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Synthesis and characterization of novel 1,2,4-triazine derivatives with antiproliferative activity

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ABSTRACT

A series of novel small molecules with a 1,2,4-triazine scaffold was obtained according to a recently published and highly efficient synthetic route. Screening for antiproliferative and cytotoxic activity revealed distinct anticancer effects against the human leukemia cell line K-562 combined with a remarkable low cytotoxicity. All compounds were in agreement with the 'rule-of-five' claims by Lipinski and calculated log P_{calc} values were experimentally confirmed (log P_{exp}). For the most active compounds, in vitro serum albumin binding was investigated and structure–activity relationships were established.

1. Introduction

In the past few years, numerous small molecules possessing a 1,2,4-triazine scaffold have been shown to exhibit a great variety of pharmacological effects. Several reports have been published on the application of these compounds, such as 5-lipoxygenase (5-LO) inhibitors,[1,2] herbicides, bactericides, fungicides, antimicrobials,[3,4] and gonadotropin-releasing hormone receptor (GnRH-R) antagonists.[5] Even for fused 1,2,4-triazine compounds not only antitumoral and antimetastatic activities against a wide range of cancer cells but also kinase inhibiting activities could be observed.[6–10] In 2007, cancer accounted for ~7.9 million death cases (around 13% of all deaths). Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030.[11]

From our experience with heterocyclic compounds containing an amidrazone scaffold,[12,13] we aimed to synthesize novel 1,2,4-triazines as efficient anticancer drugs with low cytotoxicity and good bioavailability properties. In this paper, we present the straightforward synthesis of eight 1,2,4-triazin-5-ones **3a–h** (Scheme 1), and the indirect determination of log $P_{O/W}$ values (log P_{exp}) by reversed-phase high-performance liquid chromatography (RP-HPLC) based on the logarithm of capacity factors (log *k*). The comparison of the experimentally determined log P_{exp} with the estimated log P_{calc} values calculated based on group contributions from fragments of the molecular structure,[14,15] was also done. Antiproliferative and cytotoxic activity of the 1,2,4-triazin-5-ones **3a–h** against HUVEC (human umbilical vein endothelial cells), K-562 (human leukemia cell line), and HeLa cells were determined. The established anticancer drugs, such as tyrosine kinase inhibitor imatinib (Gleevec®) and anthracycline antibiotic doxorubicin (Adriamycin®) were also tested in the same assays for comparison. As protein binding can affect the distribution of the drugs within the body and their rates of metabolism and excretion, spectrofluorimetric analysis of the four most active compounds (against K-562) was conducted to determine the in vitro dissociation constants (K_d) for human serum albumin (HSA) binding. The potential of the 1,2,4-triazin-5-ones **3a–h** as lead structures for anticancer therapy is also discussed.

2. Results and discussion

2.1. Synthesis of 1,2,4-triazin-5-one compounds

Hydrazonoyl chlorides **1a–b** as starting compounds for triazinone synthesis were prepared according to known literature procedures.[16,17] Conversion of **1a–b** with the respective amines in dioxane led to amidrazone intermediates **2a–h**. One to two hours of refluxing **2a–h** with formaldehyde in the presence of *p*-toluen-

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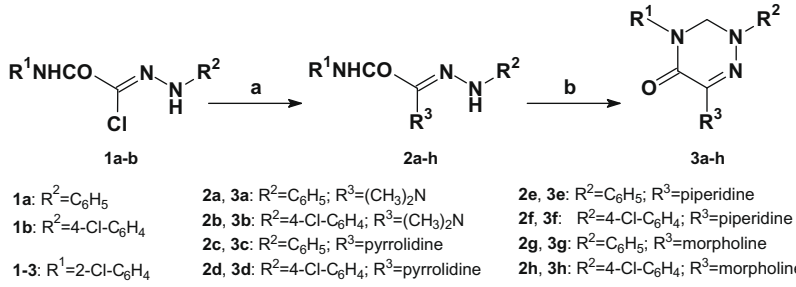


Scheme 1. Reagents and conditions: (a) dimethylamine for **2a**–**b**, pyrrolidine for **2c**–**d**, piperidine for **2e**–**f**, morpholine for **2g**–**h**, dioxane, room temperature, 12 h and (b) H_2CO, TsOH, EtOH, 1–2 h, reflux.

sulfonic acid yielded nearly pure 1,2,4-triazin-5-ones **3a–h** (Scheme 1).[18]

2.2. Determination of bioavailability parameters

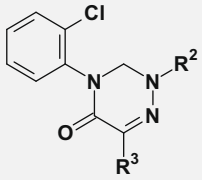
The triazinone compounds **3a–h** were subjected to the freely accessible program MIPC (Molinspiration Property Calculator) to obtain the Lipinski descriptors for bioavailability estimation.[14,15] These parameters describe molecular properties important for drug pharmacokinetics in the human body, especially their oral absorption. The rule says, that an oral active drug must not violate more than one of the following criteria: $\leqslant$5 hydrogen donors (nOHNH), $\leqslant$10 hydrogen acceptors (nON), $M_W \leqslant 500$ Da, log P_{calc} $\leqslant$5. All triazinones meet these claims (Table 1). The log $P_{O/W}$ value, defined as the logarithm of the partition coefficient between *n*-octanol and water, is an important parameter to reflect a compound's hydrophobicity and is therefore crucial for drug absorption and transport. As expected due to missing hydrogen donor residues (nOHNH), calculated log P_{calc} values of all compounds are in a quite high but still suitable range. Exclusively compound **3f** violated one of the 'rule-of-five' claims with a log P_{calc} value of 5.1. The log P_{calc} values were compared with experimental log P_{exp} values obtained from the logarithm of the capacity factors (log *k*) that were measured by reversed-phase high-performance liquid chromatography (RP-HPLC) (see Section 4).[19,20] The determination of log *k* derived from RP-HPLC retention times demonstrates a convenient technique for estimating log $P_{O/W}$ values and was carried out in this study to replace the time-consuming log $P_{O/W}$ determination by the shake-flask method.[21]

As shown in Table 1 the experimental data are in excellent agreement with the in silico data showing the applicability of the computational method for this class of compounds. Both reference compounds for pharmacological testing, imatinib and doxorubicine, were also subjected to MIPC. Most of the triazinone compounds listed in Table 1 have similar log P_{calc} values as that of imatinib, an orally bioavailable drug that does not violate the 'rule-of-five'. However, for imatinib a protonation at experimental pH conditions has to be considered in contrast to **3a–h**, which is reflected in an effective log P_{exp} value of 1.2.[22] Doxorubicin does not fulfill three out of four claims and is therefore applied intravenously.

2.3. Pharmacological activities and serum albumin binding

Antiproliferative and cytotoxic activities of compounds **3a–h** were determined as described in previous papers and in the experimental part (Section 4.4) herein.[24–26] Among the eight 1,2,4-triazin-5-ones synthesized, **3a–d** showed the highest antiproliferative effect on the human leukemia cell line K-562 with a moderate growth inhibition efficacy on the human umbilical vein endothelial cell line (HUVEC). Though the most potent compound **3c** is obviously less active against K-562 than imatinib, the preferred drug for treating chronic myeloid leukemia (CML), a comparable low growth inhibiting effect on HUVEC was found. Cytotoxicity of **3c** against HeLa cells is ranging at similar values. In contrast to doxorubicin, an established but highly cytotoxic drug for the treatment of acute myeloid leukemia (AML), lymphoma, sarcoma, and carcinoma, **3c** showed a five times lower antiprolifer-

Table 1
Substitution patterns, Lipinski's 'rule-of-five' descriptors, experimental log *P* values (log P_{exp})



	R^2	R^3	M_W[a]	nON[a]	nOHNH[a]	log P_{calc}[a]	log P_{exp}
3a	C_6H_5	$N(CH_3)_2$	328.80	5	0	3.5	3.6
3b	4-Cl–C_6H_4	$N(CH_3)_2$	363.25	5	0	4.2	4.2
3c	C_6H_5	Pyrrolidine	354.84	5	0	3.9	4.0
3d	4-Cl–C_6H_4	Pyrrolidine	389.29	5	0	4.6	4.6
3e	C_6H_5	Piperidine	368.87	5	0	4.4	4.6
3f	4-Cl–C_6H_4	Piperidine	403.31	5	0	5.1	5.0
3g	C_6H_5	Morpholine	370.84	6	0	3.3	3.5
3h	4-Cl–C_6H_4	Morpholine	405.28	6	0	4.0	4.0
Imatinib	—	—	493.62	8	2	3.9	1.2[22]
Doxorubicin	—	—	543.52	12	7	0.57	0.71[23]

n.d.: not determined.
[a] Lipinski descriptors: M_W, molecular weight; nON, number of hydrogen acceptors; nOHNH, number of hydrogen donors; log P_{calc}, log *P* values calculated by molinspiration property calculator for the neutral species; log P_{exp}, experimentally determined log *P* values by RP-HPLC.

ative activity against K-562, but a 22 times lower cytotoxicity on HeLa cells (Table 2). These results suggest that there is little correlation between cytotoxicity and antiproliferative activity for the 1,2,4-triazin-5-ones. If one compares the effect of R^3 on the antiproliferative activity against K-562 cells, an optimum exists for the pyrrolidine moiety. 4-Chloro substitution on the phenyl ring R^2 not only raises the log *P* values, but lowers the effect on K-562. Interestingly the existence of the chlorine atom in **3b** and **3d** seems to raise the growth inhibiting effect on HUVEC cells. An introduction of a morpholine group leads to a distinct decrease of any activity. Considering the estimated new cases (44,790 men and woman) and deaths (21,870 men and woman) from leukemia in the United States in 2009,[27] and the proceeding emerge of resistances against chemotherapeutic agents (multidrug resistance), there is a demand for novel, more effective anticancer agents. Taking 1,2,4-triazin-5-one as lead structure for the development of less toxic and selective anti-leukemia drugs, further chemical modifications in the substitution patterns of the aromatic groups are envisioned for optimization of pharmacological activity. An introduction of hydrogen donor groups may be considered as well.

For **3a–d**, their binding to human serum albumin (HSA), the most abundant protein (35–50 mg mL^{-1}) in plasma, was studied using an existing spectrofluorimetric in vitro method.[28–31] Exemplarily, Figure 1A shows the spectra of the intrinsic fluorescence quenching of 2.0 μM HSA (1.3 mg/10 mL) in PBS (phosphate buffered saline) buffer (pH 7.4) in the presence of varying concentrations of **3c**. The spectra exhibit a concentration dependent quenching of intrinsic HSA fluorescence emission. Figure 1B demonstrates the percentual reduction in fluorescence (% Quenching) of HSA at 332 nm as a function of compound concentration. The data were fitted to a quadratic binding equation and the average K_d value was found to be 2.46 ± 0.51 μM. With a K_d of 78.64 ± 6.71 μM imatinib showed a weak affinity towards HSA, which is not surprising if one considers the well-known relationship between lipophilicity (log P_{exp}) and HSA binding.[28]

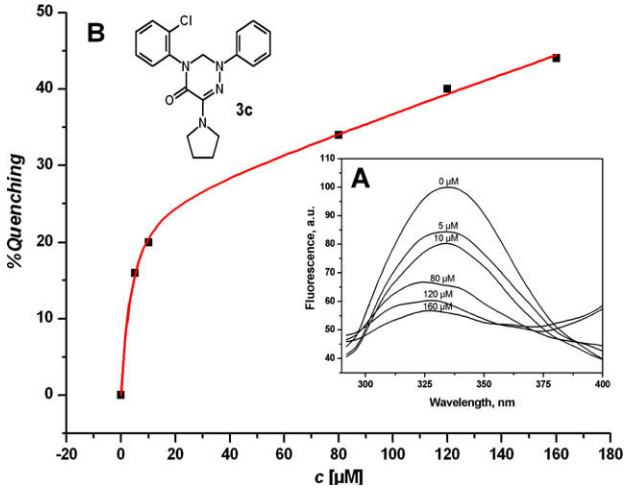


Figure 1. (A) Fluorimetric emission spectra of HSA in the absence and presence of **3c** (concentrations are indicated). (B) Reduction of fluorescence (% Quenching) of HSA as a function of **3c** concentration. The solid line presents the best fit of the quadratic binding curve.

3. Conclusions

A series of eight 1,2,4-triazin-5-ones was synthesized, which exhibit distinct antiproliferative activities against the chronic myeloid leukemia (CML) cell line K-562 combined with a low cytotoxicity. Calculated molecular properties, such as the logarithm of partition coefficients (log P_{calc}), were experimentally confirmed. The described molecular characteristics are in agreement with the Lipinski's 'rule-of-five' claims for orally bioactive drugs. Therefore we are planning to conduct kinase inhibition assays for compounds **3a–d**. Serum albumin binding for the most active compounds **3a–d** was determined revealing K_d values in the range between 2.5 and 10.2 μM. To gain a deeper inside of the plasma binding of the 1,2,4-triazin-5-ones, it is also necessary to investigate binding to α_1-acid glycoprotein (AGP), the second important plasma protein. Additionally, further lead optimization is necessary to identify significantly more potent analogs in this series.

4. Experimental

4.1. Chemicals

Human serum albumin (essentially fatty acid free, prepared from fraction V albumin, Sigma–Aldrich, Taufkirchen, Germany), reference standards for log *P* determination and solvents for reversed-phase high-performance liquid chromatography (RP-HPLC) were of the highest available purity (Sigma–Aldrich, Taufkirchen, Germany). All chemicals and solvents for synthesis were of reagent grade and used without further purification. Water was purified with a Direct-Q5 water purification system (Millipore, Eschborn, Germany).

4.2. Synthesis

4.2.1. General

Melting points were determined on a Boëtius hot-stage apparatus. Elemental analyses were performed by Leco Microlab, Inc., and determined values are within 0.4% of theory. NMR spectra were recorded on a Gemini 2000 operating at 399.96 MHz for ^{1}H NMR and

Table 2
Antiproliferative (GI_{50}) and cytotoxic (CC_{50}) effects of triazinones **3a–h** and K_d values of triazinone compounds **3a–d** and imatinib for the reduction of the intrinsic fluorescence of HSA

Compound	HUVEC GI_{50} (μg/mL)	K-562 GI_{50} (μg/mL)	HeLa CC_{50} (μg/mL)	K_d (μM)
3a	34.5 ± 2.8	5.8 ± 0.4	39.5 ± 7.6	7.96 ± 5.71
3b	16.1 ± 1.4	11.0 ± 0.7	39.4 ± 5.2	10.21 ± 5.50
3c	40.9 ± 4.9	5.2 ± 0.4	44.3 ± 5.8	2.46 ± 0.51
3d	18.9 ± 1.5	8.6 ± 0.6	>50	2.77 ± 2.06
3e	24.7 ± 2.1	42.0 ± 5.5	26.0 ± 2.3	n.d.
3f	>50	>50	>50	n.d.
3g	>50	>50	>50	n.d.
3h	45.6 ± 5.6	>50	>50	n.d.
Imatinib	10.9 ± 1.2	0.1 ± (6.7×10^{-3})	38.8 ± 1.4	78.64 ± 6.71
Doxorubicin	0.1[32]	1.0 ± 0.6	2.0 ± 0.8	n.d.

GI_{50}, concentration which inhibits cell proliferation by 50% compared to control; CC_{50}, concentration which is toxic for 50% of the cells compared to control (4 parallels per concentration); K_d, dissociation constant; n.d.: not determined.

at 100.6 MHz for ^{13}C NMR spectra in DMSO-d_6 which was also used as internal standard. Chemical shifts are given in δ units and refer to the center of the signal. EI-mass spectra were obtained with an AMD 402 mass spectrometer (AMD Intectra) at 70 eV. Reactions were monitored by TLC (Silica gel 60 F_{254}, Merck) using chloroform/ether (7:3, v/v) and heptane/ethylacetate (3:1, v/v) and compounds were detected with ultraviolet light (254 nm). Compounds **1a**, **2a**, **2b**, **2e**, **2g**,[17] **1b**,[16] and **3e**[18] were obtained by published procedures.

4.2.2. Representative procedure for the synthesis of compounds 2

A solution of **1a** (3.0 g, 10 mmol) in dioxane (~40 cm^3) was added dropwise to pyrrolidine (1.4 g, 20 mmol) in a few cm^3 of dioxane. After stirring at ambient temperature for at least 12 h, the mixture was poured into cold water (300 cm^3). The solid was collected, washed with water, and dried. Recrystallization from ethanol gave **2c** (2.5 g, 81%) as beige crystals. Mp: 101–106 °C.

4.2.2.1. *N*-(2-Chlorophenyl)-2-phenylhydrazono-2-(pyrrolidin-1-yl)acetamide 2c. δ_H = 9.66 (1H, s, CONH), 9.47 (1H, s, NNH), 8.22–6.83 (9H, Ph), 3.29 (4H, t, J = 6 Hz, CH_2NCH_2), 1.86 (4H, m, J = 6 Hz, $2CH_2$); δ_C = 160.5 (1C, C=O), 138.9 (1C, C=N), 145.1–113.9 (12C, Ph), 49.2 (2C, CH_2NCH_2), 25.9 (2C, $2CH_2$). *m/z* (EI-MS) calcd for $C_{18}H_{19}ClN_4O$ 342; found 342 (M^+, 100%).

4.2.2.2. *N*-(2-Chlorophenyl)-2-(4-chlorophenyl)-hydrazono-2-(pyrrolidin-1-yl)-acetamide 2d. Employing 3.4 g of **1b** (10 mmol) in the procedure described above gave **2d** (2.6 g, 86%) as beige crystals. Mp: 113–115 °C. δ_H = 9.63 (1H, s, CONH), 9.49 (1H, s, NNH), 8.13–6.87 (8H, Ph), 3.28 (4H, t, J = 6 Hz, CH_2NCH_2), 1.86 (4H, m, J = 6 Hz, $2CH_2$); δ_C = 159.9 (1C, C=O), 138.9 (1C, C=N), 143.7–113.9 (12C, Ph), 48.6 (2C, CH_2NCH_2), 25.2 (2C, $2CH_2$). *m/z* (EI-MS) calcd for $C_{18}H_{18}Cl_2N_4O$ 376; found 376 (M^+, 100%), 221 (41).

4.2.2.3. *N*-(2-Chlorophenyl)-2-(4-chlorophenyl)-hydrazono-2-(piperidin-1-yl)acetamide 2f. Employing 3.4 g of **1b** (10 mmol) and 1.6 g piperidine (20 mmol) in the procedure described above gave **2f** (3.5 g, 92%) as pale yellow crystals. Mp: 138–143 °C. δ_H = 9.51 (1H, s, CONH), 9.34 (1H, s, NNH), 8.12–7.10 (8H, Ph), 3.00 (4H, t, J = 6 Hz, CH_2NCH_2), 1.65 (4H, m, J = 6 Hz, $2CH_2$), 1.52 (2H, m, J = 6 Hz, CH_2); δ_C = 159.9 (1C, C=O), 138.9 (1C, C=N), 142.7–115.1 (12C, Ph), 48.4 (2C, CH_2NCH_2), 25.2 (2C, $2CH_2$), 23.9 (1C, CH_2). *m/z* (EI-MS) calcd for $C_{19}H_{20}Cl_2N_4O$ 390; found 390 (M^+, 100%), 84 (89), 235 (49).

4.2.2.4. *N*-(2-Chlorophenyl)-2-(4-chlorophenyl)-hydrazono-2-(morpholin-4-yl)acetamide 2h. Employing 3.4 g of **1b** (10 mmol) and 1.7 g morpholine (20 mmol) in the procedure described above gave **2h** (3.2 g, 81%) as pale yellow crystals. Mp: 135–142 °C. δ_H = 9.64 (1H, s, NNH), 9.56 (1H, s, CONH), 8.14–7.07 (8H, Ph), 3.77 (4H, t, J = 4.5 Hz, CH_2OCH_2), 3.08 (4H, t, J = 4.5 Hz, CH_2NCH_2); δ_C = 159.9 (1C, C=O), 137.3 (1C, C=N), 142.7–115.4 (12C, Ph), 66.0 (2C, CH_2OCH_2), 47.5 (2C, CH_2NCH_2). *m/z* (EI-MS) calcd for $C_{18}H_{18}Cl_2N_4O_2$ 392; found 392 (M^+, 25%), 237 (100), 125 (51), 266 (48).

4.2.3. Representative procedure for the synthesis of compounds 3

3.2 g of **2a** (10 mmol), a 37%-solution of formaldehyde (1.5 cm^3, 20 mmol), and *p*-toluenesulfonic acid (0.1 g) were refluxed in ethanol (~50 cm^3) until the starting amidrazone was converted completely. The mixture was cooled to room temperature and the solvent was evaporated. The solid was collected and recrystallized from ethanol yielding **3a** (1.4 g, 42%) as yellow crystals. Mp: 108–110 °C.

4.2.3.1. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-6-dimethylamino-2-phenyl-1,2,4-triazin-5-one 3a. δ_H = 7.63–6.86 (9H, Ph), 5.21 (2H, NCH_2N), 2.90 (6H, s, $2CH_3$). δ_C = 152.4 (1C, C=O), 147.6 (1C, C=N), 145.0–114.5 (12C, Ph), 63.5 (1C, NCH_2N), 39.5 (2C, $2CH_3$). *m/z* (EI-MS) calcd for $C_{17}H_{17}ClN_4O$ 328; found 328 (M^+, 100), 161 (78), 118 (74).

4.2.3.2. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-(4-chlorophenyl)-6-dimethylamino-1,2,4-triazin-5-one 3b. Employing 3.5 g of **2b** (10 mmol) in the procedure described above gave **3b** (1.3 g, 37%) as yellow crystals. Mp: 125–128 °C; δ_H = 7.63–7.28 (8H, Ph), 5.23 (2H, NCH_2N), 2.91 (6H, s, $2CH_3$). δ_C = 152.3 (1C, C=O), 147.9 (1C, C=N), 143.9–115.9 (12C, Ph), 63.3 (1C, NCH_2N), 39.4 (2C, $2CH_3$). *m/z* (EI-MS) calcd for $C_{17}H_{16}Cl_2N_4O$ 362; found 362 (M^+, 100), 195 (99), 125 (91).

4.2.3.3. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-phenyl-6-(pyrrolidin-1-yl)-1,2,4-triazin-5-one 3c. Employing 3.4 g of **2c** (10 mmol) in the procedure described above gave **3c** (2.1 g, 61%) as yellow crystals. Mp: 119–123 °C; δ_H = 7.62–6.82 (9H, Ph), 5.21 (2H, NCH_2N), 3.49 (4H, t, J = 6 Hz, CH_2NCH_2), 1.86 (4H, m, J = 6 Hz, $2CH_2$); δ_C = 153.1 (1C, C=O), 145.4 (1C, C=N), 145.4–114.4 (12C, Ph), 63.9 (1C, NCH_2N), 48.1 (2C, CH_2NCH_2), 24.6 (2C, $2CH_2$). *m/z* (EI-MS) calcd for $C_{19}H_{19}ClN_4O$ 354; found 354 (M^+, 100).

4.2.3.4. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-(4-chlorophenyl)-6-(pyrrolidin-1-yl)-1,2,4-triazin-5-one 3d. Employing 3.8 g of **2d** (10 mmol) in the procedure described above gave **3d** (2.2 g, 58%) as yellow crystals. Mp: 134–136 °C; δ_H = 7.62–7.25 (8H, Ph), 5.23 (2H, NCH_2N), 3.49 (4H, t, J = 6 Hz, CH_2NCH_2), 1.85 (4H, s, $2CH_2$); δ_C = 153.7 (1C, C=O), 146.2 (1C, C=N), 144.9–116.4 (12C, Ph), 64.3 (1C, NCH_2N), 48.9 (2C, CH_2NCH_2), 25.3 (2C; $2CH_2$). *m/z* (EI-MS) calcd for $C_{19}H_{18}Cl_2N_4O$ 388; found 388 (M^+, 100), 221 (64).

4.2.3.5. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-(4-chlorophenyl)-6-(piperidin-1-yl)-1,2,4-triazin-5-one 3f. Employing 3.4 g of **2f** (10 mmol) in the procedure described above gave **3f** (2.5 g, 63%) as yellow crystals. Mp: 197–198 °C; δ_H = 7.60–7.26 (8H, Ph), 5.21 (2H, NCH_2N), 3.25 (4H, t, J = 6 Hz, CH_2NCH_2), 1.57 (4H, m, J = 5 Hz, $2CH_2$); δ_C = 152.1 (1C, C=O), 147.4 (1C, C=N), 143.7–116.0 (12C, Ph), 63.0 (1C, NCH_2N), 48.0 (2C, CH_2NCH_2), 24.6 (2C, $2CH_2$), 23.9 (1C, CH_2). *m/z* (EI-MS) calcd for $C_{20}H_{20}Cl_2N_4O$ 402; found 402 (M^+, 100), 235 (74).

4.2.3.6. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-phenyl-6-(morpholin-4-yl)-1,2,4-triazin-5-one 3g. Employing 3.6 g of **2g** (10 mmol) in the procedure described above gave **3g** (2.3 g, 62%) as yellow crystals. Mp: 153–155 °C; δ_H = 7.64–6.88 (9H, Ph), 5.26 (2H, NCH_2N), 3.72 (4H, t, J = 4.7 Hz, CH_2OCH_2), 3.27 (4H, s, CH_2NCH_2); δ_C = 152.0 (1C, C=O), 145.9 (1C, C=N), 144.7–114.6 (12C, Ph), 66.4 (2C, CH_2OCH_2), 63.1 (1C, NCH_2N), 47.6 (2C, CH_2NCH_2). *m/z* (EI-MS) calcd for $C_{19}H_{19}ClN_4O_2$ 370; found 370 (M^+, 100).

4.2.3.7. 2,3,4,5-Tetrahydro-4-(2-chlorophenyl)-2-(4-chlorophenyl)-6-(morpholin-4-yl)-1,2,4-triazin-5-one 3h. Employing 3.9 g of **2h** (10 mmol) in the procedure described above gave **3h** (1.6 g, 39%) as yellow crystals. Mp: 186–188 °C; δ_H = 7.63–7.30 (8H, Ph), 5.27 (2H, NCH_2N), 3.71 (4H, t, J = 4.7 Hz, CH_2OCH_2), 3.28 (4H, s, CH_2NCH_2); δ_C = 151.9 (1C, C=O), 146.3 (1C, C=N), 143.6–116.1 (12C, Ph), 65.4 (2C, CH_2OCH_2), 62.9 (1C, NCH_2N), 47.5 (2C, CH_2NCH_2). *m/z* (EI-MS) calcd for $C_{19}H_{18}Cl_2N_4O_2$ 404; found 404 (M^+, 100), 237 (59).

4.3. Determination of log P_{exp} by RP-HPLC

The HPLC system consisted of a PU-980 Intelligent HPLC Pump, a UV-975 Intelligent UV–vis Detector set at 210 nm and an 851-AS Intelligent Autosampler (Jasco, Groß-Umstadt, Germany). The column was a LiChroCART® 125-4 with LiChrospher® 100 RP-18 endcapped material (5 μm) (Merck, Darmstadt, Germany). As eluent for 3,4-dihydro-2*H*-1,2,4-triazin-5-ones a mixture of methanol-PBS buffer (75/25 (v/v)) pH 7.4 was used. The flow rate of the mobile phase was 1 mL/min. The sample volume injected was 15 μL (concd ~3 μM/L). The dead time (t_0) was measured by injection of the unretained compound formamide dissolved in mobile phase. Lacking comparable data for compounds **3a–h** a more general calibration according to an OECD protocol was established using the following reference compounds: benzophenone (log $P_{O/W}$: 3.2), diphenylamine (log $P_{O/W}$: 3.4), benzyl benzoate (log $P_{O/W}$: 4.0), *n*-butylbenzene (log $P_{O/W}$: 4.6), and bibenzyl (log $P_{O/W}$: 4.8) perfectly covering the previously calculated log P_{calc} value range between 3.5 and 4.0.

Retention times (t_R) of the reference compounds were determined and according to the equation:

$$k = (t_R - t_0)/t_0$$

the capacity factor (k) was calculated which is directly related to log $P_{O/W}$ (Table 3).

A correlation curve of log $P_{O/W}$ versus log k was obtained by least-squares linear regression (Fig. 2) and log P_{exp} values of triazinone compounds were deviated from the linear equation of the graph (Table 4).[19,20]

4.4. Pharmacology

4.4.1. Cells and culture conditions

Cells of HUVEC (ATCC CRL-1730), K-562 (DSM ACC 10), and HeLa (DSM ACC 57) were cultured in DMEM (CAMBREX 12-614F), RPMI 1640 (CAMBREX 12-167F), and RPMI 1640 (CAMBREX 12-167F), respectively. All cells were grown in the appropriate cell culture medium supplemented with 10 mL/L ultraglutamine 1 (CAMBREX 17-605E/U1), 500 μL/L gentamicin sulfate (CAMBREX 17-518Z), and 10% heat inactivated fetal bovine serum (PAA A15-144) at 37 °C in high density polyethylene flasks (NUNC 156340).

4.4.2. Antiproliferative assay

The test substances were dissolved in DMSO before being diluted in DMEM. The adherent cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163; Biochrom, Berlin, Germany). For each experiment approximately 10,000 cells were seeded with 0.1 mL culture medium per well of the 96-well microplates (NUNC 167008).

4.4.3. Cytotoxic assay

For the cytotoxic assay, HeLa cells were 48 h pre-incubated without the test substances. The dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells after the preincubation time.

Table 3
Retention times (t_R), calculated logarithms of capacity factors (log k) and log $P_{O/W}$ values of the reference compounds

Reference	t_R[a] (min)	log k	log $P_{O/W}$
Benzophenone	2.97	0.24	3.2
Diphenylamine	3.42	0.34	3.4
Benzyl benzoate	4.89	0.55	4.0
n-Butylbenzene	10.63	0.95	4.6
Bibenzyl	11.12	0.97	4.8
Formamide (t_0)	1.08	—	—

[a] Retention times were determined in triplicate and the calculation of log k was done with the arithmetic mean values.

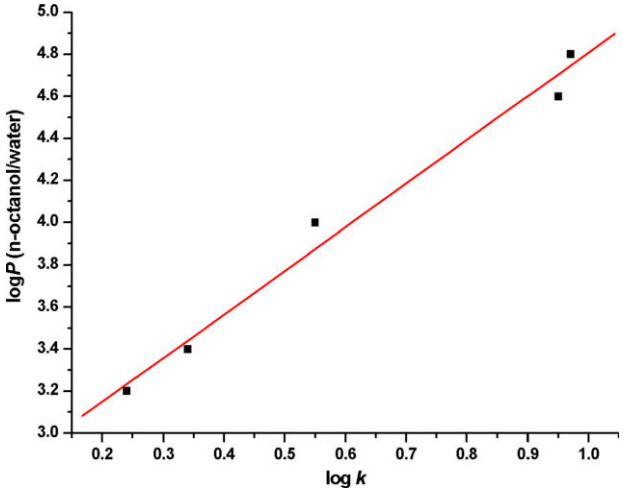


Figure 2. Calibration curve for determination of log P_{exp} values by plotting known log $P_{O/W}$ values of different reference compounds (Table 3) versus their log k values; $y = 2.74 + 2.07x$; $r = 0.9920$.

Table 4
Retention times (t_R) and the calculated logarithms of capacity factors (log k) of the 1,2,4-triazin-5-ones

Compound	t_R (min)[a]	log k
3a	3.97	0.43
3b	6.09	0.34
3c	5.80	0.64
3d	9.82	0.91
3e	8.87	0.86
3f	14.4	1.09
3g	3.53	0.36
3h	5.21	0.58

[a] Retention times were determined in triplicate and the calculation of log k was done with the arithmetic mean values.

4.4.4. Condition of incubation

The cells were incubated with dilutions of the test substances for 72 h at 37 °C in a humidified atmosphere and 5% CO_2.

4.4.5. Method of evaluation

To estimate the influence of chemical compounds on cell proliferation of K-562, we determined the numbers of viable cells present in multi-well plates via CellTiter-Blue1 assay. It uses the indicator dye resazurin to measure the metabolic capacity of cells as the indicator of cell viability. Viable cells of untreated control retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Non-viable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Under our experimental conditions, the signal from the CellTiter-Blue1 reagent is proportional to the number of viable cells. The adherent HUVEC and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing, the stain was eluted with 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN, Switzerland). The GI_{50} and CC_{50} values were defined as the value at the intersection of the dose response curve with the 50% line, compared to untreated control. These comparisons of the different values were performed with the software Magellan (TECAN).

4.5. Spectrofluorimetric determination of serum albumin binding

All fluorescence measurements were performed at room temperature on a Perkin Elmer MPF-44 Fluorescence Spectrophotometer equipped with a Xenon lamp, 4 mm excitation and emission slits. Intrinsic tryptophan fluorescence emission spectra were recorded at a wavelength of 290 nm. HSA was dissolved in PBS buffer at a concentration of 5.0 μM. Stock solutions of **3a–d** were prepared in DMSO at concentrations of 0.1 mM, 1 mM, and 10 mM. For titrations, a series of increasing concentrations of compounds **3a–d** in PBS buffered HSA solution (final concentration 2 μM) was prepared and incubated for 30 min at ambient temperature. After that, the fluorescence emission was recorded. The reduction in fluorescence (% Quenching) was plotted against total compound concentration (Fig. 1B). Determination of K_d was realized using the quadratic binding equation for fluorescence quenching as described before and the Marquardt algorithm in conjunction with a nonlinear least-squares curve fitting routine (Microcal Origin 6.0).[30]

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