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N-Benzylsalicylthioamides: Highly Active Potential Antituberculotics

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A gseries of 29 new derivatives of *N*-benzylsalicylthioamides was synthesized and the compounds were tested for *in-vitro* antimycobacterial activity against *Mycobacterium tuberculosis*, *Mycobacterium kansasii*, and *Mycobacterium avium*. The activity was analyzed by quantitative structure-activity relationship (QSAR). Activity increased with increasing lipophilicity and electron donating effect of the substituents in the acyl moiety and decreased with the electrophilic superdelocalizability of the molecules. The most active compounds are more active than isoniazid (INH) and are active against INH-resistant potential pathogenic strains of mycobacterium.

Keywords: Antimycobacterial activity / Antituberculotic activity / *N*-Benzylsalicylamides / QSAR / Salicylthioamides

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Introduction

Since 1985, the world is confronted with the return of tuberculosis to Europe and North America. In the developing countries, due to insufficient medical care, hygienic standards, and compliance of the population with the treatment, a number of mycobacterial strains became resistant to modern chemical drugs (i.e., the development of multidrug resistant strains); infection has been often transferred to Europe and North America, since migration towards developed countries is a contemporary feature. The multidrug resistant strains of *M. tuberculosis* coming to Central Europe are mainly from the countries of the former Soviet Union. In addition, the unfavorable state is also being influenced by an increase in AIDS, which is often accompanied by mycobacterial diseases. The development of new antituberculotic agents with high activity is the principal goal of our research. Our group is especially interested in the development of drugs against *M. tuberculosis* and potentially pathogenic strains like *M. avium* and *M. kansasii*. We have recently studied a number of structurally different compounds, such as salicylanilides [1], benzoxazinediones [2], alkoxyphenylcarbamic acids [3], tetrazoles [4, 5], dihydroindolethiones [6], derivatives of benzoxazine with thioxo group [7], and other heterocycles [8]. The aim of this paper is the synthesis, antimycobacterial evaluation, and quantitative structure-activity relationship (QSAR) study of *N*-benzylsalicylthioamides.

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Abbreviation: multiple linear regression (MLR)

Results

Chemistry

The synthesis of the title compounds is illustrated in Scheme 1. The synthesis of most of the starting *N*-benzylsalicylamides was described in our previous paper [9]. We completed a group of starting *N*-benzylsalicylamides with fourteen new compounds (see Table 1 and Fig. 1).

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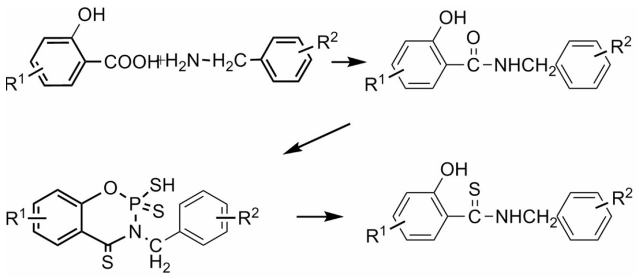

For substituents R^1 and R^2 see Tables 1 and 3.

Scheme 1. Synthetic pathway to *N*-Benzylsalicylthioamides.

Yields, melting points, and carbonyl frequencies are summarized in Table 2. The synthesis of *N*-benzylsalicylthioamides was performed by the method elaborated in our group and described in a patent [10].

The synthesis consists of the microwave-promoted thionation of the starting *N*-benzylsalicylamides. *N*-Benzylsalicylamides and P_4S_{10} produce heterocyclic compounds that hydrolyze to *N*-benzylsalicylthioamides (see Fig. 1). The structures are summarized in Table 3 and Fig. 2. The yields, melting points, and carbonyl frequencies are summarized in Table 4. The application of the Willgerodt–Kindler reaction [11] in case of *N*-benzylsalicylamides was unsuccessful. The structure of the compounds was established by IR, ^{1}H- and ^{13}C-NMR spectroscopy and verified by elemental analysis.

Biology

In-vitro antimycobacterial activity of the compounds was evaluated against *Mycobacterium tuberculosis* CNCTC My 331/88, *Mycobacterium kansasii* CNCTC My 235/80, *Mycobacterium avium* CNCTC My 330/88, and *Mycobacterium kansasii* 6509/96 using the micromethod for the determination of the minimum inhibitory concentration (MIC). All strains were obtained from the Czech National Collection of Type Cultures (CNCTC), National Institute of Pub-

Table 1. Antimycobacterial activity (minimum inhibition concentrations, MICs) of *N*-benzylsalicylamides **1**–**15**.

Compound	R^1	R^2	MIC (μmol/L)							
			M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80		*M. kansasii* 6509/96	
			14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1	H	4-*tert*-but	16	32	32	62.5	62.5	62.5	32	62.5
2	H	3-CF3	62.5	62.5	16	16	16	32	62.5	62.5
3	5-Br	3-Br	32	32	32	32	32	62.5	32	32
4	5-Br	4-Br	16	32	16	32	32	32	32	32
5	3,5-Cl_2	4-*tert*-but	32	32	62.5	62.5	62.5	62.5	62.5	62.5
6	4-Cl	4-Br	16	32	32	32	32	32	32	32
7	4-CH_3	H	62.5	125	62.5	125	125	125	125	125
8	4-CH_3	4-CH_3	125	500	62.5	250	250	500	250	500
9	4-CH_3	4-Cl	62.5	125	62.5	125	125	250	250	250
10	4-CH_3	4-*tert*-but	32	62.5	16	16	32	32	62.5	62.5
11	4-CH_3	3-NO_2	62.5	62.5	32	62.5	62.5	125	62.5	125
12	4-OCH_3	3-Cl	125	125	62.5	62.5	62.5	125	62.5	125
13[a]	3-CH_3	H	62.5	125	62.5	62.5	62.5	125	62.5	125
14	3-CH_3	4-Cl	16	32	32	62.5	62.5	62.5	62.5	62.5
15	3,5-Br_2	4-CF_3	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
Isoniazid			1	1	>250	>250	>250	>250	4	4

[a] Synthesis in ref. [18].

Table 2. Yield, melting point, and carbonyl frequency of *N*-benzylsalicylamides **1**–**15**.

Compound	Yield (%)	M.p. (°C)	$\nu_{C=O}$ (cm^{-1})	Compound	Yield (%)	M. p. (°C)	$\nu_{C=O}$ (cm^{-1})
1	73	130–131	1645	**8**	81	141–143	1642
2	91	116–118	1636	**9**	89	162–163	1641
3	51	168–170	1641	**10**	94	118–120	1650
4	49	154–156	1622	**11**	84	162–163	1640
5	76	91–92	1641	**12**	77	105–107	1636
6	75	153–156	1621	**14**	92	97–99	1643
7	76	105–107	1644	**15**	85	129–130	1630

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Table 3. Antimycobacterial activity (minimum inhibition concentrations, MICs) of *N*-benzylsalicylthioamides **16**–**45**.

Compound	R^1	R^2	MIC (μmol/L)							
			M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80		*M. kansasii* 6509/96	
			14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
16[a]	H	H	0.5	1	1	2	1	2	2	2
17	H	4-CH_3	0.25	0.5	0.25	0.5	0.5	1	0.5	1
18	H	4-Cl	1	2	0.5	0.5	1	1	0.5	1
19	H	4-OCH_3	4	4	0.98	0.98	4	4	4	4
20	H	3,4-Cl_2	0.49	0.98	0.98	0.98	2	2	2	4
21	H	4-F	0.5	1	1	2	4	4	2	2
22	H	3-CH_3	0.5	1	0.5	1	2	4	2	2
23	H	4-*tert*-but	1	2	0.25	0.25	2	4	2	2
24	H	3-Cl	1	1	1	1	1	1	1	1
25	H	3-CF3	0.5	1	0.5	1	2	2	2	2
26	5-Br	3,4-Cl_2	4	4	16	16	16	32	16	16
27	5-Br	3-Br	1	2	8	8	8	8	8	8
28	5-Br	4-Br	1	2	4	4	8	16	4	4
29	5-Cl	H	2	2	8	8	8	8	8	8
30	5-Cl	3,4-Cl_2	2	4	16	32	32	32	16	16
31	5-Cl	4-F	1	2	8	8	8	16	8	8
32	3,5-Cl_2	3,4-Cl_2	16	32	62.5	62.5	32	62.5	32	32
33	3,5-Cl_2	4-*tert*-but	32	32	62.5	62.5	62.5	62.5	62.5	62.5
34	4-Cl	4-Br	1	2	2	4	8	16	4	8
35	4-CH_3	H	0.5	1	1	2	2	2	1	2
36	4-CH_3	4-CH_3	0.125	0.25	0.25	0.5	0.25	0.5	0.5	1
37	4-CH_3	4-Cl	0.5	0.5	0.5	0.5	0.5	1	1	1
38	4-CH_3	4-*tert*-but	0.5	0.5	1	2	1	1	1	1
39	4-CH_3	3-NO_2	2	2	1	2	2	4	4	4
40	5-OCH_3	H	4	8	4	4	16	32	16	16
41	4-OCH_3	H	2	4	4	4	8	8	8	8
42	4-OCH_3	3-Cl	0.5	1	1	2	2	2	1	1
43	5-NO_2	4-CH_3	32	62.5	>125	>125	125	125	125	125
44	3-CH_3	4-Cl	2	4	1	1	2	2	2	4
45	3,5-Br_2	4-CF_3	32	32	32	32	62.5	62.5	62.5	62.5
Isoniazid			1	1	>250	>250	>250	>250	4	4

[a] Synthesis in ref. [19].

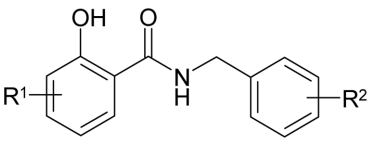

Figure 1. A survey of structures of *N*-benzylsalicylamides. For substituents R^1 and R^2 see Table 1.

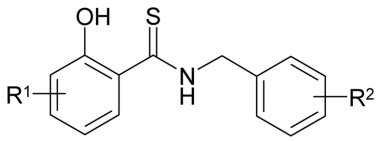

Figure 2. A survey of structures of *N*-benzylthiosalicylamides. For substituents R^1 and R^2 see Table 3.

lic Health, Prague, with the exception of *M. kansasii* 6509/96, a clinical isolate. Tables 1 and 3 summarize the MICs of the new starting compounds and the title products, respectively. The MICs were determined after incubation at 37°C for 14 and 21 days. For the sake of comparison, we also included the values of MICs of the standard isoniazid (INH). The method is described in our previous papers [1–9]. The antiproliferative activity and cytotoxicity of some compounds were investigated at the Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute in Jena. The results are summarized in Table 5. The methods were described in the previous papers [12, 13] and in the experimental part (Section 4) herein.

Calculations

The program HyperChem, Release 7.52, was used to calculate log *P* (octanol / water system), volume, surface, and polarizability of the compounds under study [14]. The program Gaussian 03, Revision C.02, was used for the quantum chemical calculations [15]. All molecular models were computed by the B3LYP/6-31G* method. The obtained wave-function data were processed in a self-

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Table 4. Yield, melting point, and frequency of hydroxyl vibration of *N*-benzylsalicylthioamides **17**–**45**.

Compound	Yield (%)	M.p. (°C)	$\nu_{C=O}$ (cm^{-1})	Compound	Yield (%)	M. p. (°C)	$\nu_{C=O}$ (cm^{-1})
17	34	73–74	3311	**32**	19	91–93	3355
18	17	101–102	3324	**33**	31	120–122	3338
19	43	92–93	3332	**34**	49	106–108	3320
20	41	96–97	3301	**35**	45	91–93	3308
21	34	64–65	3316	**36**	81	99–100	3313
22	42	85–86	3302	**37**	45	131–133	3312
23	80	89–90	3312	**38**	52	121–122	3327
24	51	96–97	3305	**39**	10	99–100	3315
25	40	29–30	3311	**40**	37	94–95	3273
26	37	117–118	3365	**41**	29	74–75	3377
27	32	136–137	3366	**42**	49	73–74	3376
28	38	131–132	3309	**43**	25	125–127	3324
29	55	109–110	3311	**44**	42	94–95	3376
30	34	133–134	3291	**45**	5	130–132	3347
31	40	112–113	3363				

developed program to compute quantum chemical descriptors. The complete set of 177 molecular descriptors consisted of 166 different quantum chemical descriptors (*e.g.*, superdelocalizabilities, indices of frontier electron densities, Mulliken charges, *etc.*), Hammett constants, Hansch constants, and several chemical shifts from ^{13}C-NMR spectra. A detailed review of quantum chemical descriptors is available in the literature [16]. The structure-activity relationships were searched for by the program STATOO (R. Doležal). The program STATOO is based on multiple linear regression (MLR). Its selection algorithm searches the best parameters in correlation equations. Statistical evaluation is given under every equation. The symbols are usual, q^2 is the cross-validated correlation coefficient determined by leave-one-out procedure.

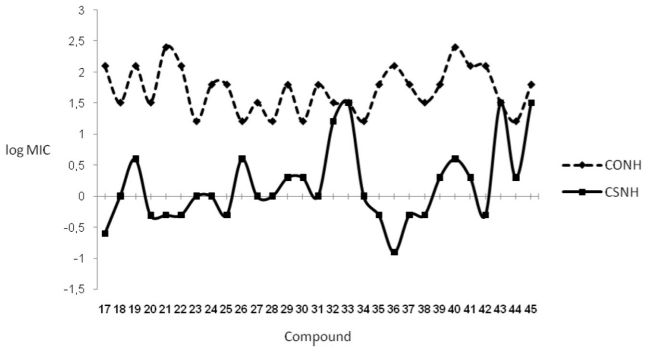

Figure 3. Comparison of antimycobacterial activity against *M. tuberculosis* (log MIC after 14 d incubation) of *N*-benzylsalicylthioamides **17**–**45** with that of *N*-benzylsalicylamides. The values of the activity of corresponding *N*-benzylsalicylamides not presented in this paper were culled from Ref. [9].

Discussion

In general, the synthesized compounds possess *in-vitro* activities against all tested mycobacterial strains, which are better than or comparable to that of INH (Tables 1 and 3). The values of MICs are generally within the range from 0.125 to 125 μmol/L, most often between 0.5 and 4 μmol/L. The most active compounds, **17** and **36**, were more active against *M. tuberculosis* 331/88 than INH. All new compounds, again, were more effective against *M. kansasii* 235/80 and *M. avium* 330/88 than INH. The replacement of an oxo group in the starting *N*-benzylsalicylamides for a thioxo group increases the antimycobacterial activity of the title compounds against all mycobacterial strains. The increase in antimycobacterial activity against *M. tuberculosis* (incubation 14 d) by replacing carbonyl oxygen for sulfur is demonstrated in Fig. 3. Interestingly, the antimycobacterial activities of *N*-benzylsalicylamides do not correlate significantly with those of the corresponding *N*-benzylsalicylthioamides. The newly synthesized compounds form a new promising group of antimycobacterials with a broad spectrum of antimycobacterial activity. Two most active compounds, **17** and **36**, were chosen for preclinical testing. Antiproliferative activity and cytotoxicity of compounds **16**, **17**, **18**, **19**, **20**, **23**, and **36** were investigated (Table 5). Unfortunately, the compounds under study are cytotoxic.

The set of 177 descriptors was used for QSAR study. The best three-parameter QSAR models were searched for using MLR technique in the STATOO program. The most important parameters were: log *P* – partition coefficient for system octanol / water; σ_{R1} – Hammett constants of substituents R^1 in the acyl moiety; $SumS_e^w$ – sum of

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Table 5. Antiproliferative activity and cytotoxicity.

Compound	Antiproliferative activity		Cytotoxicity
	Huvec GI_{50} (μg/mL)	K-562 GI_{50} (μg/mL)	HeLa CC_{50} / CC_{10} (μg/mL)
16	9.2	8.7	11.1 / 3.9
17	10.0	9.9	12.5 / 5.7
18	5.9	5.2	5.5 / 2.7
19	19.2	14.8	21.6 / 9.2
20	5.9	5.2	5.6 / 2.1
23	4.6	3.5	3.7 / 1.7
36	12.5	13.0	12.4 / 4.5
INH	>50.0	>50.0	>50.0 / 50.0
ETH	37.2	50.0	40.8 / 40.2

weighted electrophilic superdelocalizabilities for all atoms in the molecule (calculated from the output of the Gaussian); S_n3' – nucleophilic superdelocalizability on carbon 3 on the benzyl (calculated from output of the Gaussian); charge1 – Mulliken charge on carbon 1 in the acyl moiety (calculated from output of the Gaussian); S_eH – electrophilic superdelocalizability of hydrogen atoms in the thioamide group; $Sumf_e$ – sum of indices of electrophilic frontier electron density of all atoms in the molecules (calculated from output of the Gaussian); π_{R2} – Hansch constant of substituents R^2 on the benzyl; chargeH – Mulliken charge on the hydrogen atom in the thioamide group. The program STATOO determined the statistically most significant correlation equations (see Eqs. 1 – 8). In accordance with the correlations found, the antimycobacterial activities are always enhanced by electron donating substituents R^1 in the acyl moiety (*i.e.*, when s_{R1} is negative). Lipophilicity (log *P* or π_{R2}) mostly increases the activity. Other significant descriptors correspond with the local properties of certain atoms (*i.e.*, S_n3', chargeH, charge1 and S_eH) or express the overall molecular properties (*i.e.*, $SumS_e^w$ and $Sumf_e$). A higher nucleophilic superdelocalizability on carbon 3′ (S_n3') increases the activity against *M. avium* Eq. (3) and Eq. (4). Electrophilic superdelocalizability (S_eH) and Mulliken charge (chargeH) on the hydrogen atom of the thioamide group are both statistically associated with Hammett constant (σ_{R1}), and therefore, when they are lower, they also enhance the activity in the same way Eqs. (6 and 7). A lower Mulliken charge on carbon 1 (charge1) enhances the activity Eq. (6). The sum of weighted electrophilic superdelocalizabilities ($SumS_e^w$), present in most equations, should be lower in order to increase the activity. The sum of indices of electrophilic electron densities ($Sumf_e$) increases the activity when it is higher Eq. (6). All presented QSAR models show acceptable statistical significance suggesting a high importance of lipophilic and electronic properties of *N*-benzylsalicylthioamides for their antimycobacterial activities.

$$\log MIC^{14d}_{M\ tuber.} = -0.478\ (\pm 0.107)\log P + 1.551\ (\pm 0.206)\sigma_{R1} + 0.048\ (\pm 0.009)SumS_e^w - 2.46\ (\pm 0.646) \quad \text{Eq. (1)}$$

$\mathbf{r^2} = 0.811$ $\mathbf{q^2} = 0.758$ $\mathbf{s} = 0.282$ $\mathbf{F} = 37.407$ $\mathbf{n} = 30$

$$\log MIC^{21d}_{M\ tuber.} = -0.491\ (\pm 0.097)\log P + 1.581\ (\pm 0.187)\sigma_{R1} + 0.043\ (\pm 0.008)SumS_e^w - 1.725\ (\pm 0.586) \quad \text{Eq. (2)}$$

$\mathbf{r^2} = 0.828$ $\mathbf{q^2} = 0.776$ $\mathbf{s} = 0.256$ $\mathbf{F} = 41.595$ $\mathbf{n} = 30$

$$\log MIC^{14d}_{M\ avi.} = 1.819\ (\pm 0.163)\sigma_{R1} - 0.013\ (\pm 0.002)S_n3' + 0.043\ (\pm 0.007)SumS_e^w + 2.118\ (\pm 0.765) \quad \text{Eq. (3)}$$

$\mathbf{r^2} = 0.913$ $\mathbf{q^2} = 0.88$ $\mathbf{s} = 0.219$ $\mathbf{F} = 86.974$ $\mathbf{n} = 29$

$$\log MIC^{21d}_{M\ avi.} = 1.611\ (\pm 0.19)\sigma_{R1} - 0.012\ (\pm 0.002)S_n3' + 0.041\ (\pm 0.008)SumS_e^w + 2.101\ (\pm 0.887) \quad \text{Eq. (4)}$$

$\mathbf{r^2} = 0.864$ $\mathbf{q^2} = \mathbf{0.819}$ $\mathbf{s} = 0.254$ $\mathbf{F} = 52.98$ $\mathbf{n} = 29$

$$\log MIC^{14d}_{M\ kan.} = -0.472\ (\pm 0.106)\log P + 1.948\ (\pm 0.204)\sigma_{R1} + 0.04\ (\pm 0.009)SumS_e^w - 1.27\ (\pm 0.64) \quad \text{Eq. (5)}$$

$\mathbf{r^2} = 0.846$ $\mathbf{q^2} = 0.796$ $\mathbf{s} = 0.28$ $\mathbf{F} = 47.615$ $\mathbf{n} = 30$

$$\log MIC^{21d}_{M\ kan.} = 39.247\ (\pm 10.225)charge1 + 98.36\ (\pm 9.011)S_eH - 3.271\ (\pm 0.43)Sumf_e + 13.978\ (\pm 2.456) \quad \text{Eq. (6)}$$

$\mathbf{r^2} = 0.886$ $\mathbf{q^2} = 0.856$ $\mathbf{s} = 0.236$ $\mathbf{F} = 67.402$ $\mathbf{n} = 30$

$$\log MIC^{14d}_{M\ kan.\ cl.} = -0.486\ (\pm 0.103)\pi_{R2} + 177.82\ (\pm 18.757)\ chargeH + 0.046\ (\pm 0.007)SumS_e^w - 64.828\ (\pm 6.336) \quad \text{Eq. (7)}$$

$\mathbf{r^2} = 0.869$ $\mathbf{q^2} = 0.811$ $\mathbf{s} = 0.249$ $\mathbf{F} = 57.257$ $\mathbf{n} = 30$

$$\log MIC^{21d}_{M\ kan.\ cl.} = -0.401\ (\pm 0.092)\log P + 1.731\ (\pm 0.177)\sigma_{R1} + 0.034\ (\pm 0.008)SumS_e^w - 1.006\ (\pm 0.556) \quad \text{Eq. (8)}$$

$\mathbf{r^2} = 0.852$ $\mathbf{q^2} = 0.791$ $\mathbf{s} = 0.243$ $\mathbf{F} = 50.012$ $\mathbf{n} = 30$

The most active compound can be obtained by a synthesis of four compounds if the Topliss approach [17] is used.

The eight above-mentioned MLR QSAR models represent the best three-parameter linear relationships found in 177 investigated descriptors. Generally, the antimycobacterial activities of *N*-benzylsalicylthioamides against *M. tuberculosis*, *M. avium*, *M. kansasii,* and *M. kansasii cl.* are enhanced by electron-donating as well as lipophilic substituents at the acyl moiety of *N*-benzylsalicylthioamides. It seems that electronic properties of the substituents in

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the acyl moiety of *N*-benzylsalicylthioamides induce more distinctive changes in the activity than the substituents on the benzyl. For further development of *N*-benzylsalicylthioamides, we suggest to introduce potent electron-donating substituents (*e.g.*, $-N(CH_3)_2$) into the acyl moiety and to keep lipophilic substituents in the amine moiety. Contrary to isosteres of salicylanilides, the antimycobacterial activity of which has been recently found to be enhanced by electron-withdrawing substituents (paper to be published), the *N*-benzylsalicylthioamides may act with a different mechanism. We suppose that increasing the electron density on the sulfur of the CSNH group is the key factor for increasing the antimycobacterial activity.

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The authors have declared no conflict of interest.

Experimental

Melting points were determined on a Kofler block (C. Reichert, Vienna, Austria) and are uncorrected. The IR spectra were measured in KBr pellets or in $CHCl_3$ solutions on a Nicolet Impact 400 apparatus (Nicolet, Madison, WI, USA); the wave numbers are given in cm^1. The NMR spectra were recorded on a Varian Mercury–Vx BB 300 spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 300 MHz for ^1H and 75 MHz for ^{13}C in d_6-DMSO. Chemical shifts were recorded as d values in ppm, and were indirectly referenced to tetramethylsilane via the solvent signal (7.26 for ^1H and 77.0 for ^{13}C). The coupling constants *J* are given in Hz. Elemental analyses were done on a CHNS-O CE FISONS EA1110 elemental analyzer (Fisons Instruments, Italy). Analyses of the C, H, N, and S contents were within ± 0.4% of the theoretical values. To check the purity of the products, TLC was performed on silica gel plates precoated with a fluorescent indicator, Silufol UV 254 + 366 (Kavalier, Votice, The Czech Republic), in cyclohexane / acetone 3 : 1. Interpretation of NMR spectra and values of elemental analyses are in the attachment to the journal.

General procedure for preparation of *N*-benzylsalicylamides 1–15

A suspension of substituted salicylic acid (0.02 mol) and substituted benzylamine (0.02 mol) in chlorobenzene (100 mL) was heated under reflux in the presence of PCl_3 (0.01 mol) for 3 h. The reaction mixture was filtered while hot, and the solvent was evaporated under reduced pressure. The product was recrystallized from ethanol / water (yields were in the range of 49–95%).

General procedure for the preparation of *N*-benzylsalicylthioamides 16–45

A suspension of starting *N*-benzylsalicylamide (0.005 mol) and an equivalent amount of P_4S_{10} in pyridine (10 mL) was heated under reflux for 45 min in a microwave reactor. Without isolation of the intermediary product, the reaction mixture was added to a mixture of toluene (150 mL) and dilute HCl (150 mL, pH 1), and refluxed under vigorous stirring for 3 h. The toluene layer was separated, toluene was evaporated, and the residue was chromatographed (silica gel, toluene). The product was recrystallized from ethanol / water (yields were in the range of 17–81%).

Tests for cytotoxicity and antiproliferative activity

Cells and culture conditions

Cells /cell-culture medium: A: Huvec (ATCC CRL-1730) / DMEM (Cambrex 12-614F; Cambrex Bio Science at Biotech A.S., Prague, Czech Republic); B: K-562 (DSM ACC 10) / RPMI 1640 (Cambrex 12-167F); C: HeLa (DSM ACC 57) / RPMI 1640 (Cambrex 12-167F).

Cells were grown in the appropriate cell-culture medium supplemented with 10 mL/L ultraglutamine I (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; Fisher Scientific spol. s.r.o., Pradubice, Czech Republic).

Antiproliferative assay

The test substances were dissolved in DMSO before being diluted in DMEM / Dulbecco's modified Eagle medium. 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).

Cytotoxic assay

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

Condition of incubation

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

Method of evaluation

To estimate the influence of chemical compounds on cell proliferation of K-562, we determinate 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).

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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).

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