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Research Article

# High-performance liquid chromatography-based evaluation of lipophilicity of a series of novel stilbene derivatives

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## Abstract

Lipophilicity is related to physicochemical, metabolic, and toxicity properties of the drugs. The present study reports a reversed-phase high-performance liquid chromatographic method, RP-HPLC (stationary phase: LiChrosorb RP-18; mobile phase: acetonitrile-water or methanol-water) in isocratic elution mode for determination of the lipophilic behavior of thirty-one newly synthesized combretastatin CA-4 derivatives. Lipophilicity was estimated through log P using the chromatographic descriptor log $k_w$. The quadratic and linear relationships were established between the logarithm of the capacity factor for each compound and the concentration of organic modifier in the mobile phase (φ). These two regression models were evaluated using Mandel's test. The relationship between the chemical structure of newly stilbene derivatives and log P was described. The correlations between the chromatographic lipophilicity and *in vitro* cytotoxic activity against three different cell lines were examined.

## Keywords

combretastatin's analogues, log P, retention behavior

## Introduction

The efficiency of the treatment of a disease depends on the physicochemical properties of the drugs. An essential property of a compound is lipophilicity. Lipid solubility is one of the most important parameters since it considerably influences the biological activity of the compounds. The lipophilicity of the therapeutic drug plays a main role in the process of the absorption, the transport from the site of application to the next compartment and the transport to deeper compartments (distribution), biotransformation (metabolism), and elimination from the body (ADME).

The effective penetration of cell membranes is the first barrier that medications overcome. The hydrophobic effect is assumed to be one of the driving forces of the passive transport of drugs through biological membranes and a component of drug-receptor binding (Gies and Landry 2003; Poole and Poole 2003). Matyszewska et al. (2023) proved that there are other aspects than lipophilicity that determine the drug-lipid interactions, such as charge, ability to form polymers, as well as drug structure, the latter leading to a possible steric hindrance. Morak-Mlodawska et al. (2023) also confirm that other properties than lipophilicity affect the bioavailability.

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Fujita et al. first proposed the octanol-water partition coefficient ($P_{o/w}$) as a good model for biological partitioning. Since then, the logarithm of the octanol-water partition coefficient (log $P_{o/w}$) has become the most widely used lipophilicity parameter (Pliška et al. 1996; Poole and Poole 2003; Valkó 2004). Lipophilicity, log $P_{o/w}$, is also used as one of the standard properties identified by Lipinski in the "Rule of Five" for drug-like molecules (Pillai et al. 2001; Moreno et al. 2011; Benet et al. 2016; Katz et al. 2022). Log $P_{o/w}$ can be determined through practical and computational methods. The classic "shake flask" method (octanol/water partitioning), a direct method, and indirect separation experimental techniques like chromatographic methods, immobilized artificial membranes, and electrokinetic have been developed for this purpose (Poole and Poole 2003; Katz et al. 2022; Coutinho et al. 2023). The determination of the partition coefficient by the "shake-flask" equilibration method is associated with some difficulties, such as long time for experiments and poor reproducibility. The procedure was replaced by the indirect techniques such as chromatographic methods.

HPLC is usually performed with nonpolar bonded reverse-phase columns and a water-containing mobile phase. Biological partitioning of compounds in aqueous biphasic systems, such as blood and various tissues, is similar to the chromatographic partition process because of the vast area of contact surfaces in both systems (Nasal et al. 2003; Poole and Poole 2003; Valkó 2004). The partitioning from the aqueous/organic mobile phase into the stationary phase can be used as a direct measure of lipophilicity using log *k* (González et al. 1995; Grouls et al. 1997; Cimpan et al. 2000; Paschke et al. 2001; Musiol et al. 2007; Pallicer et al. 2010; Han et al. 2012; OECD 2022). The determination of log P with RP-HPLC is based on the construction of a correlation model between a retention property characteristic of the solute and the separation system for a training set of solutes with known log $P_{o/w}$ values. Further measurements of the retention property characteristic in the separation system can then be used to estimate the log P values for other compounds (Rezaee et al. 2009; Moreno et al. 2011; Coutinho et al. 2023). Other different parameters of hydrophobicity determined with HPLC methods include chromatographic hydrophobicity indices (CHI), ElogD, alphalogD, and $\varphi_0$ (Valkó 2004; Ilijaš et al. 2013; Katz et al. 2022).

Combretastatins are a class of the natural *cis*-stilbenes that consist of two substituted aromatic rings, ring A and ring B, linked by an olefinic bridge. They are isolated from the African willow tree *Combretum caffrum* (Combretaceae). It is known that they inhibit the growth of cancer cells. The most potent compound of this group is combretastatin A4, CA-4, Fig. 1. The compound is a powerful microtubule-destabilizing agent that inhibits microtubule assembly by binding to tubulin at the colchicine binding site. The main limitations of CA-4's clinical utility are its low solubility and poor bioavailability in biological media, which significantly impair its *in vivo* activity. As a result, CA-4 has served as a lead molecule for the design of combretastatin analogues as potential anticancer agents (Gerova et al. 2016; Bukhari et al. 2017; Hamze et al. 2020).

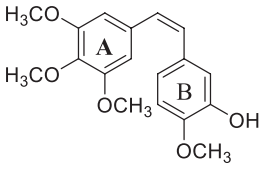


**Figure 1.** Structure of combretastatin, CA-4.

The presence of a *cis*-configuration double bond in the bridge in CA-4 is fundamental for achieving high cytotoxicity. The 3,4,5-trimethoxysubstituted A-ring is also very important for the substantial cytotoxic activity and efficient binding at the colchicine binding site of the microtubule. Therefore, the B-ring structure is the one susceptible to useful modification (Mikstacka et al. 2013; Gerova et al. 2016; Bukhari et al. 2017). The benzoxazolone heterocycle is a bioisosteric replacement of the B-ring. This scaffold is an important building block due to its wide range of physiological effects. Therefore, of interest is the investigation of the biological properties of the novel CA-4/benzoxazolones hybrids, Fig. 2.

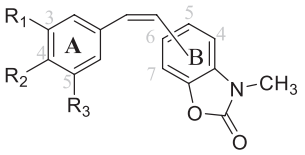


**Figure 2.** General formula of styrylbenzoxazolones. $R_i$ – H or $H_3CO$.

The structures of studied *cis*- and *trans*-styrylbenzoxazolones are shown in Table 1. These are newly synthesized compounds in the Faculty of Chemistry and Pharmacy (Sofia University). Their synthesis and structures have been reported previously. The *in vitro* cytotoxicity of the combretastatin A-4 analogues against different cell lines was examined (Gerova et al. 2016). In our study, we used code names for the newly synthesized benzoxazolones. These names indicate the position of substitution in the benzoxasolone fragment (4, 5, 6, or 7) as well as the number and position of the methoxy groups in ring A (1/2, 3/4, 5/6, 7/8).

There are numerous reports on the relationship between biological activity and lipophilicity for the different classes of compounds. The aim of this study was to assess the lipophilicity of the novel combretastatin analogues. We used the RP-HPLC method to determine the log P with methanol-water and acetonitrile-water as mobile phases. Structure-activity relationships (SAR) are discussed in this work, as well as the relationship between the lipophilicity and the chemical structure of the studied compounds. The experimental lipophilicity data have been compared with *in vitro* examined cytotoxicity of styrylbenoxazolones against different cell lines.

**Table 1.** Structure of styrylbenzoxazolones.

| Compound | Styryl fragment - position in ring B | Ring A | | |
|---|---|---|---|---|
| | | $R_1$ | $R_2$ | $R_3$ |
| S41-Z | 4 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S42-E | 4 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S43-Z | 4 | $OCH_3$ | H | $OCH_3$ |
| S44-E | 4 | $OCH_3$ | H | $OCH_3$ |
| S45-Z | 4 | $OCH_3$ | $OCH_3$ | H |
| S46-E | 4 | $OCH_3$ | $OCH_3$ | H |
| S47-Z | 4 | H | $OCH_3$ | H |
| S48-E | 4 | H | $OCH_3$ | H |
| S51-Z | 5 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S52-E | 5 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S53-Z | 5 | $OCH_3$ | H | $OCH_3$ |
| S54-E | 5 | $OCH_3$ | H | $OCH_3$ |
| S55-Z | 5 | $OCH_3$ | $OCH_3$ | H |
| S56-E | 5 | $OCH_3$ | $OCH_3$ | H |
| S57-Z | 5 | H | $OCH_3$ | H |
| S58-E | 5 | H | $OCH_3$ | H |
| S61-Z | 6 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S62-E | 6 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S63-Z | 6 | $OCH_3$ | H | $OCH_3$ |
| S64-E | 6 | $OCH_3$ | H | $OCH_3$ |
| S65-Z | 6 | $OCH_3$ | $OCH_3$ | H |
| S66-E | 6 | $OCH_3$ | $OCH_3$ | H |
| S67-Z | 6 | H | $OCH_3$ | H |
| S71-Z | 7 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S72-E | 7 | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| S73-Z | 7 | $OCH_3$ | H | $OCH_3$ |
| S74-E | 7 | $OCH_3$ | H | $OCH_3$ |
| S75-Z | 7 | $OCH_3$ | $OCH_3$ | H |
| S76-E | 7 | $OCH_3$ | $OCH_3$ | H |
| S77-Z | 7 | H | $OCH_3$ | H |
| S78-E | 7 | H | $OCH_3$ | H |

# Materials and methods

## Chemicals and materials

The compounds from the calibration set are listed in Table 2. All reagents were primary reference standard grade (≥ 96% purity HPLC) and were purchased from Merck KGaA (Darmstadt, Germany).

HPLC-grade methanol and acetonitrile were supplied by Merck KGaA (Darmstadt, Germany). Deionized water used in this study was purified using a Mili-Q system (Milipore, Bedford, MA, USA).

Stock solutions of reference substances and test benzoxazolones were prepared in acetonitrile-methanol (1:1, v/v) because of the low solubility of some compounds. The injection concentration was 0.2 mg/ml in the working organic modifier (acetonitrile or methanol).

## Methods

### *Instrumental and chromatographic conditions*

The experimental data were acquired using an HPLC system (Konik Tech 560) equipped with a thermostat ($T_{col}$ 35 °C). A Diod Array UV detector (Konik DAD 560) was employed to measure the retention of test components. DAD was operated in full acquisition mode over the 200–600 nm interval, with chromatograms simultaneously recorded at 254, 274, 292, and 305 nm analytical wavelengths. The absorption maximum of *Z*-isomers is between 274 and 300 nm, and of *E*-isomers is between 302 and 336 nm.

Retention studies were conducted using LiChrosorb RP-18 200 mm × 4.6 mm × 5 mm (Hewlett Packard). Isocratic compositions of the mobile phase were used for the experiments. The eluents were mixtures of methanol or acetonitrile as organic modifiers and water, starting with 95% to 60% organic solvent (φ) – 95, 90, 80, 70, 60% (v/v). The flow rate was 0.75 ml/min in all cases. The injection volume was 10 µL. Each measurement was performed in triplicate, and the mean was used in further calculations. The void volume was measured by injecting a solute of the unretained compound potassium nitrate, $KNO_3$.

## Retention characteristics

The most frequently used parameter of retention in HPLC is the capacity factor, *k*. The *k* value is determined from the retention time, calculated according to the expression:

$$k = (t_R - t_0) / t_0 \qquad \text{Eq. 1}$$

where $t_R$ is the retention time of the analyzed compound

$t_0$ is the void time of the column used.

In RP-HPLC there is a linear correlation (Snyder-Soczeminski equation) between the retention of the analyte expressed as log *k* and the percentage of the organic modifier in the mobile phase (φ) for binary water-organic eluents:

$$\log k = \log k_w - S\varphi \qquad \text{Eq. 2}$$

where *k* is the retention factor of the analyzed compound,

$k_w$ is a value of *k* of the analyte extrapolated to the mobile phase containing 100% water, which represents the commonly employed chromatographic hydrophobicity parameter.

S is a solute-dependent solvent strength parameter. It is considered to be related to the specific hydrophobic surface area of the solute. (Poole and Poole 2003; Valkó 2004; Han et al. 2012; Coutinho et al. 2023).

A better correlation between log *k* and φ can be further described by the quadratic relationship (Ba¸czek et al. 2000; Poole and Poole 2003; Valkó 2004; Tache et al. 2012):

$$\log k = \log k_w + B\varphi + A\varphi^2 \qquad \text{Eq. 3}$$

where *k* is the analyte retention factor

A and B are constants for a given analyte and a reversed-phase system.

φ is the volume fraction of the stronger solvent in the mobile phase.

$k_w$ is the value of *k* extrapolated to 100% water as the mobile phase (φ = 0).

The quadratic term is often explained through the perturbation of the retention behavior of the analytes on a modified hydrophobic stationary phase when using a water-rich mobile phase. For values of φ greater than 30%, this model often gives a more appropriate fitting than the linear model, especially with a mobile phase comprised of acetonitrile as the organic modifier (Valkó 2004).

Log $k_w$ is widely accepted as the most reproducible and reliable lipophilicity descriptor derived from chromatographic retention data. The values are most often used as measures of hydrophobicity from RP-HPLC. Log $k_w$ values can predict log P only for nonhydrogen-bonding or weak hydrogen-accepting substances (Ba¸czek et al. 2000).

## Statistical analysis

IUPAC recommends Mandel's test to check the best fit for the calibration curve (linear or quadratic) of each analyte (Danzer and Currie 1998; Andrade and Gomez-Carracedo 2013; ISO 8466-1:2021 2021). This test compares the residual standard deviations of nonlinear and linear models using a Fisher' F-test. All regression analyses and statistical evaluations were performed via Microsoft Excel on a custom-made calculator. The statistical data for the P value were calculated for a 95% confidence limit.

# Results and discussion

## Correlation between log $P_{o/w}$ and log $k_w$ for the reference compound

In this study, 12 compounds for mobile phase $CH_3CN/H_2O$ and 16 compounds for mobile phase $CH_3OH/H_2O$ have been selected as reference compounds (Table 2). Some of the chosen compounds of the calibration set possess similar fragments to those of the test compounds, while others have different structures that allow for building a model covering a wide range of organic fragments and log $P_{o/w}$ values (from 1.1 to 4.88). It is important that the structures of the reference set and the samples are similar; otherwise, incorrect estimates of the partition coefficient can be obtained (Poole and Poole 2003).

The solutions of each reference compound were analyzed. The chromatographic activity was performed under isocratic conditions using a non-polar C18 stationary phase. After determining the void volume with a solution of $KNO_3$, the capacity factors ($k$) of the reference compounds were calculated according to Eq. 1. The log $k$ values have been extrapolated to 0% organic modifier ($CH_3OH$ or $CH_3CN$) to determine the capacity factor represented as log $k_w$. The log $k_w$ values were computed through linear and quadratic regression models. The value of coefficient A was too small in all the nonlinear equations ($CH_3CN/H_2O$ 0.00004–0.0003 and $CH_3OH/H_2O$ 0.00008–0.0004). The log $k_w$ values for the reference compounds, determined through the two different regression models, were evaluated using Mandel's test. The test indicated that a linear model is better than a quadratic function for benzyl alcohol and 1,2-dimethoxybenzene for $CH_3CN/H_2O$ and 1,4-dimethoxybenzene, CA-4, ethylbenzene, and pyrene for the $CH_3OH/H_2O$ mobile phase, as shown in Table 2. Therefore, we used the log $k_w$ values confirmed from Mandel's test in further calculations. We supposed stronger interactions between compounds with a nonlinear model and stationary phase, probably due to the free silanol groups.

**Table 2.** Log $P_{o/w}$ and log $k_w$ of the reference compounds used for calibration. RM: regression model; lin—linear, non—nonlinear (quadratic). Source of log $P_{o/w}$ values: the open chemistry database at the National Library of Medicine. Source of log $P_{o/w}$ for combretastatin: Drug Bank Online.

| Calibration compounds | log $P_{o/w}$ | acetonitrile-water | | methanol-water | |
|---|---|---|---|---|---|
| | | log $k_w$ | type RM | log $k_w$ | type RM |
| Benzene | 2.13 | 1.89 | non | 2.83 | non |
| Toluene | 2.73 | 2.65 | non | 3.53 | non |
| Naphthalene | 3.3 | 2.97 | non | 4.66 | non |
| Anthracene | 4.45 | 3.41 | non | 6.53 | non |
| Phenanthrene | 4.46 | 3.61 | non | 4.81 | non |
| Pyrene | 4.88 | 3.53 | non | 3.98 | lin |
| Benzaldehyde | 1.48 | 3.09 | non | 2.64 | non |
| Benzyl alcohol | 1.1 | 1.69 | lin | 3.36 | non |
| Methoxybenzene (anisole) | 2.11 | 1.96 | non | 2.80 | non |
| 1,2-Dimethoxybenzene | 1.6 | 0.78 | lin | 1.98 | non |
| 1,4-Dimethoxybenzene | 2.04 | 1.77 | non | 1.55 | lin |
| Combretastatin CA-4 | 3.32 | 2.59 | non | 2.43 | lin |
| 2-Chlorophenol | 2.15 | | | 1.80 | non |
| 2,6-Dimethoxyphenol | 1.15 | | | 3.07 | non |
| Ethylbenzene | 3.15 | | | 0.86 | lin |
| Phenol | 1.46 | | | 1.29 | non |

A calibration plot of log $P_{o/w}$ of the reference compounds versus the calculated log $k_w$ values from the HPLC experiment was established for both mobile phases. The log $P_{o/w}$ values were taken from DrugBank Online (DrugBank Online 2024) for combretastatin and from the open chemistry database at the National Library of Medicine (PubChem 2024) for the other compounds in the calibration set. The correlation was calculated using the least-squares linear regression. The calibration curves were linear (Fig. 3):

For the mobile phase acetonitrile-water:

$$\log P = 1.141 * \log k_w - 0.048 \quad \text{Eq. 4}$$

For the mobile phase methanol-water:

$$\log P = 0.493 * \log k_w + 1.111 \quad \text{Eq. 5}$$

The P and R values were calculated as well. A statistically significant correlation was observed between the values from the database and the chromatographic determination:

For the mobile phase acetonitrile-water: P = 0.002, R = 0.79

For the mobile phase methanol-water: P = 0.015, R = 0.60.

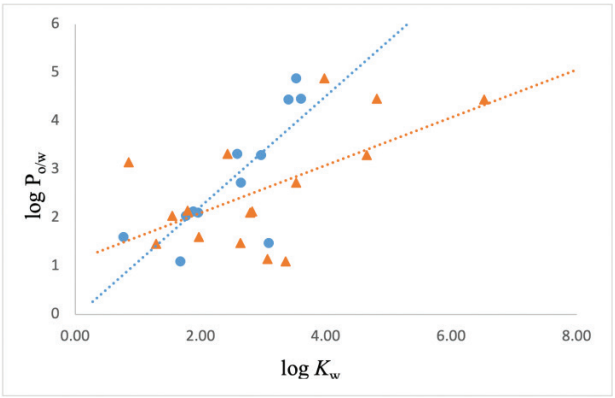


**Figure 3.** Log $P_{o/w}$ calibration curves for mobile phase $CH_3CN/H_2O$ (circles) and $CH_3OH/H_2O$ (triangles).

This good correlation indicated that our systems were suitable to estimate log P values for new compounds.

The log P values of CA-4 were calculated according to established dependencies: for $CH_3CN$-$H_2O$, it is 2.91, and for $CH_3OH$-$H_2O$, it is 3.12. The value from the database (DrugBank Online) is log $P_{o/w}$ 3.32. The result of the calculation of log P (CA-4) from the model with $CH_3OH$-$H_2O$ is more similar to the literature value than $CH_3CN$-$H_2O$.

## Determination of log P of styrylbenzoxazolones with HPLC

The 31 styrylbenzoxazolones shown in Table 1 were analyzed using the same isocratic RP-HPLC method as the calibration compounds. Log $k_w$ was determined for both mobile phases through linear and quadratic regression models. The values of log $k_w$ and the results of Mandel's test are listed in Table 3.

Log $k_w$ can be used as a lipophilicity index converted to a log P scale as well. The equations of calibration curves from reference substances were applied to determine the test compounds log P at both mobile phase conditions—Eq. 4 for acetonitrile-water and Eq. 5 for methanol-water. The results are shown in Table 3. The values of log P for acetonitrile-water were between 1.20 and 3.97 and for methanol-water between 2.14 and 4.55. They were in the recommended interval between 0 and 6 by the OECD Guideline for the Testing of Chemicals for estimated log P with RP-HPLC (Coutinho et al. 2023; OECD 2022).

## Relationship between the structure of the analyzed combretastatin analogues and log P from RP-HPLC

The value of HPLC log P was higher for the *E*-isomers than *Z*-isomers analyzed with acetonitrile-water mobile phase for all tested pairs, as shown in Table 3. The exception to this was observed in the S45-Z/S46-E, S57-Z/S58-E, and S61-Z/S62-E pairs. The *Z*-isomers in listed couples showed higher log P values. The calculated lipophilicity for S45-Z and S61-Z was significantly higher than for their respective *E*-isomers. All *E*-isomers with two methoxy groups at positions 3 and 5 in ring A showed a higher lipophilicity compared to their *Z*-geometric isomers.

**Table 3.** The chromatographic lipophilicity indices and the type of regression model of the investigated compounds. [a] RM: regression model; lin—linear, non—nonlinear (quadratic).

| Compound | $CH_3CN$-$H_2O$ | | | $CH_3OH$-$H_2O$ | | |
|---|---|---|---|---|---|---|
| | log $k_w$ | type RM[a] | log P | log $k_w$ | type RM[a] | log P |
| S41-Z | 1.10 | lin | 1.20 | 4.61 | non | 3.38 |
| S42-E | 1.19 | lin | 1.31 | 5.42 | non | 3.78 |
| S43-Z | 2.99 | non | 3.37 | 6.69 | non | 4.41 |
| S44-E | 3.36 | non | 3.79 | 6.97 | non | 4.55 |
| S45-Z | 2.58 | non | 2.90 | 4.72 | non | 3.44 |
| S46-E | 1.24 | lin | 1.37 | 5.68 | non | 3.91 |
| S47-Z | 3.12 | non | 3.51 | 5.75 | non | 3.95 |
| S48-E | 3.33 | non | 3.75 | 6.49 | non | 4.31 |
| S51-Z | 2.88 | non | 3.23 | 5.09 | non | 3.62 |
| S52-E | 2.96 | non | 3.33 | 5.76 | non | 3.95 |
| S53-Z | 3.35 | non | 3.78 | 6.11 | non | 4.12 |
| S54-E | 3.43 | non | 3.87 | 6.36 | non | 4.25 |
| S55-Z | 2.75 | non | 3.09 | 2.08 | lin | 2.14 |
| S56-E | 2.94 | non | 3.31 | 2.20 | lin | 2.19 |
| S57-Z | 3.32 | non | 3.75 | 6.09 | non | 4.12 |
| S58-E | 3.13 | non | 3.53 | 6.59 | non | 4.36 |
| S61-Z | 2.91 | non | 3.27 | 5.24 | non | 3.70 |
| S62-E | 1.39 | lin | 1.53 | 5.42 | non | 3.79 |
| S63-Z | 3.27 | non | 3.69 | 6.06 | non | 4.10 |
| S64-E | 3.52 | non | 3.97 | 6.23 | non | 4.18 |
| S65-Z | 2.77 | non | 3.12 | 5.19 | non | 3.67 |
| S66-E | 2.81 | non | 3.16 | 5.91 | non | 4.03 |
| S67-Z | 1.38 | lin | 1.53 | 5.85 | non | 4.00 |
| S71-Z | 2.52 | non | 2.83 | 4.92 | non | 3.54 |
| S72-E | 3.13 | non | 3.53 | 5.75 | non | 3.95 |
| S73-Z | 3.18 | non | 3.58 | 5.15 | non | 3.65 |
| S74-E | 3.45 | non | 3.89 | 6.78 | non | 4.46 |
| S75-Z | 2.68 | non | 3.01 | 4.61 | non | 3.38 |
| S76-E | 2.89 | non | 3.25 | 6.23 | non | 4.19 |
| S77-Z | 3.18 | non | 3.58 | 5.43 | non | 3.79 |
| S78-E | 3.38 | non | 3.81 | 5.79 | non | 3.97 |

All *E*-isomers showed higher lipophilic indices than their *Z*-benzoxazolones derivatives with a methanol-water mobile phase. The values of log P for 3,5-dimethoxy substituted ring A with *Z*-structure were close to those for *E*-geometric isomers. The most significant differences in lipophilicity between the *Z*- and *E*- isomers were observed for the analogues with the styryl fragment at position 7 in ring B. We supposed less steric hindrance between the two cyclic fragments (phenyl and benzoxazone) and the most stable isomers in these compounds.

The lipophilicity of a drug is related to its ability to cross cell membranes by means of passive diffusion. Higher lipophilicity is associated with easier passage through membranes. The retention in RP-HPLC is related to the penetration of the molecules between the nonpolar chains of the stationary phase. This process is greatly facilitated if the molecules have a more planar, more elongated structure or nonpolar fragments. Greater retention and higher values for HPLC log P were observed for the *E*-isomers of the tested styrylbenzoxazolones in both chromatographic systems. Analytes with two methoxy groups at the third

and the fifth position in ring A showed the highest relative lipophilicity in both mobile phases used. This fact is probably caused by the absence of the methoxy group at C4 in ring A. All CA-4 analogues with three methoxy groups are less lipophilic than compounds with one or two polar groups at C3 and C5 positions in ring A in our study, Table 3. The higher polarity of a compound with three methoxy groups is related to their lower lipophilicity in both chromatographic systems.

## Correlation between RP-HPLC log P values for Z-isomers and biological activity

*Z*-isomers of CA-4 analogues were used for biological assays. The *in vitro* toxicity of styrylbenzoxazolones against different cell lines—HepG2, EA.hy926, and K562 cells—was examined. The results were reported previously; see Table 3 in Gerova et al. (2016). The *E*-styrylbenzoxazolones were not active at concentrations below 50 μM. Stilbene derivative S61-Z, (*Z*)-3-methyl-6-(3,4,5-trimethoxystyryl)-2(3H)-benzoxazolone, showed the highest antiproliferative and proapoptotic potential of the series. This compound was found to exhibit a stronger cytotoxic effect than CA-4, even against cells that are relatively resistant to other anticancer agents and CA-4.

The natural stilbene CA-4 is part of our calibration set, Table 2. The HPLC-calculated log P ($CH_3CN$) value was 2.59, and the log P ($CH_3OH$) value was 2.43. The log P values for S61-Z determined with both chromatographic systems were similar, for acetonitrile-water 3.27 and for methanol-water 3.70, Table 3. Log P values for S61-Z were greater than those of chromatographic log P for CA-4. The higher lipophilicity of S61-Z may be correlated with better bioavailability and stronger action against tumor cells, in our opinion. These results suggest that the synthesis of this new compound and replacement of ring B in CA-4 with a benzoxazolone scaffold is a useful approach.

Since the log $P_{o/w}$ is an important parameter of biological activity, a correlation with the antitumor activity of the studied compounds is looked for. The relationship between $IC_{50}$ for all tested cell lines and log P from two HPLC methods is shown in Fig. 4. The P and R values were calculated. It was verified that there is not a strong relationship between lipophilicity and activity in this study.

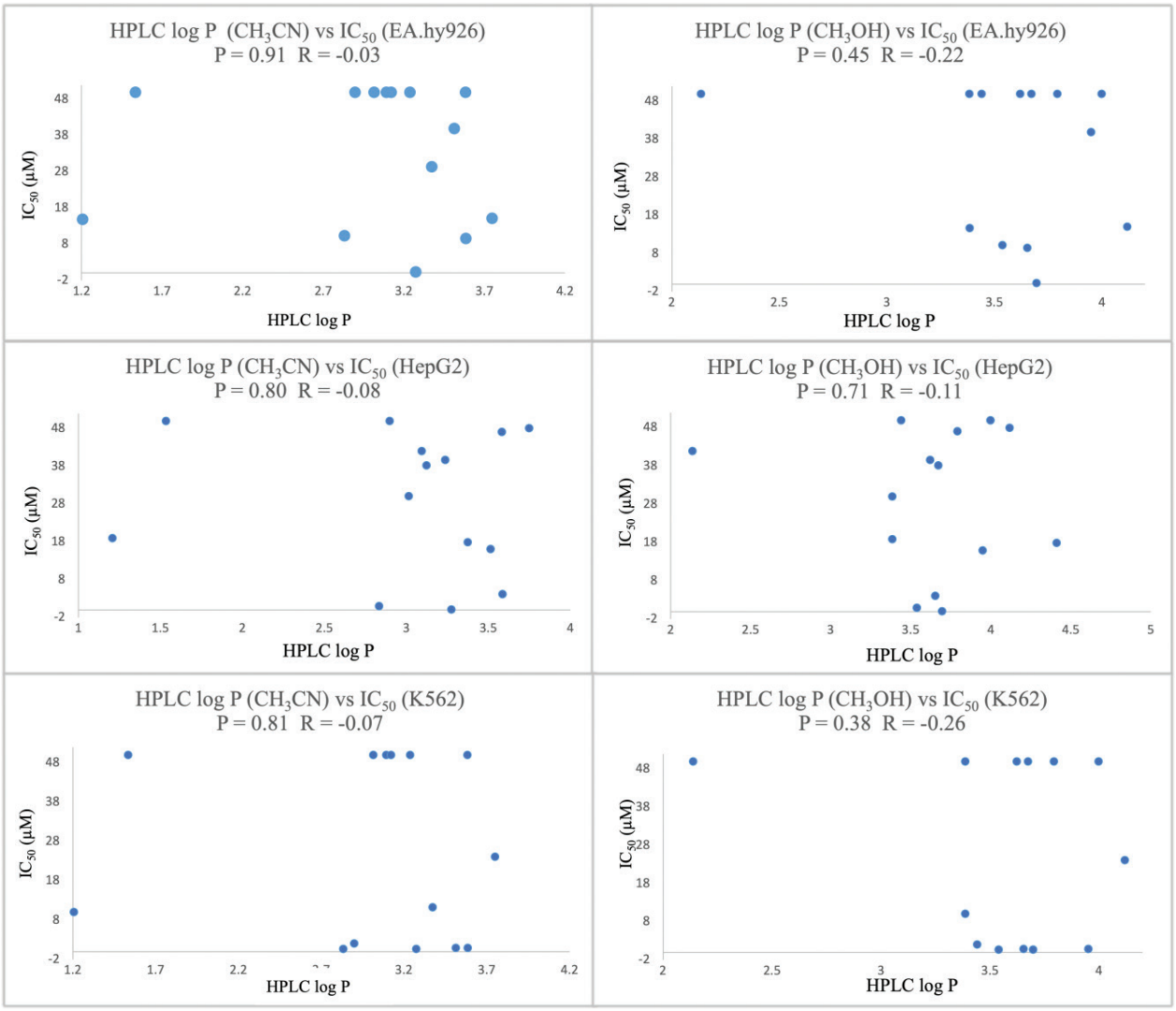


**Figure 4.** Correlation between $IC_{50}$ against three cell lines and lipophilicity measured HPLC log P for *Z*-isomers of CA-4 derivatives; the level of significance is shown for each correlation.

The natural *cis*-stilbene CA-4 is a powerful inhibitor of tubulin polymerization and a potential anticancer agent. CA-4 binds itself to the colchicine pocket of β-tubulin and inhibits the formation of the mitotic spindle and then causes mitotic catastrophe in cancer cells. *In silico* model showed S61-Z can be expected to bind itself to tubulin at the same site as CA-4; see fig. 5 in Gerova et al. (2016). The main role of the biological activity is steric overlapping of S61-Z with the colchicine pocket and acting as a tubulin binding agent. The assays proved that S61-Z is a promising anticancer agent. There were other compounds with higher lipophilicity compared to that of CA-4 in both models in our study, but their bioactivities were less effective. Therefore, the lipophilicity is not the main factor for determining the biological activity of the tested CA-4 analogues. The specific interaction between analyte and colchicine pocket cannot be imitated by the HPLC system.

## Conclusion

The chromatographic lipophilicity of thirty-one newly synthesized compounds was estimated using acetonitrile-water and methanol-water mixtures as mobile phases and octadecyl-modified silicagel as the stationary phase. Evaluation of the mechanism of the retention—linear or quadratic—was performed in accordance with Mandel's test. The values of log P were higher for the *E*-isomers than those of the *Z*-isomers analyzed with both mobile phases. Exceptions to this were observed in the pairs S45-Z/S46-E, S57-E/S58-E, and S61-Z/S62-E for acetonitrile-water. Analytes with two methoxy groups at positions 3 and 5 in ring A showed the highest relative lipophilicity in both mobile phases used. Stilbene derivatives with three methoxy groups were less lipophilic than compounds with one or two groups in ring A in our study.

The correlations between the chromatographic lipophilicity and $IC_{50}$ for all tested cell lines were not strong. The cytotoxic activity *in vitro* of the other *Z*-isomers with one or two methoxy groups in 3,5-positions in ring A was lower compared to that of S61-*Z*, but these compounds have a higher chromatographic lipophilicity. The log P values of S61-Z from both chromatographic systems were higher than calculated for CA-4. The stronger effect of S61-*Z* as a tubulin-binding agent relates to its structure. The correlation of the HPLC study compared with the *in vitro* tests shows that log P does not provide valuable information. However, lipophilicity determined in our study may assist in the interpretation of the pharmacokinetic mechanism *in vivo*.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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### Data availability

All of the data that support the findings of this study are available in the main text.

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