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Original article

Determination of permeability and lipophilicity of pyrazolo-pyrimidine tyrosine kinase inhibitors and correlation with biological data

Elena Dreassi [a], Alessandra Tania Zizzari [a], Federico Falchi [a], Silvia Schenone [b], Annalisa Santucci [c], Giovanni Maga [d], Maurizio Botta [a,*]

[a] Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via Aldo Moro, I-53100 Siena, Italy
[b] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Genova, Viale Benedetto XV 3, I-16132 Genova, Italy
[c] Dipartimento di Biologia Molecolare, Università degli Studi di Siena, Via Fiorentina 1, I-53100 Siena, Italy
[d] Istituto di Genetica Molecolare, IGM-CNR, Via Abbiategrasso 207, I-27100 Pavia, Italy

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ABSTRACT

A library of 23 pyrazolo-pyrimidine compounds Src tyrosine kinase (TK) inhibitors, that reduced proliferation of a human osteogenic sarcoma cell line, was taken to investigate lack of correlation between inhibition of cellular viability (CV%) and enzymatic inhibition constants (K_i Src). With the aim of understanding this behaviour, we focused on physico-chemical parameters which characterize partition coefficient and diffusion through membrane. Parallel artificial membrane permeability assay (PAMPA) has been frequently used for the evaluation of *in vitro* permeability of new chemical entities and, in this paper, a new approach for determining permeability of low soluble compounds was obtained. Goodness of PAMPA methodology was confirmed by log K_w and computational approaches, by VolSurf, Cerius^2 and QikProp software programs. The results suggest that the lipophilicity and passive diffusion across the membranes do not significantly influence the activity of the compounds. This trend can be explained by a different target for some of the compounds in our set. In fact some compounds resulted also to be active toward Abl enzyme, another cytoplasmatic TK.

1. Introduction

Physico-chemical properties of drugs, from first steps of drug development, are of primary importance. Among these, lipophilicity is one of the most important parameter because it is a mainly involved in pharmacokinetic processes such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) and in ligand–target interactions [1]. Moreover, lipophilicity is the molecular parameter of choice in numerous quantitative structure–activity relationships (QSAR) of different classes of compounds [2].

Poor solubility and poor permeability are among the main causes of failure during drug development. Therefore it is important to determine these properties associated with a drug. The partition coefficient, log *P*, is an indispensable tool in predicting the transport and activity of drugs [3].

A total of 23 new pyrazolo[3,4-*d*]pyrimidines were selected for this study. Chemical synthesis and characterization of these compounds have been previously reported [4]. The selected pyrazolo-pyrimidine derivatives showed inhibitory properties toward Src phosphorylation, and some of them significantly reduced the growth of osteogenic sarcoma (SaOS-2) cells [4]. (Table 1)

A lack of correlation between cellular data on SaOS-2 cells and enzymatic results (K_i Src) was observed for these compounds. One of the possible explanations is that different compounds may vary in cell membrane permeability and PAMPA technique was used to examine the permeability of these compounds [5].

The PAMPA (parallel artificial membrane permeability assay), proposed by Kansy in 1998, is a high throughput *in vitro* assay system that evaluates transcellular permeation [5] and is used also to simulate human intestinal absorption in pharmaceutical research. In fact several studies indicate that PAMPA permeability is correlated with both Caco-2 cell permeability and human intestinal absorption [6–12]. Different authors have used this method to study the behaviour of lipophilic compounds crossing the membranes [13,14].

In this paper a modified approach to evaluate permeation of highly lipophilic and poorly soluble compounds is reported and validated with a set of drugs well described in the literature; some modifications were brought to the original method because of low solubility of these kind of compounds.

* Corresponding author. Tel.: +39 0577 234306; fax: +39 0577 234333.
E-mail addresses: dreassi@unisi.it (E. Dreassi), zizzari2@unisi.it (A.T. Zizzari), schensil@unige.it (S. Schenone), santucci@unisi.it (A. Santucci), maga@igm.cnr.it (M. Maga), botta@unisi.it (M. Botta).

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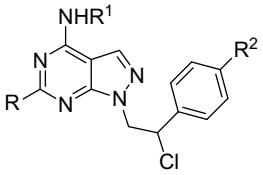
Table 1
Structure and biological data of tested compounds.



Compd	R	R^1	R^2	Src enzyme K_i[a] (μM)	CV[b] (%) after 24 h
1	MeS	$C_6H_5CH_2$	H	3.7 ± 0.9	92.8 ± 1.3
2	MeS	C_6H_5	H	1.2 ± 0.3	100.1 ± 1.2
3	MeS	*m*-F-$C_6H_4CH_2$	H	5.2 ± 1.1	106.2 ± 3.0
4	MeS	$C_6H_5(CH_2)_2$	H	0.7 ± 0.1	102.7 ± 1.9
5	MeS	*o*-F-$C_6H_4CH_2$	H	21.0 ± 4.0	104.1 ± 1.3
6	MeS	$C_6H_5CH_2$	F	35.0 ± 8.0	62.7 ± 0.7
7	MeS	*p*-F-$C_6H_4CH_2$	H	4.6 ± 1.7	102.1 ± 1.4
8	MeS	*p*-MeO-$C_6H_4CH_2$	H	4.0 ± 1.3	101.0 ± 3.0
9	MeS	*o*-Cl-$C_6H_4CH_2$	H	3.1 ± 1.4	101.7 ± 0.9
10	MeS	$C_6H_5CH_2$	Cl	3.0 ± 1.0	52.2 ± 2.4
11	MeS	*m*-F-$C_6H_4CH_2$	H	1.4 ± 0.3	94.8 ± 2.7
12	EtS	$C_6H_5(CH_2)_2$	H	7.5 ± 2.7	107.3 ± 3.5
13	MeS	*m*-F-$C_6H_4(CH_2)_2$	H	13.0 ± 3.0	101.1 ± 1.1
14	MeS	*o*-Cl-$C_6H_4(CH_2)_2$	H	4.0 ± 1.7	102.9 ± 1.3
15	MeS	$C_6H_5(CH_2)_2$	Cl	2.8 ± 1.2	99.9 ± 0.9
16	MeS	*m*-Cl-C_6H_4	H	0.6 ± 0.1	17.1 ± 1.3
17	MeS	*p*-Me-$C_6H_4(CH_2)_2$	H	15.0 ± 2.0	101.9 ± 1.9
18	MeS	*p*-Cl-$C_6H_4(CH_2)_2$	H	25.0 ± 4.0	85.2 ± 0.7
19	MeS	*m*-Br-C_6H_4	F	4.1 ± 0.8	63.1 ± 1.1
20	MeS	*m*-Cl-$C_6H_4(CH_2)_2$	H	6.0 ± 2.1	80.6 ± 0.7
21	MeS	*m*-Br-C_6H_4	H	1.8 ± 0.2	100.1 ± 1.1
22	EtS	*m*-Cl-C_6H_4	H	0.5 ± 0.1	76.5 ± 1.9
23	PrS	*m*-Cl-C_6H_4	H	1.2 ± 0.4	77.8 ± 3.0

[a] K_i (expressed as a μM concentration) toward recombinant human Src was calculated according to the following equation: $K_i = (ID_{50} - E_0/2)/\{E_0 - [S_0/K_{m(ATP)} - 1]/E_0\}$, where S_0 is the concentration of the competing substrate (ATP) and E_0 is the concentration of the enzyme. Each experiment was in triplicate, and mean values were used for the interpolation. Curve fitting was performed with the program GraphPad Prism.

[b] Antiproliferative activity of tested compounds (12.5 μM) toward SaOS-2 cells (MTT assay) after 24 h of treatment, expressed as cellular viability (CV, %) with respect to control (100%). Values are means ± SD of three independent experiments performed in duplicates.

The lipophilicity parameters, log K_w, for all the set of compounds were obtained by a chromatographic method. Chromatography provides an easy, reliable and accurate way to determine the molecular lipophilicity of compounds based on their retention factors. The partition coefficients, *P*, were correlated with lipophilicity parameter, log K_w, obtained through RPLC (reversed-phase liquid chromatography) technique. Traditionally, lipophilicity is measured by the distribution of a compound in a biphasic system, either liquid–liquid (octanolwater partition coefficient) or solid–liquid (retention on RPLC) [15] but the advantages of RPLC techniques for fast lipophilicity measurements are well recognized [16,17]; in particular, they are characterized by low sample consumption, insensitivity to impurities, and automation possibilities.

Comparison with computational data gave us further confirmation of the goodness of the method applied to verify permeability.

The lack of correlation between K_i (Src), PAMPA, log K_w and cellular data (CV% on SaOS-2 cell line) for compounds **1**–**23** suggests that other factors could be involved in the cell activity and the hypothesis that the compounds could act also on a different tyrosine kinase is put forth.

Abl shares significant sequence homology with Src and, in its active conformation, bears remarkable structural resemblance with most members of the Src family kinases (SFKs). Consequently compounds were tested also on Abl tyrosine kinase. As a result, some compounds originally developed as Src inhibitors, exhibited potent inhibition of Abl kinase [18,19].

2. Materials and methods

2.1. Chemicals and instruments

A total of 23 novel tyrosine kinase inhibitors, reported in Table 1, were selected for this study. Chemical synthesis, characterization, biological data on Src inhibition (K_i) and antiproliferative activity on human osteogenic sarcoma (SaOS-2) cells (expressed as cellular viability, CV%) have been previously reported [4].

All solvents (Chromasolv HPLC grade), acetaminophen, caffeine, carbamazepine, cephalexin, chloramfenicol, ketoprofen, furosemide, propanolol, warfarin, theophyllin, and L-α-phosphatidylcholine were from Sigma–Aldrich Srl (Milan, Italy). Dodecane was purchased from Fluka (Milan, Italy).

Milli-Q quality water (Millipore, Milford, MA, USA) was used.

LC analysis were performed with a Varian Prostar LC system (Varian, Inc., Mitchell Drive, USA) equipped with an injector valve, a 20 μl sample loop (Mod. Rheodyne) and Prostar UV detector (Varian). LC separation was achieved on Varian Microsorb C18 column (150 × 4.6 mm, 5 μm particle size) maintained at room temperature.

Hydrophobic filter plates (MultiScreen-IP, Clear Plates, 0.45 μm-diameter pore size), 96-well microplates, and 96-well UV-transparent microplates were obtained from Millipore (Bedford, MA, USA).

Recombinant human Abl was purchased from Upstate Biotechnology (Waltham, MA).

2.2. Artificial membrane permeability assay

Donor solution (0.5 mM) was prepared by diluting 1 mM DMSO compound stock solution using phosphate buffer (pH 7.4, 0.025 M). Filters were coated with 5 μl of a 1% (w/v) dodecane solution of phosphatidylcholine sonicated to ensure complete dissolution. Donor solution (150 μl) was added to each well of the filter plate. To each well of the acceptor plate was added 300 μl of solution (50% DMSO in phosphate buffer). All compounds were tested in three different plates on different days. The sandwich was incubated for 24 h at room temperature under gentle shaking. After the incubation time, the sandwich plates were separated and samples were taken from both receiver and donor sides and analysed using LC with UV detection.

Permeability (P_{app}) for PAMPA, was calculated according to the following equation, obtained from Wohnsland and Faller [20] and Sugano et al. [21] equation with some modification in order to obtain permeability values in nM/s,

$$P_{app} = \frac{V_D V_A}{(V_D + V_A)At} \ln(1 - r)$$

where V_A is the volume in the acceptor well, V_D is the volume in the donor well (cm^3), *A* is the "effective area" of the membrane (cm^2), *t* is the incubation time (s) and *r* the ratio between drug concentration in the acceptor and donor well respectively. Drug concentration is estimated by using the peak area integration.

2.3. Methods for lipophilicity determination

2.3.1. *log* P *determination using LC assay (log* K_w*)*

log K_w was obtained by extrapolation of log retention factor (*K*) to 0% organic measured in reversed-phase liquid chromatography. K_w is the solute retention factor for water as mobile phase and

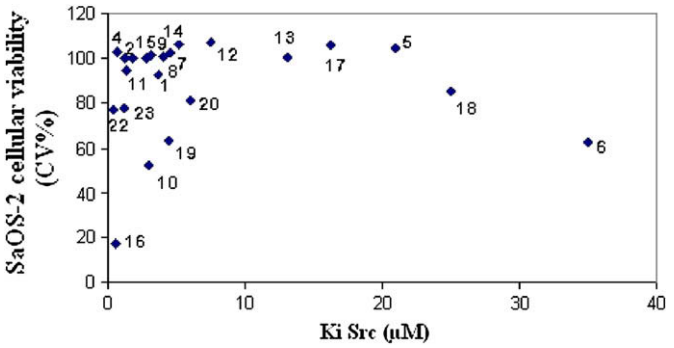


Fig. 1. Correlation between cellular data on SaOS-2 cells expressed as cellular viability (CV%) and enzymatic results (μM) on Src tyrosine kinase.

allows to measure experimental molecular lipophilicity. The LC experiments were performed on a Microsorb RP-column using methanol–water mixtures maintained at a flow rate of 1 ml/min. The substances were prepared as 0.1 mg/ml solutions in methanol. Each capacity factor determination was performed in triplicate and the mean value was used for calculation [22].

Extrapolated retention factors log K_w were derived according to the following equation using at least seven isocratic log K values determined at different concentrations of methanol.

$$\log K = -Sj + \log Kw$$

where j is the methanol fraction. The slope S and the intercept log K_w were generated by linear regression analysis.

2.3.2. Computed lipophilicity

The log P for the compounds was calculated using VolSurf and QikProp software programs and Cerius^2 [AlogP98] descriptor [23–26]. VolSurf log P is computed by mean of linear equation derived by fitting VolSurf descriptor to experimental data on water/octanol partition coefficient [27]. The AlogP98 descriptor is calculated using the method described by Ghose et al. [27]. In this atom-based approach, each atom of the molecule is assigned to a particular class with additive contributions to the total value of log P.

2.4. Cell-free assay with recombinant Abl

Recombinant human Abl was used to investigate the mechanism of kinase inhibition, as previously reported [28]. In detail, activity was measured in a filter-binding assay using an Abl specific peptide substrate (Abltide, Upstate Biotechnology) in the presence of 0.012 μM [γ-^{32}P]ATP, 50 μM peptide, and 0.005 μM c-Abl. The apparent affinity (K_m) of Abl for the peptide and ATP substrates was determined separately (1.5 and 10 μM, respectively). Each experiment was done in triplicate and mean values were used for the interpolation. Curve fitting was performed with the program GraphPad Prism. K_i values toward isolated Abl were calculated according to the following equation: $K_i = ID_{50}/\{E_0 + [E_0(K_{m(ATP)}/S_0)]\}/E_0$, where E_0 and S_0 are the enzyme and the ATP concentrations (0.005 and 0.012 μM, respectively).

Table 2
Experimental PAMPA permeability (P_{app}) and literature data of ten reference drugs.

Compound	$P_{app} \times 10^6$ (cm/s) exp.	$P_{app} \times 10^6$ (cm/s) lit.
Acetaminophen	0.300	3.50
Caffeine	1.770	10.80
Carbamazepine	1.580	11.30
Chloramphenicol	0.092	1.70
Cephalexin	0.015	0.10
Furosemide	0.002	0.60
Ketoprofen	0.410	2.84
Propanolol	0.210	0.96
Theophylline	0.560	4.80
Warfarin	0.015	0.50
Correlation between exp. and literature		0.985

3. Results

In Fig. 1 correlation between the biological data on Src inhibition (K_i) and antiproliferative activity on human osteogenic sarcoma (SaOS-2) cells (expressed as cellular viability, CV%) for the 23 tyrosine kinase inhibitors is reported. One of the possible explanations for lack of correlation between data is that compounds may vary in cell membrane permeability and we started our study by examining whether cellular permeability using PAMPA system and other physico-chemical properties could explain this behaviour.

3.1. Artificial membrane permeability assay

Many *in vitro* systems have been used for understanding the mechanism of membrane permeability. In contrast to the Caco-2 system, the PAMPA system represents a simpler passive permeability model. Its utility has been suggested in predicting human absorption, as a screening model for blood–brain barrier permeability and as a model for evaluating the permeability indicating that PAMPA is a relative new tool in drug discovery and the complexity of the passive membrane permeability could be easily overcome [29,30].

Some well known experiences of PAMPA are described in literature; for example Kansy proposed a model membrane permeation assay consisting of filters coated with alkane (dodecane, hexadecane, 1,9-decadiene) solution of phosphatidylcholine [5].

In this paper we describe an approach to measure membrane permeability of our drug candidates.

To overcome the poor water solubility of the compounds under study we increased the amount of DMSO in the donor wells; the same amount of DMSO was added also in acceptor wells. The procedure was validated considering a set of well-described drugs from literature (correlation between experimental and literature data = 0.985) [7]. Results reported in Table 2 showed a decrease in permeability when the co-solvent was added, as demonstrated by the shift of trend of obtained data. This result can be due to the increase of DMSO in the donor and in the acceptor wells; in fact, as suggested by Sugano, the permeability was decreased by all co-solvents in the case of some different drugs [31].

One reason is suggested to be the increased affinity of these hydrophobic compounds to the water phase by the addition of co-solvents, resulting in a decrease in the partitioning to the lipophilic part of the lipid membrane. However applicability of permeability determination by PAMPA method with high percentage of DMSO for the hydrophobic chemicals is confirmed by various authors [32].

Implementation of new methods enabling rapid screening of membrane penetration properties of drugs is highly desirable in drug development. From this point of view, the proposed method for *in vitro* membrane permeation assay, using a lipophilic artificial membrane with high DMSO percentage in both acceptor and donor wells, is a useful tool for a rapid screening for the same family of compounds.

Permeability through PAMPA was then calculated for each pyrazolo-pyrimidine compound as amount of product diffused from donor side to receiver side as a function of time. The results of the PAMPA experiments in term of P_{app} and $-\log P_{app}$ are reported in

Table 3. The permeability coefficient of the 23 compounds used in this study ranged from 5.40 to 7.07 ($-\log P_{app}$).

3.2. Methods for lipophilicity determination

log *P* was determined using the RPLC method. RPLC retention data, expressed either as log K_w or K_w, have been shown to correlate well with absolute and relative lipophilicity values [33]. The classical shake-flask method, or variations of this method which have been described [34], are neither rugged nor rapid enough for medium to high-throughput applications, generally more sensitive to impurities and less amenable to automation than the RPLC methods.

With a judicious choice of conditions [35], it is possible developing a method that would be accurate, rapid and reproducible. log K_w was obtained by extrapolation of log retention factor (k) to 0% organic modifier measured in RPLC. log K_w values were obtained from the slopes and intercepts of these relationships. The slopes S of Eq. (1) usually correlate with the extrapolated retention factors log K_w.

$$\log K = -Sj + \log K_w \quad (1)$$

The good linearity between S and log K_w may be considered as an indicative measure of uniformity in the retention mechanism. The reversed-phase retention parameters and the retention factor obtained are listed in Table 4. Correlation coefficients were higher than 0.981. Standard deviations of extrapolated log K_w values were within ±0.03. log *P* was calculated with three software programs, Cerius^2 [23], VolSurf [24] and QikProp [25], and the results are reported in Table 3 with log K_w data.

3.3. Correlation analysis

Regression analysis was performed to evaluate the relationship between experimental data obtained by PAMPA, log K_w and the computed log *P*.

Table 3
Permeability values (apparent permeability P_{app}), experimental (log K_w) and *in silico* (log *P* VolSurf, AlogP98 Cerius^2, log $P_{o/w}$ QikProp) lipophilicity, for the tested compounds.

Compd	$P_{app} \times 10^6$ (cm/s)	$-\log P_{app}$	log K_w	log *P*	AlogP98	log $P_{o/w}$
				VolSurf	Cerius^2	QikProp
1	3.98	5.40	5.49	5.39	5.23	6.13
2	3.31	5.48	5.34	5.36	5.39	6.00
3	3.09	5.51	6.07	4.99	5.44	6.73
4	2.57	5.59	6.01	5.77	5.55	6.34
5	2.51	5.60	5.88	5.08	5.44	6.69
6	2.39	5.62	5.48	4.75	5.44	6.58
7	1.99	5.70	6.05	5.02	5.44	6.73
8	1.50	5.82	5.80	5.50	5.54	6.80
9	1.44	5.84	5.94	5.63	5.90	6.75
10	1.35	5.87	6.03	5.33	5.90	6.55
11	1.26	5.90	5.87	4.98	5.60	6.26
12	1.26	5.90	6.28	5.89	5.90	7.06
13	0.87	6.06	6.44	5.51	5.76	6.94
14	0.77	6.11	6.16	6.02	6.22	6.98
15	0.74	6.13	6.54	6.10	6.22	6.94
16	0.72	6.14	6.76	5.32	6.05	6.33
17	0.56	6.25	7.04	6.02	6.04	7.02
18	0.56	6.25	7.41	6.25	6.22	7.20
19	0.49	6.31	6.69	5.62	6.34	6.81
20	0.46	6.34	5.94	6.17	6.22	7.20
21	0.44	6.35	6.37	5.80	6.14	6.59
22	0.26	6.58	6.75	5.90	6.40	6.87
23	0.08	7.07	7.40	6.08	6.93	7.26

Table 4
Reversed-phase retention parameters and retention factors obtained.

Compd	Equation $\log K = -Sj + \log K_w$		
	S	log K_w	r^2
1	−0.06	5.49	0.998
2	−0.06	5.34	0.995
3	−0.07	6.07	0.993
4	−0.06	6.01	0.993
5	−0.06	5.88	0.990
6	−0.06	5.48	0.990
7	−0.07	6.05	0.992
8	−0.06	5.80	0.996
9	−0.06	5.94	0.996
10	−0.06	6.03	0.992
11	−0.06	5.87	0.998
12	−0.07	6.28	0.997
13	−0.07	6.44	0.990
14	−0.06	6.16	0.998
15	−0.07	6.54	0.997
16	−0.07	6.76	0.989
17	−0.07	7.04	0.986
18	−0.08	7.41	0.983
19	−0.07	6.70	0.997
20	−0.06	5.94	0.993
21	−0.07	6.37	0.997
22	−0.07	6.75	0.997
23	−0.07	7.39	0.999

High r^2 values ($p < 0.05$) were observed between these parameters suggesting a statistically good correlation (Table 5).

The good correlation between PAMPA and log K_w suggests that only one of these variables is necessary to describe the passive permeation across the membranes for the selected compound.

Our results also suggest that parameters calculated by Cerius^2 software program are more predictive, at least in this particular family of compounds, than those calculated through Volsurf program or QikProp.

Regression analysis between the above mentioned data and K_i (Src) vs. antiproliferative activity on SaOS-2 cells (CV%) showed a lack of correlation (Table 6). Our results suggest that the lipophilicity and passive diffusion across the membranes do not significantly influence activity of the tested compounds. This trend could be explained by different target of some compounds in our set.

3.4. Cell-free assay with recombinant Abl

The compounds were therefore tested also on Abl tyrosine kinase and some of them turn out to be very active on Abl enzyme (Table 7), some seem to be active on both the enzymes.

In an attempt to correlate all independent variables (K_i Src, K_i Abl, P_{app}, log K_w and log *P*) with the activity on SaOS-2 cells (CV%) multivariate regression analysis was done but no correlation was found, suggesting that other factors are involved in the mechanism of action of pyrazolo-pyrimidine compounds.

Table 5
Correlation between experimental data $-\log P_{app}$, log K_w and the computed log *P*.

	$-\log P_{app}$	log K_w	log *P*	AlogP98	log $P_{o/w}$
			VolSurf	Cerius^2	QikProp
$-\log P_{app}$	1.00				
log K_w	0.81*	1.00			
log *P* VolSurf	0.67*	0.62*	1.00		
AlogP98 Cerius^2	0.96*	0.80*	0.74*	1.00	
log $P_{o/w}$ QikProp	0.65*	0.64*	0.67*	0.63*	1.00

*Significant correlation $p < 0.05$.

Table 6
Correlation between experimental and *in silico* data ($-\log P_{app}$, $\log K_w$, $\log P$ VolSurf, AlogP98 Cerius^2 and $\log P_{o/w}$ QikProp) vs. K_i (Src) and cellular data (activity on SaOS-2 cell expressed as cellular viability, CV%).

	K_i (Src)	CV%
$-\log P_{app}$	−0.16	−0.28
$\log K_w$	0.01	−0.23
$\log P$ VolSurf	−0.18	0.13
AlogP98 Cerius^2	−0.19	−0.29
$\log P_{o/w}$ QikProp	0.25	0.14

Our hypothesis that some compounds could be selective for other kinases was confirmed by results observed on Abl. Regarding Abl inhibition, the most active compounds in the enzymatic assay are **5**, **9**, **21** and **22**.

4. Discussion

In this study permeability of a set of 23 tyrosine kinase inhibitors was examined using PAMPA method. The appropriate conditions for determining the permeability of poorly soluble compounds were examined and $\log K_w$ was set out to develop a method that would be accurate, rapid and reproducible, applicable to a variety of drug-like molecules.

The general trend in the examined compounds was that hydrophobic compounds had lower permeability whereas polar compounds had higher permeability in the PAMPA system.

Our study is consistent with previous work, where such unusual behaviour has been already reported by different authors when very lipophilic compound ($\log P_{oct\text{-}buffer,}$ pH 7.4 > 3.5) have been tested with PAMPA and Caco-2 systems [31,36–38].

Analysis of the data obtained by $-\log P_{app}$, $\log K_w$ and the computed $\log P$ shows good correlation between experimental and *in silico* data. Our results suggest that parameters calculated by Cerius^2 software are more predictive on this kind of compounds than those calculated through VolSurf and QikProp. When Cerius^2 AlogP98 data were compared with the PAMPA permeability by linear regression analysis, a good correlation was found ($r^2 = 0.96$, $p < 0.05$).

The set of compounds was therefore tested also on Abl tyrosine kinase and some of them turn out to be very active on Abl enzyme (Table 6), resulting to be Src–Abl dual inhibitors.

Our study suggests therefore the existence of compounds not only specific for Src tyrosine kinase but also for Abl tyrosine kinase, even if more detailed studies are necessary to clarify the mechanism of action of pyrazolo-pyrimidine compounds in cells and the reason why high permeability of some compounds, even if with good enzymatic activity, did not express good cellular activity.

The differences in permeability obtained by PAMPA assay did not justify the poor cellular activity of those compounds showing good enzymatic activity. Through PAMPA was individuated a range (5.9–6.3) in which these tyrosine kinase inhibitors showed a log P_{app} which are important for the cellular activity purpose. In an attempt to correlate all variables (K_i Src, K_i Abl, P_{app}, $\log K_w$ and $\log P$) with the activity on SaOS-2 cells (CV%) multivariate regression analysis was done but no correlation was found, suggesting that other tyrosine kinases may be involved in the mechanism of action of these compounds, as suggested by us in other papers [4,28,39,40], mainly based on the structural homology among kinases [16]. Correlation, using inhibition data from a panel of the following tyrosine kinases, HER-1, KDR, Flt-3, IGF-1R, Tek, c-Met, Ret, JAK-2, EphB4, FGFR-3-K650E, Axl, FAK, PKA, CDK2/A, Akt, PDK1, and B-Raf-V599E on compounds **1**, **3**, **5**, **9**, **15**, **19**, **20** and **22**, did not afford the desired results, but it is well known that many more TKs exist in a cell and the network among them is very complicated and not fully understood at the moment.

In conclusion, this careful study on very lipophilic dual Src–Abl inhibitors, going from solubility to PAMPA, does not explain satisfactorily the addressed question (lack of correlation between enzymatic an cell data), suggesting that our products, at least in this particular environment (SaOS2 cells) somehow prefer to remain in the cell membrane (not clear where, but studies are ongoing) than to completely cross it and finally address the TKs present in the cell.

However, despite the identification of different signalling cascades activated by TKs in tumor cells, the total biological effects deriving from the inhibition of a single enzymatic pathway are not predictable; this will be the major focus of system biology and will be subject matter of studies in the near future.

Table 7
Activity on Abl tyrosine kinase of tested compounds.

Compd	K_i [a] Abl (μM)
1	0.26 ± 0.03
2	0.40 ± 0.05
3	0.32 ± 0.04
4	7.03 ± 1.20
5	0.08 ± 0.02
6	0.25 ± 0.03
7	0.10 ± 0.01
8	0.30 ± 0.10
9	0.09 ± 0.01
10	0.11 ± 0.02
11	0.40 ± 0.07
12	0.13 ± 0.03
13	0.23 ± 0.05
14	0.32 ± 0.05
15	0.11 ± 0.03
16	0.60 ± 0.04
17	0.37 ± 0.02
18	0.35 ± 0.03
19	0.15 ± 0.01
20	0.20 ± 0.02
21	0.06 ± 0.01
22	0.04 ± 0.01
23	0.15 ± 0.06

[a] K_i (expressed as a μM concentration) toward isolated Abl was calculated according to the following equation: $K_i = ID_{50}/\{E_0 + [E_0(K_{m(ATP)}/S_0)]/E_0\}$, where E_0 and S_0 are the enzyme and the ATP concentration (0.005 and 0.012 μM, respectively). Each experiment was in triplicate, and mean values were used for the interpolation.

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