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R176502, a New Bafilolide Metabolite with Potent Antiproliferative Activity from a Novel *Micromonospora* Species

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During the course of a screening program intended to identify new antiproliferative agents, a new bafilolide metabolite was discovered. R176502 (**1**) was isolated from the liquid fermentation cultures of a novel *Micromonospora* species found in African river bottom sediment. It was purified from ethyl acetate extracts using a series of countercurrent chromatographic steps. The structure was determined using 1- and 2-D NMR experiments. Three previously described bafilomycins (bafilomycins A_1 (**2**), B_1 (**3**), and B_2 (**4**)) were also isolated (from other microbial strains). R176502 exhibited potency for inhibition of tumor cell proliferation in the nM range of concentrations.

The progress that has been made in the field of major cellular signaling pathways has resulted in recent discoveries of promising "targeted therapeutics" to help fight cancer (*e.g.*, inhibitors of farnesyltransferase, growth factor receptor tyrosine kinases, Bcr/abl, *etc.*). Despite these advances, more classical cytotoxics still remain an important part of the foundation of an oncology product portfolio in the modern pharmaceutical industry. In the course of our screening program designed to identify new antitumor compounds (cytotoxics), we isolated a new bafilolide metabolite — R176502 (**1**) — from liquid fermentation cultures of a novel actinomycete of the genus *Micromonospora*. Bafilomycins A_1 (**2**), B_1 (**3**), and B_2 (**4**)[1] were also isolated from several different microbial cultures (Figure 1). In this report, we detail the production, isolation, structure determination, and biological activity of **1**.

Results and Discussion

Fermentation and Isolation

Liquid fermentation cultures of *Micromonospora* sp. JS1035 were extracted with ethyl acetate to yield the crude extract that was subsequently purified using counter current partition chromatography (CPC). Pure material for structure elucidation was obtained after two consecutive CPC fractionations using different conditions. Mass spectral analysis (see Table 1) of this material indicated a molecular weight of 853 and a molecular formula of $C_{47}H_{67}NO_{13}$. The NMR data for **1** was initially obtained in $CDCl_3$. However, it was soon discovered that **1** was not stable in this solvent. Subsequent NMR work was performed using acetone-d_6 and yielded a complex proton NMR spectrum. A full set of NMR experiments including ^{13}C NMR, DEPT, COSY, HMQC, and HMBC was obtained.

Concurrently, extracts from four other actinomycete

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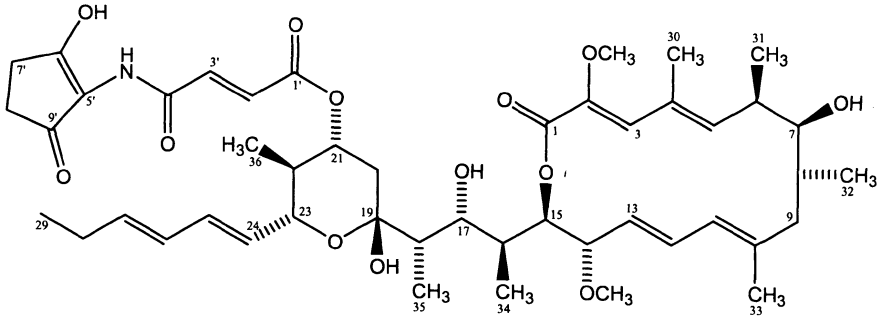
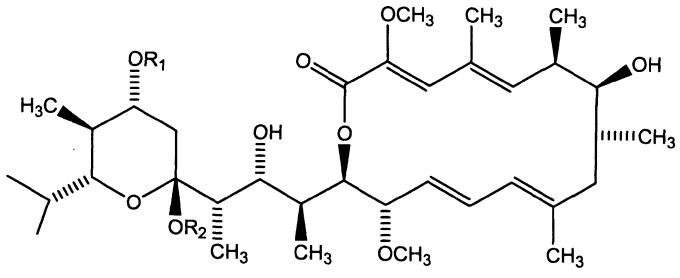
Fig. 1. Structures of R176502 (**1**), bafilomycins A_1 (**2**), B_1 (**3**) and B_2 (**4**).



R176502 (**1**)



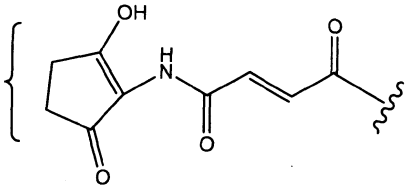
	R_1	R_2
Bafilomycin A_1 (**2**)	H	H
Bafilomycin B_1 (**3**)		H
Bafilomycin B_2 (**4**)		CH_3

Table 1. Physico-chemical properties of R176502 (**1**).

Appearance	Light brown solid
MP	114-120°C
$[\alpha]^{25}_D$	-38.0 (*c* 0.1, MeOH)
Molecular Formula	$C_{47}H_{67}NO_{13}$
Pos. ESI-MS	854 $(M+H)^+$
HRFAB-MS (*m/z*)	Found: 876.4514
	Calcd: 876.4510 (for $C_{47}H_{67}NO_{13}Na$)
UV (CH_3CN) λ_{max} nm	233, 286, 345
IR ν_{max} (film) cm^{-1}	3408, 2923, 1717, 1687, 1614, 1247, 1097, 751

strains with similar activity profiles were examined. Each of these extracts contained the same three major components based on HPLC retention times and UV spectra. The three components were isolated from one of the extracts using CPC and HPLC. Low resolution MS of these three compounds yielded molecular weights of 622, 815, and 829. These molecular weights matched published data for three bafilomycins: **2**, **3**, and **4**.[1] In addition, the ^{1}H NMR ($CDCl_3$) and UV data for these three compounds matched the data published for **2**, **3**, and **4**. The authors of this bafilomycin article believe that **4**, which is methylated at the hemiacetal carbon (C19), is an artifact formed during the isolation procedure. ^{1}H NMR spectra for **2**~**4** (in acetone-d_6) bore marked resemblance to the ^{1}H NMR spectrum of **1**. In fact, it appeared that **1** was most closely related to **3**. The UV spectrum of **1** (see Table 1) was also most closely related to that of **3** as both displayed an additional shoulder (compared to bafilomycin A_1) at approximately 345 nm.

Structure Determination

Assembling the partial structures for **1** was straightforward once the dereplication of **2**, **3**, and **4** was completed. The NMR data (Table 2) for the macrolide ring (C1~C15), C16~C23, and C1′~C4′ were in agreement with published data,[2] although, some differences in chemical shift were noted which could be attributed to the use of different NMR solvents or structural differences. In addition, our assignments were confirmed by COSY, HMQC, and HMBC data. Attachment of the new hexadiene sidechain (C24~C29) was accomplished by careful study of the correlations between relevant atoms. For instance, H24 showed an HMBC correlation to C22 and C23, and H25 also showed an HMBC correlation to C23. The COSY data were also consistent with placement of the new sidechain at C23. Several ^{13}C NMR signals (C5′, C6′, and C9′) corresponding to the cyclopentenone ring were obscured in the ^{13}C NMR spectrum, probably due to the presence of resonance structures between the vinylic OH and the carbonyl group. Although no other bafilolide researchers reported this problem, there were also no reports of these compounds being unstable in $CDCl_3$, which we found for **1** and **3**.

The stereochemistry of **2** has been rigorously established by X-ray crystallography;[3] the solution confirmation was also studied and found to correspond well to the crystalline structure.[4] Other published reports detail the stereochemistry for other bafilomycins and other members of this class.[5] Molecules in this class have the same stereochemistry for the lactone ring, the tetrahydropyran ring, and the C16~C18 linkage. All appear to possess a hydrogen-bonding network that includes C19OH, C17OH, and C1=O. This network effectively defines the configuration of the side chain. Based on the analysis of coupling constants, chemical shifts, and ROESY data, the proposed stereochemistry of **1** is consistent with this conformation. The new hexadiene sidechain at C23 was assigned as *alpha* to the ring based on two key ROESY correlations. There is a correlation between H23 and CH_3-36 and also between H23 and H21. These correlations would be unlikely unless H23 was positioned above the ring.

Biological Activity

A panel of cell proliferation assays (referred to herein as "bioassays") based on the inhibition of cell proliferation in selected human tumor cell lines (mammary MCF-7, colon HT-29, leukemia K562/C1,000 and melanoma Malme-3M) was used to identify antiproliferative activities in the different samples throughout the purification procedures (data not shown). Extracts from strain JS1035 grown in four different media were tested in bioassays; the one that contained the most antiproliferative activity was selected for further study. A 1 : 1,000-dilution of the active extract resulted in an almost complete inhibition of cell proliferation in the three cell lines tested (melanoma Malme-3M cell line was omitted at this stage). From this initial crude extract, collected fractions from CPC were individually analyzed on MCF-7 cell proliferation. A major single fraction was shown to contain antiproliferative activity, which was then further purified by HPLC. A single HPLC peak was found to contain antiproliferative activities as measured by inhibition of cell proliferation in our bioassay. This peak was used to initiate structure elucidation of the main active compound, **1**. Subsequent structure work was completed using material isolated with two consecutive CPC steps.

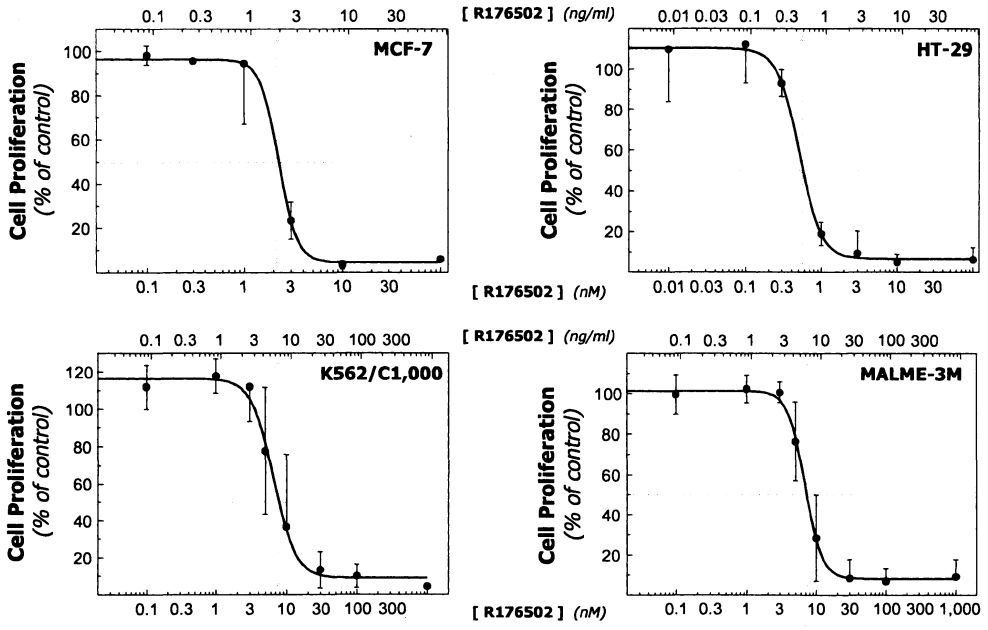
The purified compound was extensively characterized for its ability to inhibit tumor cell growth. As indicated in Figure 2, the dose-dependent inhibition of **1** in the four selected tumor cell proliferation assays was typical for classical cytotoxics, with very steep and narrow dose-response curves. Considering their structural similarities, **1**, **2**, and **3** were also compared side by side in our bioassay (Table 3). The three compounds exhibited similar potency for inhibition of tumor cell proliferation in the nM range of concentrations, with a slightly higher activity on colon HT-29 carcinoma cells (sub-nanomolar concentrations). Initial observation on inhibition of tumor cell proliferation was

Table 2. ^{1}H and ^{13}C NMR chemical shifts for R176502 (**1**)[a].

	δ_C	M	δ_H
1	167.57	s	
2	141.97	s	
3	133.35	d	6.63 (1H, s)
4	132.74	s	
5	145.55	d	5.94 (1H, d, 8.7)
6	37.85	d	2.52 (1H, m)
7	80.17	d	3.28 (1H, br d, 5.4)
8	41.93	d	1.86 (1H, m)
9	42.27	t	2.12 (1H, m), 2.03 (1H, m)
10	144.98	s	
11	125.19	d	5.80 (1H, d, 10.7)
12	134.53	d	6.67 (1H, dd, 15.0, 10.8)
13	126.95	d	5.12 (1H, dd, 14.9, 9.2)
14	82.98	d	4.07 (1H, m)
15	77.01	d	4.96 (1H, dd, 8.4, 1.3)
16	38.23	d	2.13 (1H, m)
17	70.80	d	4.11 (1H, m)
18	42.27	d	1.84 (1H, dq, 7.2, 1.2)
19	100.26	s	
20	40.22	t	2.35 (1H, dd, 11.7, 4.8), 1.34 (1H, m)
21	75.11	d	5.08 (1H, ddm, 11.1, 4.8)
22	41.81	d	1.50 (1H, m)
23	75.34	d	4.14 (1H, m)
24	130.81	d	5.50 (1H, dd, 15.1, 7.6)
25	133.51	d	6.18 (1H, dd, 15.5, 15.4)
26	129.72	d	5.99 (1H, dd, 15.1, 10.5)
27	137.21	d	5.70 (1H, dm, 15.2)
28	26.16	t	2.09 (2H, m)
29	13.73	q	0.97 (3H, d, 2.8)
30	14.13	q	1.94 (3H, br s)
31	17.62	q	1.03 (3H, d, 7.0)
32	22.20	q	0.90 (3H, d, 6.8)
33	20.33	q	1.93 (3H, br s)
34	10.02	q	0.85 (3H, m)
35	7.2	q	1.00 (3H, d, 2.5)
36	11.61	q	0.85 (3H, m)
2-OCH_3	59.94	q	3.50 (3H, s)
14-OCH_3	55.63	q	3.23 (3H, s)
1'	168.14	s	
2'	134.97	d	7.57 (1H, d, 15.3)
3'	132.97	d	6.82 (1H, d, 15.3)
4'	165.00	s	
5'	[b]	s	
6'	[b]	s	
7'	27.75	t	2.52 (2H, m)
8'	37.31	t	2.52 (2H, m)
9'	[b]	s	

[a]δ in ppm, J value in Hz. Data were recorded in acetone-d_6 at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. [b]Signal not observed.

Fig. 2. Dose-dependent inhibition of tumor cell proliferation by R176502 (**1**).



Human tumor cells (MCF-7, HT-29; K562/C1,000 and Malme-3M) were treated with indicated concentrations of R176502 for 4 days. Results, expressed as % of control untreated cells, are the mean±SE of at least 3 different experiments.

Table 3. Inhibition of tumor cell proliferation by bafilomycin A_1 (**2**), bafilomycin B_1 (**3**) and R176502 (**1**) (IC_{50} values±S.E. in nM).

Cell Lines	Bafilomycin A_1	Bafilomycin B_1	R176502
Breast MCF-7	1.55 ± 0.89	1.81 ± 0.63	1.92 ± 0.35
Colon HT-29	0.57 ± 0.10	0.93 ± 0.63	0.61 ± 0.04
Leukemia K562/C1,000	7.51 ± 3.58	5.88 ± 3.46	9.89 ± 7.35
Melanoma Malme-3M	17.30 ± 2.48	14.37 ± 4.54	7.96 ± 2.73

extended for **1** to a wide variety of human tumor cell lines — from ovary, prostate, pancreas, colon and lung origins — with observed IC_{50} values in the nM range of concentrations (data not shown).

Multi-drug resistance is considered a major element in the failure of anticancer therapeutics in clinical trials. Tumors become resistant to therapeutic agents by overexpression of both P-gp and MRP (major elements of the MDR system). Moreover, the constitutive expression of P-glycoprotein in the intestinal epithelium will, in case of oral administration, markedly reduce absorption when the compound is a substrate for the P-glycoprotein. The activity of **1** and **2** on P-gp- or MRP-expressing tumor cell lines and respective parental cells, K562/C1,000+ and K562A7, COR-L23/R and COR-L23, was compared to that of a known substrate for P-gp/MRP, adriamycin. As indicated in Table 4, both **1** and **2** inhibited cell proliferation in the nM range of concentrations regardless of the P-gp and/or MRP expression status (no significant difference between IC_{50} values). On the contrary, K562/C1,000+ and CORL23/R cells exhibited a 250-fold and 20-fold resistance to cell killing by adriamycin, respectively, as compared to their

Table 4. Multi-drug resistance: Inhibition of cell proliferation by bafilomycin A_1 (**2**), R176502 (**1**), and adriamycin (IC_{50} values±S.E. in nM).

Cell Line	Bafilomycin A_1	R176502	Adriamycin
K562A7	8.11 ± 1.93	14.08 ± 1.25	26.78 ± 5.82
K562/C1,000+	34.79 ± 12.33	29.34 ± 1.80	7,829 ± 806
COR-L23	4.26 ± 1.55	5.13 ± 0.91	36.57 ± 19.09
COR-L23/R	5.26 ± 1.81	6.90 ± 0.24	676 ± 261

respective parental cell lines. These results strongly suggested that there is no substantial recognition of **1** and **2** by the P-gp and MRP pump proteins.

R176502 is a new bafilolide derivative isolated from a novel *Micromonospora* sp. The hexadiene sidechain at C23 is novel among members of this class. There was, however, a recent report of a new bafilomycin with a methylpentadiene sidechain at C23.[6] Taken together, these results indicate that R176502 is a potent inhibitor of human tumor cell proliferation *in vitro* with activity in the nM range of concentrations in a wide panel of tumor cell lines. Although preliminary animal data suggest some acute general toxicity, the potential of the compound as a novel cancer therapeutic agent will have to await full characterization of its toxicity/efficacy profile in relevant preclinical models.

Experimental

General

^1H and ^{13}C NMR spectra were recorded at 300 K on a Bruker Avance DRX 500 spectrometer operating at 500 and 125 MHz, respectively. Standard pulse sequences were used for DEPT, COSY, HMQC, and HMBC experiments. ROESY data was acquired using a spinlock pulse of 300,000 μs (at 22 dB). Melting points were determined using an Electrothermal 9100 melting point apparatus and are uncorrected. IR data was acquired using a Perkin Elmer 1600 Series FTIR spectrophotometer. A Perkin-Elmer 243B polarimeter was used to procure optical rotation data. High resolution MS data was obtained using a Micromass 70SEQ Tandem Hybrid Mass Spectrometer. CPC was performed on a P.C., Inc. high speed countercurrent chromatograph, equipped with an Ito multilayer coil column interfaced with a Waters 991 Photodiode Array detector (monitoring at 270 nm).

Fermentation and Isolation

The actinomycete strain was isolated from river bottom sediment collected near Sombo, Cameroon. The strain was identified as a new *Micromonospora* species and deposited as LMG P-21525 at the Laboratorium voor Microbiologie en Microbiele Genetica (Gent, Belgium). The seed medium contained glucose 20 g, Pharmamedia (Trader's Protein) 15.0 g, yeast extract 5.0 g, $CaCO_3$ 4.0 g, $(NH_4)_2(SO_4)$ 3.0 g, $ZnSO_4 \cdot 7H_2O$ 0.03 g in 1 liter distilled water, pH adjusted to 6.5. The production medium contained glucose 20.0 g, dextrin 50.0 g, Pharmamedia (Trader's Protein) 30.0 g, yeast extract 1.0 g, $CaCO_3$ 5.0 g, $CoCl_2 \cdot 6H_2O$ 0.001 g in 1 liter distilled water, pH adjusted to 7.0. The media were dispensed in 25 or 30 ml aliquots to 250 ml flasks and autoclaved for 15 minutes. Frozen spore stocks (1.0 ml) were used to inoculate starter cultures (25 ml seed media per 250 ml Erlenmeyer flask). Starter cultures were incubated at 28°C and ~75% humidity on an orbital shaker (2″ throw, 250 rpm) for 48 hours. The starter cultures (1 ml) were used to inoculate 200 production media flasks (30 ml production media per 250 ml Erlenmeyer flask), which were then incubated for 6 days at the above conditions.

Ethyl acetate (22.5 ml) was added to each flask, and the flask contents were pooled into 500 ml centrifuge bottles. Each bottle was shaken vigorously and centrifuged at 6000×g for 8 minutes. The ethyl acetate was removed from each bottle and dried over anhydr Na_2SO_4. Ethyl acetate was again added to the centrifuge bottles to a total volume of approximately 450 ml, and the process was repeated. The extract was dried under reduced pressure to yield a crude extract (5.4 g).

An aliquot (400 mg) of the crude extract was subjected to CPC. The solvent system consisted of an equilibrated mixture of *n*-hexane, EtOAc, MeOH, and water (1 : 3 : 3 : 3, v/v/v/v). The lower phase was used as the stationary phase, and the upper phase served as the mobile phase which was

pumped at 3 ml/minute. The column rotation speed was 800 rpm. The crude extract was prepared by dissolving it in a mixture of upper and lower phases. The material was then loaded onto the column. The first peak to elute was collected and dried to yield 303 mg of purified material. This purified material was again subjected to CPC chromatography using modified conditions. The modified solvent system consisted of an equilibrated mixture of water, MeOH, and *n*-hexane (1 : 9 : 10, v/v/v). Again, the lower phase was used as the stationary phase, and the upper phase served as the mobile phase. After 90 minutes, the upper and lower phases were switched and the first peak to elute after the solvent change was collected and dried to yield 9.2 mg of pure **1**.

Initially, **1** was isolated using HPLC following the first CPC separation. In that case, the active CPC fraction was further separated using reversed-phase HPLC (LiChrospher, 10 μm, 10×250 mm, C_{18}). A linear gradient starting at 95 : 5 CH_3CN : H_2O to 100% CH_3CN over 25 minutes was used to isolate **1**. A 5 mg HPLC injection yielded 0.5 mg of **1**.

Drugs

All samples tested were dissolved in DMSO and further dilutions were made in culture medium, with final DMSO concentrations never exceeding 0.1% (v/v) in cell proliferation assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt] and PMS [phenazine methosulfate] were dissolved in phosphate-buffered saline (PBS).

Cell Lines and Cell Culture

The human MCF-7 mammary adenocarcinoma cells were cultured in DMEM medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml gentamicin and 5% fetal calf serum. The human HT-29 colon carcinoma cells were cultured in McCoy's 5a medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin and 5% fetal calf serum. The human Malme-3M melanoma cells were cultured in DMEM medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml gentamicin and 10% fetal calf serum. All three cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA. Cells were routinely kept as monolayer cultures at 37°C in a humidified 5% CO_2 atmosphere. The human K562/C1,000 leukemia cell line was a kind gift by Dr. H. HEYLIGEN [Dr. WILLEMS Instituut, Diepenbeek, Belgium] and is a P-gp expressing cell line obtained by culturing the cells at increasing concentrations of colchicine. The cells were kept as a suspension culture at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin and 5% fetal calf serum. The human K562A7 leukemia cell line was a kind gift by Dr. H. HEYLIGEN and is the parent, drug sensitive cell line of the K562/C1,000 cells. The K562/C1,000+ cell line was obtained by culturing the K562/C1,000 cells for an additional 2 weeks with 1 μM colchicine. This treatment induces higher levels of P-gp protein and mRNA and a more stable resistance profile. The cells were kept as a suspension culture at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin and 5% fetal calf serum. The human large cell lung cancer cell lines were obtained from the European Collection of Cell Cultures, Salisbury, U.K. COR-L23 is the parent, drug sensitive cell line and COR-L23/R is the MRP-expressing MDR-variant, developed by continuous step-wise *in vitro* incubation of COR-L23 cells with increasing concentrations of doxorubicin. The culture medium for both cell lines consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin and 10% fetal calf serum. For maintenance cultures of COR-L23/R, the medium was supplemented with 0.2 μg/ml doxorubicin. Cells were routinely kept as monolayer cultures at 37°C in a humidified 5% CO_2 atmosphere.

All media and supplements were obtained from Life Technologies, Merelbeke, Belgium. Cells were free of mycoplasma contamination as determined using the Gen-Probe *Mycoplasma* Tissue Culture kit [BioMerieux, Brussels, Belgium].

Cell Proliferation Assays[7]

In case of adherent cell lines, cells were seeded in Falcon® 96-well culture plates [Life Technologies, Merelbeke, Belgium] and allowed to adhere to the plastic for 18~48 hours. Medium was then changed and drugs and/or solvents were added. Following 4-day incubation, cell density was assessed using an MTT-based assay measuring absorbance at 540 nm using a Molecular Devices Emax 96-well spectrophotometer [Sopachem, Brussels, Belgium]. For the experiments with the suspension culture, K562A7, K562/C1,000 and K562/C1,000+ cells were seeded in Falcon® 96-well culture plates. Test drugs and/or medium were added immediately after seeding. Following 4-day incubation, cell growth was assessed using a MTS/PMS based assay measuring absorbance at 490 nm using an Emax 96-well spectrophotometer.

The antiproliferative activity was calculated as the

percentage of remaining viable cells in treated *versus* control (untreated cells) conditions. Results are expressed as IC_{50} values which represent the concentration of compound required to inhibit cell proliferation by 50%. Within an experiment, the result for each experimental condition is the mean of 3 replicate wells.

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