program of BioByte Corp. was utilized to calculate ClogP and CM_R [33]. Briefly, ClogP is the calculated partition coefficient in n-octanol / water and is a measure of hydrophobicity. CM_R is the calculated molar refractivity for the whole molecule. M_R is calculated from the Lorentz-Lorenz equation and is described as follows: $[(n^2 - 1/ n^2 +2) MW/\delta]$. Where n is the refractive index, MW is the molecular weight and δ is the density of a substance. Polar surface area, PSA, Dipole moment, Dm, Balaban index, J, and Wiener index, WI, were determined from structure of the compounds using chem3D ultra 6.0 modeling package [34]. The data are reported in Table **2**.

Data Analysis

QSAR Models were well developed using PLS regression, as implemented in the Simca (Simca-P Version 10, *Umetric AB and Erisoft AB*) software [35]. The condition for

Table 1. IC_{50} Values of N-arylsubstituted Hydroxamic Acids



Hydroxamic Acids	R_1	R_2	IC_{50}(μM)
1	Phenyl	Phenyl	118.00
2	Phenyl	4 - Methoxyl,3-nitrophenyl	131.00
3	Phenyl	2-Iodophenyl	181.00
4	3 -Chlorophenyl	2 - Methoxyphenyl	81.89
5	4 -Chlorophenyl	4 -Chlorophenyl	152.84
6	4 -Chlorophenyl	4 -Bromophenyl	63.00
7	4 -Chlorophenyl	4 -Nitrophenyl	12.00
8	2 - Methylphenyl	2 -Chlorophenyl	88.00
9	2 - Methylphenyl	4 -Chlorophenyl	142.30
10	2 - Methylphenyl	4-Ethoxyphenyl	137.08
11	4 - Methylphenyl	4-Ethoxyphenyl	142.30
12	4 - Methylphenyl	3 -Nitrophenyl	135.71

IC_{50} value of hydroxyurea > 400μM.

the computation was based on the default option of the software. The criterion used to determine the model dimensionality and the number of significant PLS components is cross validation (CV). The obtained QSAR model is considered to have a good prediction ability when the cumulative cross-validated regression coefficient (Q^2) for the extracted components, Q^2cum, is larger than 0.5. Model adequacy was mainly measured as the number of PLS principal components (A), Q^2cum, the correlation coefficient software observed values and fitted value (R) and significance level (p).

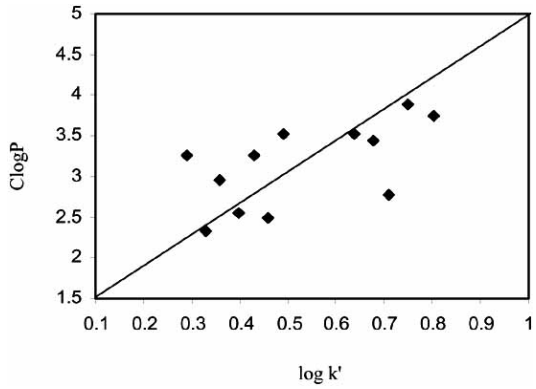


Fig. (1). Relationship between calculated hydrophobicity values (ClogP) and logk[1] of hydroxamic acids.

RESULTS AND DISCUSSION

RP- HPLC is a technique that can correlate the hydrophobicity of the molecules with retention parameters. In RP-HPLC systems, the retention capacity factor k' of a compound is a reliable indirect descriptor of lipophilicity of a compound [36].

The retention capacity factor (k') was determined as defined by,

$$k' = (t_R - t_O)/ t_O \quad (1)$$

where t_R and t_O are the retention times of the solute and the unretained compound, respectively. k' values of hydroxamic acids are reported in Table **2**.

Correlation between logk' and ClogP

Hydrophobic character of a molecule is the most important physico-chemical parameter in accounting the variation in biological activity. ClogP is the software predicted hydrophobicity of the molecules which predict these parameters as fragment constant on the basis of the chemical structure of the molecule processed. The ClogP program is based on Hanch-Leo's logP calculation method. Hydrophobicity values predicted on this basis were calculated for 12 hydroxamic acids. These calculated ClogP values were compared with the experimentally determined logk', retention capacity factor of the same compound. The tendency of logk' and ClogP values are in good agreement. Comparison of the logk' and ClogP values of compounds revealed that good linear relationship exists between these two.

$$\text{ClogP} = 2.730\ (0.651)\ \text{logk'} + 1.717\ (0.362) \quad (2)$$

$n = 10$, $r = 0.829$, $s = 0.331$, $F = 17.596$

QSAR Analysis

To develop the QSAR, several descriptors of size, electronic, lipophilic and topological characteristics were used to

Table 2. Molecular Descriptors of N-arylsubstituted Hydroxamic Acids

HAs	logk'	ClogP	CM_R	PSA	Dm (Debye)	J x 10^3	WI	O	I_{Cl}	H
1	0.330	2.320	6.21	40.540	4.546	80.530	448	1.313	0	4
2	0.458	2.482	7.296	92.350	6.766	228.913	825	1.385	0	7
3	0.677	3.443	7.527	40.54	3.064	108.722	538	1.369	0	4
4	0.360	2.952	7.329	49.770	6.755	176.292	702	1.381	1	5
5	0.804	3.746	7.204	40.540	4.829	144.243	595	1.321	2	4
6	0.750	3.896	7.490	40.54	4.998	144.243	639	1.401	1	4
7	0.787	2.770	7.324	92.350	2.771	234.496	617	1.409	1	7
8	0.638	3.532	7.176	40.540	3.596	134.560	595	1.321	1	4
9	0.491	3.532	7.176	40.540	3.247	139.397	617	1.368	1	5
10	0.290	3.267	7.766	49.770	5.692	240.898	870	1.425	0	5
11	0.429	3.267	7.766	49.770	5.443	247.933	896	1.445	0	5
12	0.398	2.562	7.296	92.350	4.082	234.608	846	1.401	0	7

characterize the compound. Polar surface area and ovality were chosen to characterize the size components. Lipophilicity was represented by logk' and ClogP. The electronic character of the compounds was captured by molecular refractivity and dipole moment. The topological characters were taken as Balaban index, Weiner index. Total no. of hydrogen bond donor and acceptor atoms, H, and chlorine atoms attached in upper or/and lower phenyl rings, I_{Cl} , were used as variable indicators.

In a PLS model, variable importance in the projection (VIP) is a parameter that shows the importance of a variable. According to the manual of Simca - P (Version 10), VIP is the sum of over all model dimensions of the contributions of variable influences (VIN) for a given PLS dimension (a) and a given X term (k), VIN^2 is computed form the squared PLS weight of that X term multiplied by the percent explained the sum of squares (SS) by that PLS dimension. VIP value is calculated from the accumulated value over all PLS dimensions, divided by the total percent explained SS by the PLS model and

$$VIP_k = \sum_{a=1}^{A} (VIP)_k^2 \qquad (3)$$

multiplied by the number of terms in the model. These terms with values of VIP are the most relevant for explaining depended variable.

To obtain an optimal model the following PLS analysis procedure was adopted. First, a PLS model with all the predictor variables was calculated. Then the variable with the lowest VIP value was eliminated and a new PLS regression was performed, leading to a new PLS model. This procedure was repeated till only main predictor variables were recommended. The optimal PLS model was selected with respect to the statistics Q^2_{cum}, R and p.

Following the analysis methods described above, models I and II were obtained for log $1/IC_{50}$ for which the model fitting results are in Table **3** where, R^2X (adj) cum and R^2Y(adj)cum stand for cumulative variance of all the X's and Y's, respectively explained by all extracted components. Eig stands for the eign value which denotes the PLS principal components. It thus be seen from Table **3** that the one PLS principal component selected in model I, which explained 48.9% of the variance of the predictor variables, and 85.7% of the variance of the dependent variables.

Table 3. Model Fitting for Models I and II

Models	n	A	$R^2_{X(adj)(cum)}$	$R^2_{y(adj)(cum)}$	Eig	Q^2_{cum}	R	p	SE
I	8[a]	1	0.489	0.857	2.43	0.693	0.926	$9.54x10^{-4}$	0.156
II	8[a]	1	0.551	0.735	2.2	0.588	0.909	$1.78x10^{-2}$	0.178
		2	0.881	0.912	1.32	0.826			

[a] Outliers compound no. 2, 4 , 6 & 12.

Table 4. The VIPs and PLS Weight (W*[1] and W*[2]) for the Molecular Structural Descriptors Including in Models I and II

Model I		Model II			
Variables	VIP	Variables	VIP	W*[1]	W*[2]
PSA	1.465	PSA	1.479	0.795	0.830
H	1.300	Dm	0.908	-0.494	0.020
Dm	0.909	log k'	0.697	0.254	-0.497
log k'	0.467	log k'^2	0.672	0.244	-0.480
I_{Cl}	0.348				

There is totally five and four predictor variables included in the model I and II, respectively. VIP values for the variables are listed in Table **4**. Based on the unscaled pseudo-regression coefficient of the independent variables and constants transformed from PLS results, analytical QSAR equations are obtained, as follows:

MODEL I

$$\log 1/IC_{50} = 3.922 \times 10^{-1} (PSA) + 3.480 \times 10^{-1} (H) - 2.432 \times 10^{-1} (Dm) + 1.251 \times 10^{-1} (\log k') + 9.316 \times 10^{-2} (I_{Cl}) + 10.472 \quad (4)$$

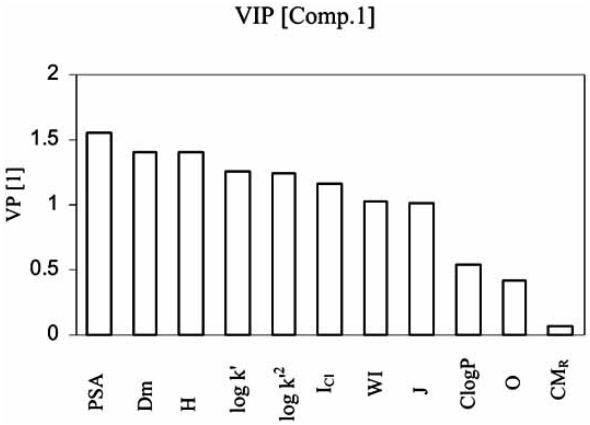


Fig. (2). VIP plot from PLS analysis using the 10 descriptors and the 12 hydroxamic acids.

MODEL II

$$\log 1/IC_{50} = 8.012 \times 10^{-1} (PSA) - 3.005 \times 10^{-1} (Dm) - 2.462 \times 10^{-2} (\log k') - 2.465 \times 10^{-1} (\log k'^{2}) + 10.472 \quad (5)$$

Plots of observed and predicted values for the two models are shown by Figs. (**4** -**5**), respectively. For the hydroxamic acids under study, all the correlation between observed and predicted dependent values in model I and II are significant as observed in figures. Based on models I and II log $1/IC_{50}$ values for hydroxamic acids were predicted. As the cross-validated Q^2cum values of models I and II are remarkably above 0.50, the two models are surely stable and have good prediction ability (Table **3**). The results obtained from the two models show those molecular structural characteristics of hydroxamic acids which govern the antitumor activity of these molecules.

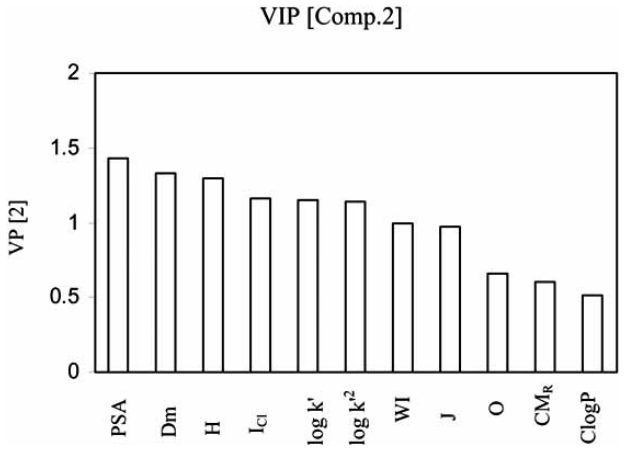


Fig. (3). VIP plot from PLS analysis using the 10 descriptors and the 12 hydroxamic acids.

In model I, five predictor variables are involved which are condensed into one PLS component. The VIPs values for

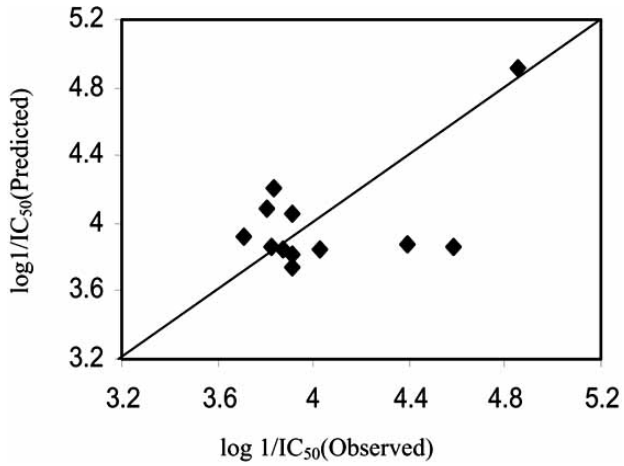


Fig. (4). Plot of predicted vs. observed log $1/IC_{50}$ values of hydroxamic acids: Model I.

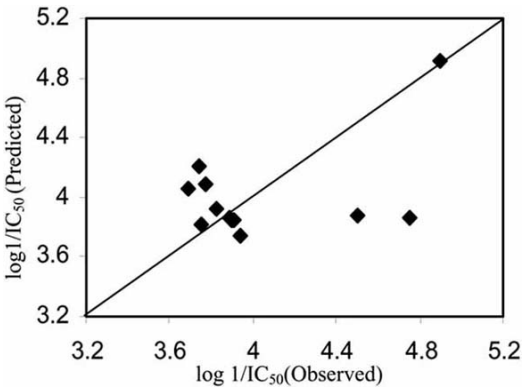


Fig. (5). Plot of predicted vs. observed log $1/IC_{50}$ values of hydroxamic acids: Model II.

the two predictors, PSA and H are larger than 1.0, indicating that they are more significant in explaining log$1/IC_{50}$ than other predictors. As indicated by the pseudo-regression coefficients for the molecular descriptors, increasing log k', PSA, H and I_{Cl} values of hydroxamic acids leads to increase in log $1/IC_{50}$ values. Increase of dipole moment value of hydroxamic acids leads to decrease log $1/IC_{50}$ values.

In model II, two PLS components were selected. The first PLS component is mainly related to Dipole moment. Increasing dipole moment values of hydroxamic acids leads to decrease the log $1/IC_{50}$ values. The second PLS component is loaded primarily on PSA logk' and $logk'^2$ for which the w*[2] values are larger than 0.480 and larger than the absolute values of w*[2] for the other descriptor. As indicated by the pseudo-regression coefficient for molecular descriptors, increasing PSA values of HAs leads to increase the log$1/IC_{50}$ values. logk' and $logk'^2$ characterizes the lipophilicity of HAs. The negative coefficients logk' and $logk'^2$ suggest that less lipophilic substituents in hydroxamic acids' moiety are crucial for antitumor activity.

CONCLUSION

The RP-HPLC method is applicable for fast analysis of hydoxamic acids library. Separation of 12 hydroxamic acids possessing similar chemical structure could be obtained within 22min. Furthermore, good correlation is found between the experimentally determined hydrophobic parameters logk' and compound predicted one ClogP. The good correlation confirms that in our case both parameters can be well used for the characterization of lipophilicity of hydroxamic acids.

The most potent compound found is N-*p*-cholorophenyl-4-nitrobenzo hydroxamic acid. By PLS regression, two QSAR models revealed that, logk', PSA, Dm, H and chlorine atoms attached in upper or/and lower phenyl rings (I_{Cl}) are important determinants for the antitumor activity of hydroxamic acids.

Table. 5. Observed and Predicted Biological Activity of N-arylsubstituted Hydroxamic Acids

HAs	log $1/IC_{50}$				
	Obsd	Calcd. (eq.4)	Obsd -Calcd	Calcd. (eq.5)	Obsd-Calcd
1	3.928	3.713	0.215	3.830	0.098
2	3.883	4.396*	-0.513	4.497*	-0.615
3	3.742	3.876	-0.029	3.895	-0.048
4	4.087	3.806*	0.281	3.778*	0.309
5	3.816	3.907	-0.091	3.755	0.061
6	4.201	3.833*	0.368	3.746*	0.455
7	4.921	4.850	0.071	4.894	0.027
8	4.056	3.911	0.145	3.900	0.366
9	3.047	4.029	-0.182	3.908	-0.061
10	3.863	3.821	0.042	3.884	-0.021
11	3.847	3.876	-0.029	3.895	-0.048
12	3.867	4.585*	-0.718	4.755*	-0.889

* Not included in the regression analysis

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