Full Paper

Synthesis and Cytotoxic Activity of Imidazo[1,2-*a*]-1,3,5-triazine Analogues of 6-Mercaptopurine

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A series of 2-substituted imidazo[1,2-*a*]-1,3,5-triazines with various aliphatic and aromatic amines has been prepared and characterized by IR, ^{1}H-NMR, and elemental analysis. The initial *in-vitro* cytotoxicity studies with five human cancer cell lines showed that all but one of the compounds are without cytotoxic activity. The one active compound, 2-(indolin-1-yl)-7,8-dihydroimidazo[1,2-*a*]-1,3,5-triazine-4(6*H*)-thione **12,** was tested on 12 human cancer cell lines. Of these, two lines, RT-4 and MCF-7, were the most sensitive to the compound, with IC_{50} values of 6.98 μM and 8.43 μM, respectively. When compared with the reference anticancer drug 6-mercaptopurine, only the RT-4 urinary bladder and KYSE-70 oesophagus cancer cell lines were more sensitive to the new compound. The antimetabolite thioguanine was more cytotoxic than **12** for all common cell lines tested.

Keywords: Aliphatic and aromatic amines / Cytotoxic activity / 6-Mercaptopurine / 2-Substituted imidazo[1,2-*a*]-1,3,5-triazines / Thioguanine

Received: August 20, 2007; accepted: September 20, 2007

DOI 10.1002/ardp.200700176

Introduction

Our research interests have been focused on the synthesis and evaluation of the cytotoxic activity of variously substituted imidazo[1,2-*a*]-1,3,5-triazines, which can be regarded as 5-aza-7-deazapurine derivatives. Depending on the nature of the substituents, representatives of this group have been found to possess antidepressant [1], herbicidal [2], antiviral [3 – 5] activities as well as to affect the circulatory system [6 – 8] and the thrombocyte aggregation [9]. In attempting to develop a new class of antitumor agents, we used the imidazo[1,2-*a*]1,3,5-triazine-2,4-dithione scaffold, prepared according to a previously described procedure [10], since this structure bears resemblance to the well known chemotherapeutic drug 6-mercaptopurine. The structure of the imidazo[1,2-*a*]-1,3,5-triazines can be compared to already known antimetabolites and analogues of purines, i. e. 6-mercaptopurine and thioguanine (Fig. 1), which have been used in the anticancer therapy successfully for years [11]. Therefore, we investigated their potential cytotoxic activity on a panel of 12 human cancer cell lines.

Results and discussion

Chemistry

The substrate 7,8-dihydroimidazo[1,2-*a*]-1,3,5-triazine-2,4(3*H*,6*H*)-dithione **1**, 2-(alkylthio)-7,8-dihydroimidazo [1,2-*a*]-1,3,5-triazine-4(6*H*)-thiones **2 – 6** and 2-amino(hydrazine)7,8-dihydroimidazo[1,2-*a*]-1,3,5-triazin-4(6*H*)-thiones **10**, **11**, **16**, and **17** were prepared according to the procedure previously described [7, 8, 10]. Thus, the reaction of 2-chloro-4,5-dihydroimidazolium hydrogen sulfate with ammonium thiocyanate in acetone led to the forma-

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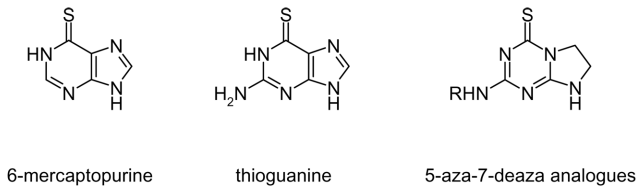
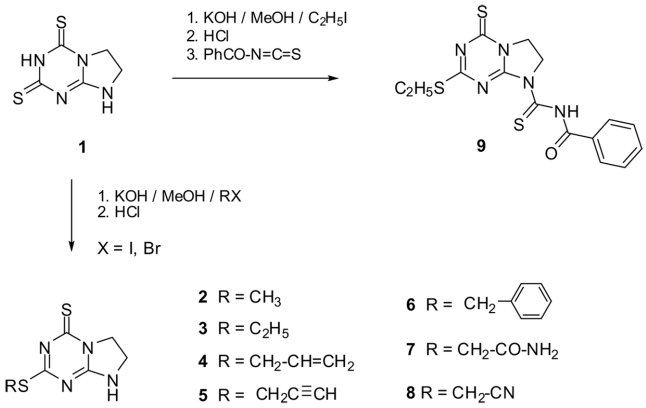
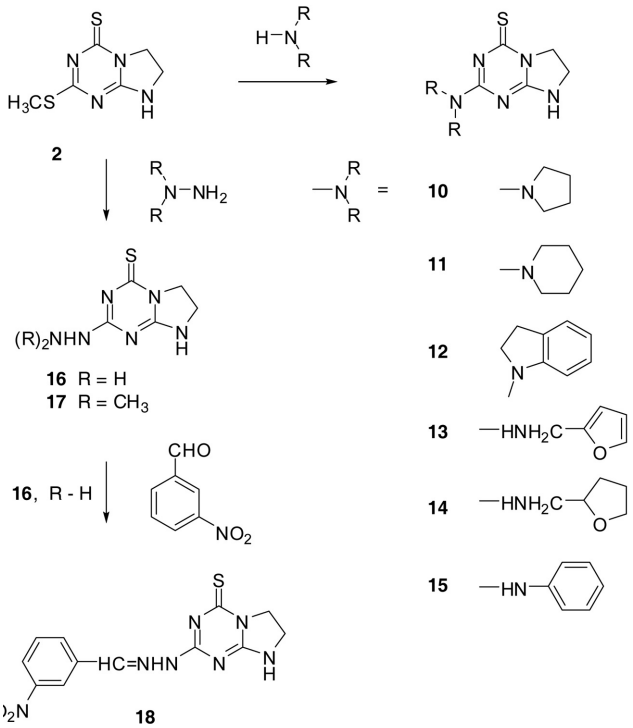


Figure 1. Structure of 6-mercaptopurine and thioguanine and their 5-aza-7-deaza analogues.



Scheme 1. General procedure for preparation of 7,8-dihydro-2-alkylthio-imidazo[1,2-*a*]-1,3,5-triazine-4(*6H*)-thiones **2**-**9**.



Scheme 2. Synthesis of 2-amino(hydrazino)-imidazo[1,2-*a*]-1,3,5-triazines **10**–**18**.

tion of the desired dithione **1**. Then, the substrate **1** was *S*-alkylated regioselectively with corresponding iodide or bromide under alkaline conditions, as shown in Scheme 1.

The identity of the newly obtained compounds **7** and **8** was confirmed by elemental analysis and IR as well as ^{1}H-NMR spectroscopic data. For example, the IR spectrum of **8** exhibited absorption at 3210, 2248 and 1661 cm^{-1}, corresponding to amide NH, nitrile C≡N, and imine C=N groups, respectively. The ^{1}H-NMR spectrum run in DMSO-d_6 revealed the presence of two multiplets at 3.6-3.8 and 4.1–4.3 ppm attributable to the 1,2-disubstituted imidazoline ring, a singlet integrating the two protons of the CH_2-C≡N group, and a singlet at 9.6 ppm, corresponding to the exchangeable proton of the NH group.

Then, we turned our attention to the reaction of imidazotriazines with isocyanates. Previously, it was found that compounds of type **2** react with aryl isocyanates at the N8-H group to give the corresponding *N*-arylcarbamoyl derivatives [7]. As shown in Scheme 1, the reaction of **3** with benzoyl isothiocyanate, carried out in acetone at room temperature, gave the N_8-benzoylthiocarbamoyl, compound **9.**

In the ^{1}H-NMR spectrum of **9**, the S-C_2H_5 group appears as a triplet of the CH_3 group at 1.4 ppm and quartet of the CH_2 group at 3.3 ppm; the imidazoline CH_2-CH_2 moiety gives a multiplet in the range of 4.0–4.1 ppm; aromatic protons are found as a multiplet at 7.2–7.6 ppm and the proton of the thioureido NH group is found at 10.6 ppm.

Compound **2** served as a substrate for further transformations by means of the nucleophilic displacement of methylthio-leaving group with aliphatic and aromatic amines as well as hydrazines (Scheme 2). According to this procedure, a series of known **10**, **11**, **16, 17** and novel compounds **12**–**15** were prepared.

Hydrazine derivative **16** obtained in this manner was then reacted with 3-nitrobenzaldehyde in boiling ethanol in the presence of catalytic amount of piperidine to give the corresponding hydrazone **18** (Scheme 2). All the compounds obtained gave satisfactory results of elemental analyses, and their structure was further confirmed by IR and ^{1}H-NMR spectral data (Experimental, Section 4).

It is noteworthy, that, for the first time, we succeeded in reacting compound **2** with an aromatic amine. Upon heating **2** with excess aniline at reflux for 1 h, the expected 2-(phenylamino)-7,8-dihydroimidazo[1,2-*a*]-1,3,5-triazine-4(*6H*)-thione **15** was obtained in 58% yield (Scheme 2). Interestingly, the ^{1}H-NMR spectra of **15** run at 200 and 500 MHz exhibited broadened signals attributable to protons 2,6-H of the aromatic ring in the range of 7.6–7.9 ppm and four broad signals of NH protons in the range of 8.6–10.2 ppm. Such a pattern indicates that a dynamic process takes place and two different tautomeric forms exist in DMSO solution.

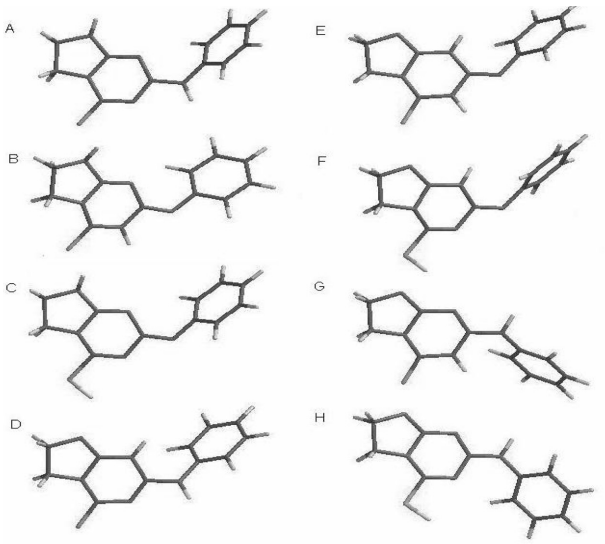


Figure 2. Theoretical structures of possible tautomers of imidazo[1,2-*a*]-1,3,5-triazine **15,** optimized by semi-empirical AM1 calculations.

Table 1. Heat of formation (ΔH_f), ΔE, and dipole moments (μ) of possible tautomers **15A**–**H**.

	Heat of formation (kcal/mol)	ΔE (kcal/mol)	Dipole moment (Debye)
A	130.92	0	6.71
B	131.46	0.54	5.49
C	139.46	8.54	6.54
D	136.37	5.45	6.25
E	134.08	3.16	3.11
F	138.34	7.42	3.17
G	134.23	3.31	3.68
H	137.35	6.43	4.66

To shed light on this phenomenon, we have calculated the heats of formation (ΔH_f) of eight possible tautomeric forms **15A-H** (Fig. 2) using quantum chemical calculations optimized by the semi-empirical AM1 method. The comparison of the heats of formation (ΔH_f) and dipole moments (μ) for tautomers **15A-H** shows that the most stable structure appears to be tautomer **A** with ΔH_f = 30.92 kcal/mol and μ = 6.71 Debye followed by tautomer **B** (ΔH_f = 131.46 kcal/mol, μ = 5.49 Debye).

Biology

Cytotoxic activity

The cytotoxic activities of imidazo[1,2-*a*]-1,3,5-triazine derivatives were examined in the primary testing on five human cancer cell lines: the breast cancer cell line MCF-7, two urinary bladder cancer cell lines RT-4 and 5637 and two non-small cell lung cancer cell lines – LCLC-103H and A-427 (Table 2). A well established microtiter assay based on the staining of cells with crystal violet was used to determine the antiproliferative activity of the compounds [12]. The compounds were added to all cancer cell lines at the same concentration of 20 μM.

Table 2. The relative percent of cell growth (%) compared to untreated control at a concentration of 20 μM. (N = 1–2 determinations)

	Cell lines				
Compound	MCF-7[a)]	RT-4[a)]	LCLC-103H	A-427	5637
2	91.56	99.86	56.85[a)]	76.08[a)]	78.29[a)]
3	108.43	107.86	91.48	108.15	92.17
4	112.70	105.13	92.79	110.78	102.28
5	100.03	103.24	93.93	101.58	98.37
6	113.56	104.05	76.07	95.14	82.06
7	112.03	106.44	66.70[a)]	66.00[a)]	64.47[a)]
8	86.76	82.37	86.72	126.02	97.39
9	102.69	106.50	94.65	91.42	86.58
10	105.34	107.69	112.81	106.37	102.03
11	97.36	110.12	118.33	102.08	97.66
12	22.00	28.56	66.37	65.15	49.77
13	113.30	106.76	105.87	105.33	99.53
14	68.99	67.37	84.52	79.45	70.36
15	97.97	103.51	112.28	103.64	105.46
16	94.06	93.20	81.32	80.49	73.48
17	106.60	104.70	103.02	107.28	98.28
18	112.17	68.82	106.94	92.07	94.70

[a)] Average of two determinations

Primary screening indicates whether a substance is active enough to inhibit the cell growth by 50% at 20 μM, which is a concentration that can typically be attained in patients. Substances that would inhibit the growth of these five lines by more than 50% at the concentration of 20 μM would be considered active. As shown in Table 2, only the indoline derivative **12** was active in this screen. Thus, compound **12** was examined further for potency of action in the secondary screening on twelve human cancer cell lines.

Compound **12** was tested at five successive concentrations on the cell lines: LCLC-103H, A-427, 5637, KYSE-510, KYSE-520, YAPC, RT-4, RT-112, DAN-G, KYSE-70, SISO, MCF-7. The concentration ranges were adjusted depending on the activity of the tested compound. The results of the secondary screening tests are shown in Table 3 as the average IC_{50} values with standard deviation, calculated from the results of three independent experiments.

The relatively high values of standard deviations may be the result of poor solubility of compound **12** in the culture medium. As a result, the compound may have been tested in some cases as a suspension in the medium. In fact, the low water solubility of all tested compounds might have influenced their activity.

Table 3. IC_{50} values (μM) ± S.D. in twelve human cancer cell lines (N = 3 determinations)

	Cell lines											
	5637	A-427	Kyse-520	SISO	RT-4	YAPC	LCLC-103H	Kyse-510	RT-112	MCF-7	DAN-G	Kyse-70
Compd. 12	22.43 ± 1.6	15.1 ± 5.1	21.7 ± 1.2	26.9 ± 5.8	6.98 ±1.3	37.57 ± 2.4	21.2 ± 5.6	26.67 ± 2.8	21.8 ± 0.9	8.43 ± 2.0	28.8 ± 1.6	23.4 ± 2.8
6-Mercaptopurine	Nd[a)]	0.37 ± 0.13	Nd[a)]	2.82 ± 0.64	15.8 ± 3.5	Nd[a)]	2.18 ± 2.24	1.78 ± 0.88	Nd[a)]	2.79 ± 0.69	15.2 ± 7.4	33.3 ± 9.5
6-Thioguanine	3.53 ± 1.40	0.31 ± 0.28	Nd[a)]	1.50 ± 0.23	3.98 ± 0.50	Nd[a)]	1.06 ± 0.74	0.016 ± 0.015	Nd[a)]	2.64 ± 1.86	0.72 ± 0.15	8.69 ± 1.43

[a)] Nd, not determined

Although the MCF-7 and RT-4 cell lines are the most sensitive to compound **12**, the results show no great selectivity towards any one specific cancer cell line (Table 3). For example, this compound **12** is only 5.4 fold less active on the YAPC cell line than on the RT-4 cell line and 4.5 fold less active than on the MCF-7 cell line. Compound **12** shows relatively low selectivity over all cell lines (relative standard deviation of mean IC_{50} is only 39%).

The IC_{50} values of known anticancer drugs like 6-mercaptopurine and 6-thioguanine are in general much lower than for **12**, with the exception of the RT-4 and KYSE-70 cell lines (Table 3). Compound **12** is twice as active as 6-mercaptopurine on the RT-4 cell line. However, **12** is only half as active in comparison to 6-thioguanine in the same cell line. Imidazotriazine **12** also shows somewhat better cytotoxic activity on the KYSE-70 cancer cell line than 6-mercaptopurine. On the other hand, thioguanine was more active than **12** in all the common cell lines tested.

Conclusions

Here, we have synthesized a series of imidazo[1,2-*a*]-1,3,5-triazines by substituting an aliphatic or aromatic amine at the ring position 2. Five novel compounds were obtained, among them a derivative with an aromatic substituent was synthesized for the first time. This group of compounds possessed a general lack of cytotoxic activity on human cancer cells in culture. Only 2-(indolin-1-yl)-7,8-dihydroimidazo[1,2-*a*]-1,3,5-triazine-4(6*H*)-thione **12** was active enough to enter the secondary screening and was tested on 12 cancer cell lines. The lines MCF-7 and RT-4 were the ones most sensitive to this compound. In comparison to the established cytotoxic activity of 6-mercaptopurine, **12** is more active only on the RT-4 and KYSE-70 cancer cell lines.

The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were defined on a Boetius 545 apparatus (Franz Kiistner, Dresden, Germany) and are uncorrected. The IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer as KBr pellets (Perkin-Elmer, Norwalk, CT, USA). The ^{1}H-NMR spectra were measured with the Varian Gemini Plus spectrometer (Varian Inc., Palo Alto, CA, USA) in DMSO-$_{d6}$ solution at 200 Hz or 500 MHz. The chemical shift values δ are expressed in ppm units towards tetramethylsilane used as internal standard. Molecular modeling studies were performed using Spartan programs v. 5.0 distributed by Wavefunction Inc. (Irvine, CA, USA) and installed on a Silicon Graphics *02* workstation.

*2-(4-thioxo-4,6,7,8-tetrahydroimidazo[1,2-a]-1,3,5-triazin-2-ylthio)acetamide **7***

Compound **1** was treated with 1.5 molar excess of chloroacetamide in methanol in the presence of KOH and the reaction mixture was left at room temperature for 1 h. The solid that precipitated was filtered off, dried, and dissolved in water. After acidification with aqueous solution of HCl the precipitated product **7** was collected by suction and recrystallized from DMF. Yield 0.27 g (26%), mp. 218–225°C. IR (KBr) ν (cm^{-1}): 3420, 3170 (NH), 1654 (C=O), 1517, 1446, 1423, 1347, 1290, 1268, 1227. ^{1}H-NMR (DMSO) δ = 3.6–4.0 (m, 4H, CH_2), 4.1–4.3 (t, 2H, CH_2), 7.2 (s, 2H, NH_2), 9.3 (s, 1H, NH). Anal. calcd. for $C_7H_9N_5OS_2$ (243.309): N, 28.78. Found N, 28.52.

*2-(4-thioxo-4,6,7,8-tetrahydroimidazo[1,2-a]-1,3,5-triazin-2-ylthio)acetonitrile **8***

Prepared as described for compound **7**.

Mp. 246–253°C. IR (KBr) ν (cm^{-1}): 3210 (NH), 2248 (CN), 1661, 1634, 1510, 1434, 1340, 1269, 1225. ^{1}H-NMR (DMSO) δ = 3.6–3.8 (m, 2H, CH_2 imidazoline), 4.2 (s, 2H, CH_2-CN), 4.1–4.3 (m, 2H, CH_2 imidazoline), 9.6 (s, 1H, NH). Anal. calcd. for $C_7H_7N_5S_2$ (225.294): C, 37.32, H, 3.13, N, 31.09. Found C, 37.57, H, 3.17, N, 31.99.

*{[2-(ethylthio)-4-thioxo-6,7-dihydroimidazo[1,2-a][1,3,5]-triazin-8-(4H)-yl]carbonthioyl}benzamide **9***

To a suspension of compound **3** in anhydrous acetone was added an equimolar amount of benzoyl isothiocyanate. The reaction mixture was stirred at room temperature for 12 h and the precipitating yellowish solid **9** was collected by filtration. Yield 79%, mp. 214–216°C. IR (KBr) ν (cm^{-1}): 3335, 3128 (NH), 1674 (C=O), 1517, 1438, 1427, 1376, 1245, 1234, 1216. ^{1}H-NMR (DMSO) δ = 1.4 (t, 3H, CH3), 3.3 (q, 2H, CH_2), 4.0–4.1 (m, 4H, CH_2CH_2), 7.2–7.6 (m, 5H, aromat), 10.6 (s, 1H, NH). Anal. calcd. for $C_{15}H_{15}N_5OS_3$ (377.507): N, 18.55. Found N, 18.54.

*2-(alkyl(aryl)amino)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thiones **12–15***

Compound **2** was dissolved in an excess of corresponding amine and heated at reflux for 1 h. After cooling to room temperature,

the precipitate was separated by suction and purified by recrystallization from a suitable solvent. According to this procedure, the following novel compounds were obtained.

2-(Indolin-1-yl)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thione ***12***

Yield 59%, mp. 336 – 338°C (EtOH). IR (KBr) ν (cm^{-1}): 3221 (NH), 1636 (C=C), 1522, 1465, 1280. ^{1}H-NMR (DMSO) δ = 3.2 – 3.3 (t, 2H, CH_2), 3.6-3.7 (t, 2H, CH_2), 4.1 – 4.3 (m, 4H, CH_2CH_2), 7.0 – 7.1 (m, 2H, Ar-H), 7.2 – 7.35 (m, 2H, Ar-H), 9.0 (s, 1H, NH). Anal. calcd. for $C_{13}H_{13}N_5S$ (271.341): N, 25.81. Found: N, 26.06.

2-(Furan-2-ylmethylamino)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thione ***13***

Yield 69%, mp. 253 – 259°C (DMF). IR, (KBr) ν (cm^{-1}): 3420, 3360, 3202 (NH), 3096, 2917, 1660, 1602, 1508, 1341. Anal. calcd. for $C_{10}H_{11}N_5OS$ (249.292): N, 28.09. Found: N, 28.17.

2-((Tetrahydrofuran-2-yl)methylamino)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thione ***14***

Yield 76%, mp. 259 – 263°C (EtOH). IR (KBr) ν (cm^{-1}): 3366, 3214, 3098, 1658, 1644, 1577, 1514, 1428, 1372, 1114. Anal. calcd. for $C_{10}H_{15}N_5OS$ (253.324): N, 27.65. Found: N, 27.34.

2-(Phenylamino)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thione ***15***

Yield 58%, mp. 223 – 227°C (DMF / H_2O). IR (KBr) ν (cm^{-1}): 3403, 3252, 1654, 1599, 1532, 1490, 1446, 1337, 1277. ^{1}H-NMR (DMSO) δ = 3.6 – 3.75 (t, 2H, CH_2), 4.2 – 4.35 (t, 2H, CH_2) 7.0 – 7.15 (t, 1H, aromat), 7.25 – 7.4 (t, 2H, aromat), 7.6 – 7.8 (br s, 2H, aromat), 9.0 (br s1H, NH) 10.1 (br s, 1H, NH). Anal. calcd. for $C_{11}H_{11}N_5S$ (245.304): N, 28.55. Found: N, 28.89.

2-(2-(3-nitrobenzylideno)hydrazinyl)-7,8-dihydroimidazo[1,2-a]-1,3,5-triazine-4(6H)-thione ***18***

To a solution of the hydrazine compound **16** (0.5 g, 3 mmol) and *m*-nitrobenzaldehyde (0.45 g, 3 mmol) in ethanol (10 mL) was added five drops of piperidine. The reaction mixture was heated at reflux for 10 h. Upon cooling to room temperature, a precipitate formed; it was separated by filtration and purified by crystallization from DMF. Yield 0.6 g (69.8%) of **18** as a yellow powder, mp. 299 – 305°C. IR (KBr) ν (cm^{-1}): 3196 (NH), 3091, 1654 (C=C), 1578 (NO_2), 1348 (NO_2), 1120. ^{1}H-NMR (DMSO) d = 3.6 – 3.8 (t, 2H, CH_2), 4.1 – 4.3 (t, 2H, CH_2), 7.7 – 7.84 (m, 4H, aromat.), 8.0 – 8.3 (s, 1H, =CH). Anal. calcd. for $C_{12}H_{11}N_7O_2S$ (317.326): C, 45.42, H, 3.49, N, 30.90. Found: C, 45.15, H, 3.75, N, 20.64.

In-vitro cytotoxicity studies

The method used for cytotoxicity testing has been described in detail elsewhere [12]. All cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). The cells were grown in RPMI-1640 medium that contained 2 g/L $NaHCO_3$, 10% fetal calf serum (FCS) and supplemented with penicillin G/streptomycin. The exception was for the MCF-7 cell line where the medium contained pyruvate (1 mM), MEM salts and amino acid. The cells were incubated 7 days in 75 mL plastic culture flasks. The atmosphere components were 95% air and 5% of CO_2 at 37°C.

The cytotoxicity testing was carried out in 96-well microtiter plates, whereby 100 µL of cell suspensions per each well were seeded at a density of 1000 cells per well (with an exception for the LCLC-103H line with only 500 cells).

For the secondary screening, cells were treated with substance at five successive concentrations; i. e. the 1000-fold concentrated stock solution in DMF was serially diluted by 50% in DMF to give feed solutions, which were diluted 500-fold into the culture medium. Controls wells received just 100 µL of culture medium containing 0.1% DMF. One plate was untreated for each cell line and fixed immediately with glutaraldehyde. Later, this plate served as the ‚C,0' control.

After 96-hours of exposure to the drug, the medium was removed and 1% glutaraldehyde buffer solution (GBS) was added to fix the cells for 20 min. Cells were stored at 4°C under PBS. Staining with crystal violet was done as previously described [11]. The optical density (OD) was measured at λ = 570 nm with an Anthos 2010 microplate reader (Salzburg, Austria). To calculate the T/C values, the equation was used as explained below:

$$T/C\ corr. = (OD_T - OD_{C,0})/(OD_C - OD_{C,0}) \cdot 100$$

where OD_T is mean OD of the treated cells; OD_C the mean OD of the controls, and $OD_{C,0}$ the mean OD at the time the drug was added. IC_{50} values were obtained by linear regression of the T/C values that ranged between 90 and 10%. The reported IC_{50} values are the average of three independent experiments.

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