FULL PAPER

Synthesis and Cytotoxicity of 1,4-Dihydropyridines and an Unexpected 1,3-Oxazin-6-one

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Eight heterocycles have been prepared in a one-pot reaction manner based on the *Hantzsch* dihydropyridine synthesis. The synthesis afforded seven dihydropyridines (DHP) and one unexpected 1,3-oxazin-6-one. Their structures were confirmed based on NMR spectroscopy and mass spectrometry. The obtained products have been evaluated for their cytotoxicity against eight cancer cell lines and one normal cell line. Two halogenated DHPs (**7** and **8**) displayed cytotoxicity toward all the nine tested cancer cell lines with IC_{50} values from 4.10 to 58.90 μM, while others showed selective activities. DHPs (**7** and **8**) bearing a Me group at C(2) and C(6) as well as a halogenated substituent at C(4′) were more antiproliferative than the others.

Keywords: Halogenated dihydropyridines, 1,3-Oxazin-6-one, *Hantzsch* DHP synthesis, Cytotoxic activity.

Introduction

Cancer is a group of diseases that affects organ tissues and is characterized by abnormal growth of cells with possibility of invading other healthy organs of the body through metastasis [1]. By 2020, about 15 million of new cancer cases will be diagnosed among the world population and 80% of those patients may die from that disease [2]. This dramatic increase is caused by the development of multidrug resistance of some cancer cells leading to a high and continuous demand of new antiproliferative substances. Previous studies had reported 1,4-dihydropyridines (DHP) as cytotoxic [3], reducer of DNA damage and stimulator of DNA repair in human cells [4], antimicrobial [5], antioxidant [6], hypotensive [7], and antidiabetic [8] agents.

1,4-Dihydropyridines, such as amlodipine, felodipine, nitrendipine, and nifedipine (**1** – **4**, *Fig. 1*), known as calcium channel blockers [9] used in cardiovascular and angina pectoris therapy, were reported as inhibitors of LDL oxidation and as antiproliferative agents against cholangiocarcinoma cells [10]. Therefore, the abovementioned observation aroused interest in synthesizing heterocycles, including seven DHP and one unexpected 1,3-oxazin-6-one, and to assess their cytotoxicity. Different substituted substrates used for the heterocycles preparation afforded products with moderate to no cytotoxicity against the eight cancer cell lines studied.

Results and Discussion

Chemistry

Different DHP analogs were prepared from an arylaldehyde, two equivalents of a *β*-ketoester (ethyl acetoacetate and ethyl benzoylacetate), and one equivalent of $AcONH_4$ as NH_3 source. The reaction catalyzed by $BiCl_3$ was carried out in THF under reflux and was stirred for 6 h (*Scheme 1*). Products (**5** – **11**) were obtained with yields from 10% to 92% and their structures were confirmed by NMR and MS data in addition to those reported.

An unexpected 1,3-oxazin-6-one was obtained from the similar reaction involving 3-nitrobenzaldehyde, ethyl benzoylacetate (2 equiv.), and $AcONH_4$ (1.5 equiv.) in THF (*Scheme 2*). $BiCl_3$ was also used as catalyst and the mixture was heated at 85° under stirring. After 6 h, the TLC of the mixture revealed a new entity formed in small quantity. After 24 h, the product afforded, after the workup, was identified by NMR and MS data as the oxazinone derivative **12**. The 1,3-oxazin-6-one ring was determined by comparing its C-shifts with those previously

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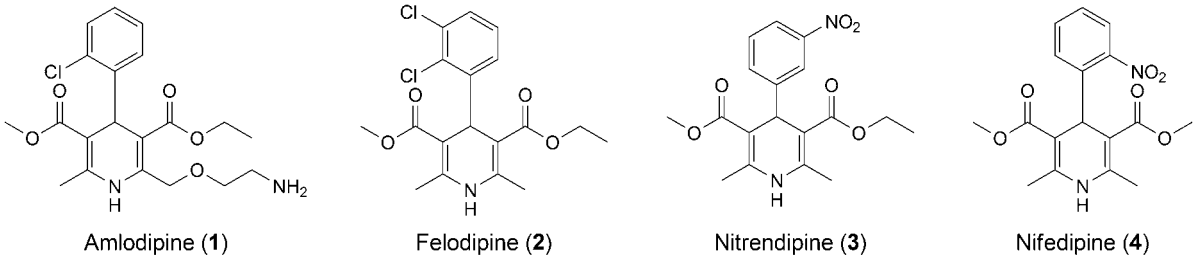
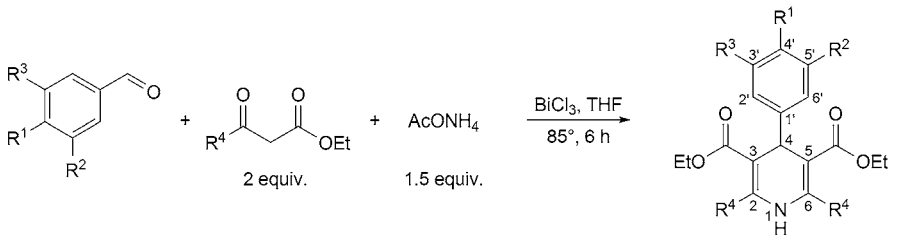


Fig. 1. Drugs with structures based on DHP scaffold.

Scheme 1. Synthesis of DHPs **5** – **11**.



	R^1	R^2	R^3	R^4	Yield [%]
5	Br	H	H	Ph	90
6	Cl	H	H	Ph	91
7	Cl	H	H	Me	88
8	Br	H	H	Me	92
9	MeO	H	H	Ph	10
10	MeO	MeO	MeO	Ph	88
11	H	H	NO_2	Me	87

reported for similar heterocycles [11] and pyrimidin-6-ones (*Fig. 2*) [12]. The conditions used for this synthesis turns to be a new method for oxazinone preparation and the mechanism is tentatively explained in *Scheme 3*. A preliminary aldol condensation took place forming **A** which was converted into the imine **B** by reacting with $AcONH_4$. The imine **B** undergoes a condensation with 3-nitrobenzaldehyde followed by a rearrangement of the H-atom through the intermediates **C** and **D** to afford the 1,3-oxazin-6-one. Previously, 1,3-oxazin-6-ones analogs were prepared either by converting *N*-thioacylisoxazolones with triphenylphosphine [13] or by rearranging *N*-acyl-4-acyloxy-*β*-lactam derivatives under basic conditions [14]. Moreover, isoxazolone and oxazoline derivatives also served as precursor for 1,3-oxazin-6-ones synthesis. Thus, after the treatment with a 1,1-dichloro-containing compound and DBN, the first intermediate led to expected product [15], whereas the same heterocycle was obtained by CO insertion into the second precursor in the presence of a cobalt catalyst [16].

The structures of **6** – **9** and **11** were previously reported [17 – 19], while **5**, **10**, and **12** are reported for the first time.

Antiproliferative Activity

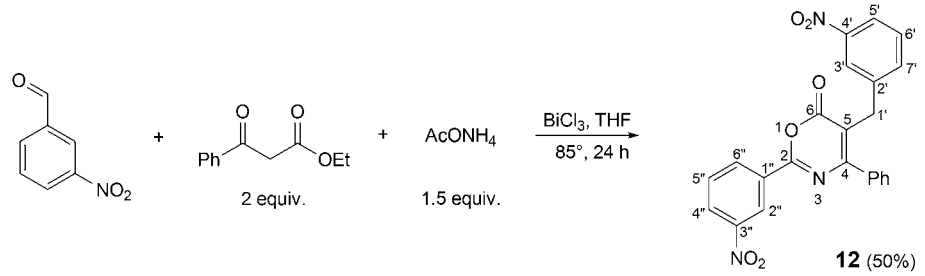
The cytotoxicity of compounds (**5** – **12**) was evaluated against nine cancer cell lines, including CCRF-CEM, CEM/ADR5000, MDA-MB231, MDA-MB231/BCRP, HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$), U87MG, U87MG.ΔEGFR, and HepG2 (*Table S1*). Compounds **7** and **8** displayed cytotoxicity toward all the tested cancer cell lines with the lowest IC_{50} values at 4.63 and 4.10 μM, respectively. These sensitivities were observed with the leukemia cancer cell lines CCRF-CEM and CEM/ADR5000, respectively. Interestingly, both DHPs were more antiproliferative against CEM/ADR5000 cell line than doxorubicin (reference drug). Halogenated DHPs bearing a Me group at C(2)/C(6) of the heterocyclic moiety (*Scheme 1*) turned to be more cytotoxic than those containing a phenyl group on the same C-atoms. However, DHPs with phenyl residues at C(2)/C(6), such as **5** and **6**, showed selective antiproliferative activity (IC_{50} at 6.21 and 5.75 μM, respectively) against CCRF-CEM when they contain a halogen atom. In general, the normal hepatocyte cell line AML12 was weakly sensitive to all the tested compounds. DHP **10** displayed moderate cytotoxicity against all cancer lines except for HCT116 ($p53^{+/+}$) with IC_{50} comprised between 21.86 and 59.93 μM.

Weak antiproliferative effect against MDA-MB and HT29 were previously reported on DHP **11** with IC_{50} values at 244.1 and 500 μM, respectively [20]. Nevertheless, it was described as an inhibitor of capacitative calcium entry in HL60 as its analogs nifedipine and nitrendipine [21]. Therefore, replacement of substituents in the DHP core by bioisosteres could be of interest in developing potent cytotoxic compounds.

Conclusion

Seven 1,4-dihydropyridine derivatives and an unexpected 1,3-oxazin-6-one were prepared from an arylaldehyde, two equivalents of a *β*-ketoester (ethyl acetoacetate and ethyl benzoylacetate), and one equivalent of $AcONH_4$ as NH_3 source. The same synthetic condition afforded a new 1,3-oxazin-6-one derivative. The cytotoxicity of all compounds was evaluated against seven cancer cell lines

Scheme 2. Synthesis of 1,3-oxazinone-6-one **12**.



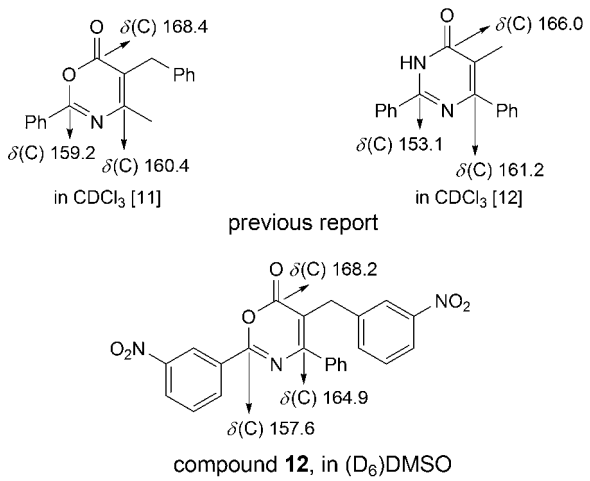


Fig. 2. Comparison of oxazinone and pyrimidone C-atom shifts [11] [12].

and one normal cell line. It turned out that the presence of a bulky substituent, such as Ph, at C(2) and C(6) might reduce the cytotoxicity, while the presence of a 4-halogenophenyl group at C(4) improve the activity. Therefore, modifying **7** and **8** structures could lead to more potent cytotoxic derivatives.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Cytotoxicity of compounds **5** – **12** toward sensitive and drug-resistant cancer cell lines and normal cells as determined by the resazurin assay.

Experimental Part

General

TLC*:* Silica gel GF_{254}. Column chromatography (CC): silica gel *60H* (particle size 90% < 45 mm, 200 – 300 mesh). M.p.: *MQAPF-301* apparatus; uncorrected. 1D- and 2D-NMR: *Bruker DRX*-400 MHz instrument; δ in ppm rel. to Me_4Si as internal standard, J in Hz. HR-ESI-MS: *Waters Xevo G2-S QToF* mass spectrometer equipped with an ESI probe; in m/z.

Synthesis of Products **5** – **11**

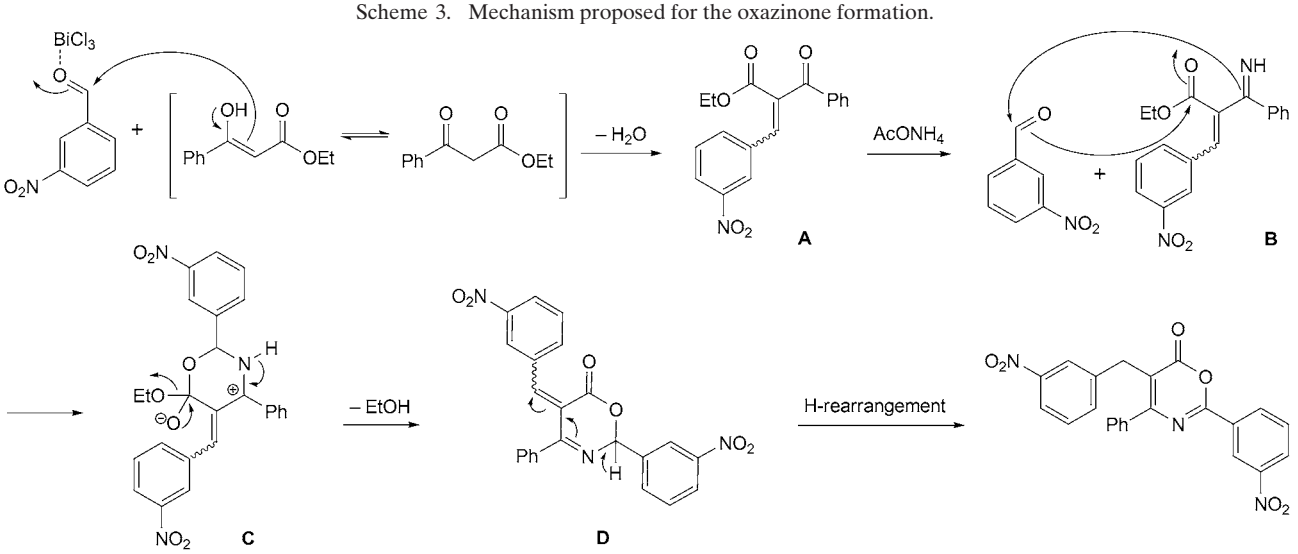
Each aldehyde (400 mg) was treated with 2 equiv. of ethyl benzoylacetate (or ethyl acetoacetate) and 1.5 equiv. of $AcONH_4$. After adding $BiCl_3$ (3 equiv.) and THF (2 ml), the medium was stirred and heated at 85° for 6 h. The halogenated products were recovered by adding CH_2Cl_2 which caused the precipitation of $BiCl_3$. Furthermore, its separation was performed by filtration, and the filtrate was evaporated *in vacuo*. The obtained residue was recrystallized in EtOH and filtered to yield yellow bright solid. Reactions from non-halogenated aldehydes afforded a brown mixture, separated from $BiCl_3$ by filtration after adding CH_2Cl_2. The solvent was evaporated by rotary evaporation and purified by CC (SiO_2) to give the non-halogenated products.

Synthesis of Product **12**

3-Nitrobenzaldehyde (400 mg) was mixed with 2 equiv. of ethyl benzoylacetate, 1.5 equiv. of $AcONH_4$, and $BiCl_3$ (3 mol equiv.). THF (2 ml) was used as a solvent and the mixture was heated at 85° under stirring for 24 h. Then, CH_2Cl_2 was added to the mixture inducing the precipitation of $BiCl_3$ and the product. Therefore, the product was poured onto H_2O and extracted three times with CH_2Cl_2. The oxazinone was obtained after evaporation of the solvent.

Diethyl 4-(4-Bromophenyl)-1,4-dihydro-2,6-diphenylpyridine-3,5-dicarboxylate (**5**). Yield: 90% (1.044 g). Yellow bright crystals. M.p. 204.8 – 205.1°. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.88 (*t*, J = 7.1, 2 Me); 3.88 (*q*, J = 7.1, 2 CH_2); 7.34 – 7.36 (*m*, 4 H, Ph); 7.38 – 7.40 (*m*, 4 H, Ph); 7.39 – 7.41 (*m*, 2 H, Ph); 7.43 (J = 8.75, 2 H, 4-BrC_6H_4); 7.45 (J = 8.75, 2 H, 4-BrC_6H_4); 5.18 (*s*, H–C(4)); 5.99 (*s*, H–N(1), exchangeable). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.8 (2 *Me*CH_2); 60.0 (2 Me*C*H_2); 166.8 (2 C=O); 136.6 (2 C, Ph); 128.2 (4 C, Ph); 128.6 (4 C, Ph); 129.5 (2 C, Ph); 146.6 (C(1′)); 129.9 (C(2′,6′));

Scheme 3. Mechanism proposed for the oxazinone formation.



131.5 (C(3′,5′)); 120.5 (C(4′)); 145.9 (C(2,6)); 104.1 (C(3,5)); 29.9 (C(4)). HR-ESI-MS: 532.1115 ([*M* + H]$^+$, $C_{29}H_{27}{}^{79}BrNO_4^+$; calc. 532.1123); 534.1100 ([*M* + H]$^+$, $C_{29}H_{27}{}^{81}BrNO_4^+$; calc. 534.1103).

Diethyl 4-(4-Chlorophenyl)-1,4-dihydro-2,6-diphenylpyridine-3,5-dicarboxylate (**6**) [17]. Yield: 91% (1.27 g). Yellow bright crystals. M.p. 186.9 – 187.1°. HR-ESI-MS: 488.1626 ([*M* + H]$^+$, $C_{29}H_{27}{}^{35}ClNO_4^+$; calc. 488.1629); 490.1601 ([*M* + H]$^+$, $C_{29}H_{27}{}^{37}ClNO_4^+$; calc. 490.1599).

Diethyl 4-(4-Chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate (**7**) [18]. Yield: 88% (0.913 g). Yellow bright crystals. M.p. 149.5 – 150.5°. HR-ESI-MS: 364.1309 ([*M* + H]$^+$, $C_{19}H_{23}{}^{35}ClNO_4^+$; calc. 364.1316); 366.1284 ([*M* + H]$^+$, $C_{19}H_{23}{}^{37}ClNO_4^+$; calc. 366.1286).

Diethyl 4-(4-Bromophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate (**8**) [19]. Yield: 92% (0.818 g). Yellow bright crystals. M.p. 163.4 – 164.5°. HR-ESI-MS: 408.0811 ([*M* + H]$^+$, $C_{19}H_{23}{}^{79}BrNO_4^+$; calc. 408.0810); 410.0796 ([*M* + H]$^+$, $C_{19}H_{23}{}^{81}BrNO_4^+$; calc. 410.0790).

Diethyl 1,4-Dihydro-4-(4-methoxyphenyl)-2,6-diphenylpyridine-3,5-dicarboxylate (**9**) [17]. Yield: 10% (0.142 g). Brown gum. HR-ESI-MS: 484.2121 ([*M* + H]$^+$, $C_{30}H_{30}NO_5^+$; calc. 484.2118).

Diethyl 1,4-Dihydro-2,6-diphenyl-4-(3,4,5-trimethoxyphenyl)pyridine-3,5-dicarboxylate (**10**). Yield: 88% (0.975 g). Yellowish solid. M.p. 189.7 – 190.1°. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90 (*t*, *J* = 7.1, 2 Me); 3.91 (*q*, *J* = 7.1, 2 CH_2); 7.35 – 7.41 (*m*, 10 H, Ph); 6.82 (*s*, H–C(2′,6′)); 5.20 (*s*, H–C(4)); 3.86 (*s*, MeO–C(3′,5′)); 3.84 (*s*, MeO–C(4′)); 6.00 (*s*, H–N(1), exhangeable). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.9 (2 *Me*CH_2), 60.2 (2 Me*C*H_2); 167.8 (2 C=O); 136.9 (2 C, Ph); 128.3 (4 C, Ph); 128.6 (4 C, Ph); 129.4 (2 C, Ph); 143.1 (C(1′)); 104.8 (C(2′,6′)); 153.2 (C(3′,5′)); 136.8 (C(4′)); 145.6 (C(2,6)); 104.4 (C(3,5)); 40.3 (C(4); 56.2 (*Me*O–C(3′,5′)); 61.0 (*Me*O–C(4′)). HR-ESI-MS: 544.2335 ([*M* + H]$^+$, $C_{32}H_{34}NO_7^+$; calc. 544.2330).

Diethyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (**11**) [18]. Yield: 87% (0.862 g). Yellow bright crystals. M.p. 162.4 – 163.1°. HR-ESI-MS: 375.1557 ([*M* + H]$^+$, $C_{19}H_{23}N_2O_6^+$; calc. 375.1551).

2-(3-Nitrophenyl)-5-[(3-nitrophenyl)methyl]-4-phenyl-6*H*-1,3-oxazin-6-one (**12**). Yield: 50% (0.568 g). Brownish solid. M.p. 247.7 – 247.9°. ^{1}H-NMR (400 MHz, C_5D_5N): 7.52 – 7.54 (*m*, 2 H, Ph); 7.56 – 7.58 (*m*, 2 H, Ph); 7.80 – 7.82 (*m*, 1 H, Ph); 4.30 (*s*, CH_2 (1′)); 8.31 (*t*, *J* = 2.0, H–C(3′)); 8.06 (*ddd*, *J* = 1.0, 2.4, 8.0, H–C(5′)); 7.39 (*t*, *J* = 8.0, H–C(6′)); 7.65 (*d*, *J* = 8.0, H–C(7′)); 9.31 (*t*, *J* = 2.0, H–C(2″)); 8.37 (*ddd*, *J* = 1.2, 2.4, 8.2, H–C(4″)); 7.65 (*d*, *J* = 8.0, H–C(5″)); 8.81 (*dt*, *J* = 1.2, 8.0, H–C(6″)). ^{13}C-NMR: 139.7 (C, Ph); 129.6 (2 CH, Ph); 129.3 (2 CH, Ph); 130.0 (CH, Ph); 32.7 (C(1′)); 143.3 (C(2′)); 124.2 (C(3′)); 149.1 (C(4′)); 121.9 (C(5′)); 130.2 (C(6′)); 135.4 (C(7′)); 138.2 (C(1″)); 123.6 (C(2″)); 149.3 (C(3″)); 126.1 (C(4″)); 130.5 (C(5″)); 134.7 (C(6″)); 157.6 (C(2)); 164.9 (C(4)); 119.2 (C(5)); 168.2 (C(6)). HR-ESI-MS: 429.0963 (M^+, $C_{23}H_{15}N_3O_6^+$; calc. 429.0961).

Cytotoxicity Assays

The resazurin reduction assay [22] was performed to assess the cytotoxicity of compounds **5** – **12** and doxorubicin as control drug toward various sensitive and drug-resistant cancer cell lines, including the CCRF-CEM and CEM/ADR5000 leukemia, MDA-MB231 breast cancer cells and its resistant subline MDA-MB231/*BCRP*, HCT116*p53*$^{+/+}$ colon cancer cells and its resistant subline HCT116*p53*$^{-/-}$, U87MG glioblastoma cells and its resistant subline U87MG.*ΔEGFR* and HepG2 hepatocarcinoma cells and normal AML12 hepatocytes The assay is based on the reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Non-viable cells rapidly lose their metabolic capacity to reduce resazurin and, thus,

do not produce fluorescent signals anymore. Briefly, adherent cells were detached by treatment with 0.25% trypsin/EDTA (*Invitrogen*, Darmstadt, Germany) and an aliquot of 1×10^4 cells was placed in each well of a 96-well cell culture plate (*Thermo Scientific*, Langenselbold, Germany) in a total volume of 200 µl. Cells were allowed to attach overnight and then were treated with different concentrations of compounds. For suspension cells, aliquots of 2×10^4 cells per well were seeded in 96-well-plates in a total volume of 100 µl. The studied compound was immediately added in varying concentrations in an additional 100 µl of culture medium to obtain a total volume of 200 µl/well. After 72 h, resazurin (*Sigma-Aldrich*, Schnelldorf, Germany) (20 µl, 0.01% *w*/*v*) in distilled H_2O was added to each well and the plates were incubated at 37° for 4 h. Fluorescence was measured on an *Infinite M2000 ProTM* plate reader (*Tecan*, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least twice with six replicates each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent the compound concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel.

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