SF002-96-1, a new drimane sesquiterpene lactone from an *Aspergillus* species, inhibits survivin expression

Silke Felix¹, Louis P. Sandjo², Till Opatz*² and Gerhard Erkel*³

**Abstract**

Survivin, a member of the IAP (inhibitor of apoptosis) gene family, is overexpressed in virtually all human cancers and is functionally involved in the inhibition of apoptosis, regulation of cell proliferation, metastasis and resistance to therapy. Because of its upregulation in malignancy, survivin has currently attracting considerable interest as a new target for anticancer therapy. In a screening of approximately 200 strains of imperfect fungi for the production of inhibitors of survivin promoter activity, a new drimane sesquiterpene lactone, SF002-96-1, was isolated from fermentations of an *Aspergillus* species. The compound inhibited survivin promoter activity in transiently transfected Colo 320 cells in a dose dependent manner with IC₅₀ values of 3.42 µM (1.3 µg/mL). Moreover, it also reduced mRNA levels and protein synthesis of survivin and triggered apoptosis.

**Introduction**

Survivin, a member of the inhibitor of apoptosis (IAP) protein family is one of the most prominent cancer-associated proteins identified to date, being upregulated in almost all human cancer types while usually undetectable in normal terminally differentiated adult tissues [1,2]. It plays a key role in both apoptosis and control of cell cycle progression and high expression of survivin in tumors correlates with increased drug resistance, an accelerated rate of recurrence and poor patient survival [3,4]. Survivin blocks apoptosis by protein–protein interactions via its characteristic baculovirus IAP repeat domain (BIR) resulting in the formation of a complex with hepatitis B X-interacting protein bound to caspase 9 that prevents recruitment of apop-
Table 1: $^1$H (600 MHz) and $^{13}$C NMR (150 MHz) data of SF002-96-1 in CD$_3$CN.

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_{\text{H}}$ (multiplicity, coupling constant)</th>
<th>$\delta_{\text{C}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.02 (dd, 4.4, 11.9 Hz, 1H)</td>
<td>70.7</td>
</tr>
<tr>
<td>2</td>
<td>1.57 (m, 1H), 1.66 (m, 1H)</td>
<td>28.2</td>
</tr>
<tr>
<td>3</td>
<td>1.34 (m, 1H), 1.37 (m, 1H)</td>
<td>42.7</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>34.0</td>
</tr>
<tr>
<td>5</td>
<td>1.94 (overlapped with the solvent peak)</td>
<td>45.7</td>
</tr>
<tr>
<td>6</td>
<td>5.72 (dd, 4.0, 4.7 Hz, 1H)</td>
<td>67.0</td>
</tr>
<tr>
<td>7</td>
<td>6.57 (d, 4.0 Hz, 1H)</td>
<td>133.5</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>133.8</td>
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<tr>
<td>9</td>
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<td>77.0</td>
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<tr>
<td>10</td>
<td></td>
<td>44.7</td>
</tr>
<tr>
<td>11</td>
<td>4.23 (d, 10.5 Hz, 1H), 4.48 (d, 10.5 Hz, 1H)</td>
<td>77.4</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>169.8</td>
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<tr>
<td>13</td>
<td>1.15 (s, 3H)</td>
<td>24.9</td>
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<tr>
<td>14</td>
<td>0.97 (s, 3H)</td>
<td>32.6</td>
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<tr>
<td>15</td>
<td>1.04 (s, 3H)</td>
<td>12.8</td>
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<tr>
<td>1'</td>
<td></td>
<td>173.5</td>
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<tr>
<td>2'</td>
<td>2.32 (m, 2H)</td>
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<td>3'</td>
<td>1.59 (m, 2H)</td>
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<td>1.30 (m, 2H)</td>
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<tr>
<td>5'</td>
<td>1.28 (m, 2H)</td>
<td>31.9</td>
</tr>
<tr>
<td>6'</td>
<td>0.87 (t, 7.0 Hz, 3H)</td>
<td>14.2</td>
</tr>
<tr>
<td>OH-1</td>
<td>2.96 (br s, 1H)</td>
<td>–</td>
</tr>
<tr>
<td>OH-9</td>
<td>3.76 (br s, 1H)</td>
<td>–</td>
</tr>
</tbody>
</table>
The proton signal at δ 4.02 showed a COSY contact with methylene protons at δ 1.57 and 1.66 while the latter presented the same correlation with methylene protons at δ 1.34 and 1.37. The protons of two geminal methyl groups (δ 0.97, 1.15) displayed HBMC correlations with the methine carbon at δ 45.7, a quaternary carbon at δ 34.0 and the methylene carbon at δ 42.7 bearing protons at δ 1.34 and 1.37. Furthermore, a methyl group at δ 1.04 correlated with the oxymethine at δ 70.7, a methine at δ 45.7, and two quaternary carbons at δ 44.6 and 77.0. The correlations from the COSY spectrum between the proton signals at δ 1.94 and 5.72, as well as between those at δ 5.72 and 6.57 in conjunction with HMBC correlations observed between the proton signal at δ 6.57 and quaternary carbons at δ 77.0 and 133.8 suggested a decalin core for SF002-96-1. Further HMBC correlations disclosed between the signals at δ 4.23 and 4.48 and the carbons at δ 44.6, 77.0, 133.8, and 169.8 suggested SF002-96-1 to be a drimane sesquiterpene (Figure 1).

Besides, the protons of the second geminal methyl group (δ 0.97) showed spatial correlations with a methine proton at δ 1.94 and two downfield protons at δ 4.02 and 5.72. Based on the absolute configuration reported for drimane sesquiterpenes [14,15], the trans-decalin core of SF002-96-1 was tentatively assigned the configurations R, S, R, S, and R, at C-1, C-5, C-6, C-9, and C-10, respectively. The foregoing data led to identify SF002-96-1 as 9,9b-dihydroxy-6,6,9a-trimethyl-3-oxo-1,3,5,5a,6,7,8,9,9a,9b-decahydroprathol[1,2-c]furan-5-yl hexanoate.

**Biological activity**

For the identification of active secondary metabolites and the characterization of their influence on survivin expression, we employed a human survivin-promoter dependent transcriptional reporter in the transiently transfected human colorectal carcinoma cell line Colo 320. Due to the overexpression of survivin in many human cancers, including colon cancer, a strong constitutive luciferase activity could be observed 24 h after transfection caused by the binding of transcription factors to the regulatory sites of the survivin gene [16]. As shown in Figure 4A, SF002-96-1 inhibited survivin promoter activity in a dose dependent manner with an IC₅₀ value of 3.42 µM (1.3 µg/mL), whereas the constitutive activity of the CMV promoter was not affected at concentrations up to 10.5 µM (4 µg/mL) indicating that the compound does not interfere with transcription in a general manner.

The expression of the survivin gene is regulated by a number of transcription factors including Stat3, NF-κB and the β-catenin activated T-cell factor (TCF) [17]. Constitutive activation of Stat3 by paracrine and autocrine mechanisms has been detected in diverse human cancer cell lines and tissues which contribute to oncogenesis by promoting cell proliferation and inhibiting apoptosis by increasing the expression of anti-apoptotic genes such as survivin [18]. We therefore investigated the effect of SF002-96-1 on Stat3-driven expression of the reporter gene luciferase in IL-6 stimulated Colo 320 cells. The compound...
Figure 4: Effect of SF002-96-1 on survivin promoter activity, survivin mRNA levels and expression. (A) Colo 320 cells were transiently transfected with a human survivin-, CMV-, or β-catenin/TCF-dependent reporter construct for 24 h with or without test compound. Control (100%): untreated cells only. For NF-κB- and Stat3-driven reporter gene expression, cells were transfected with the indicated reporter gene construct and stimulated with 10 ng/mL TNF-α, 5 ng/mL IL-1β (for pNF-κB-luc) or 10 ng/mL IL-6 (for Stat3 dependent pMW-IRF7) with or without test compound for 24 h. Control: (100%): stimulation only. Results represent the mean ± SD of at least three independent experiments. The expression of the reporter gene was determined as described in the experimental section. (B) Effect on survivin mRNA levels. Colo 320 cells were treated with different concentrations of SF002-96-1 for 8 h. mRNA levels of survivin were measured by real-time PCR as described in the experimental section. Control (100%): untreated cells. Results represent the mean ± SD of at least three independent experiments. (C) Western blot analysis for survivin. Colo 320 cells were treated with different concentrations of SF002-96-1 for 8 h. Subsequently total cell extracts were prepared and analyzed by Western blot analyses. β-Actin was used as internal control.

strongly inhibited the Stat3-dependent luciferase expression with an IC$_{50}$ value of 1.6 µM (0.6 µg/mL). In addition to Stat3, the response of the survivin promoter construct also depends on the transcription factor NF-κB [19] and as shown in Figure 4A, SF002-96-1 inhibited the inducible NF-κB-dependent reporter gene expression in Colo 320 cells with an IC$_{50}$ value of 2.63 µM (1 µg/mL). Previous studies in colon cancer cells have shown that regulation of survivin expression is, in addition, TCF/β-catenin dependent mediated by three TCF/β-catenin consensus sequences within the survivin promoter [20]. Dysregulation of TCF/β-catenin dependent gene regulation originating from aberrantly stabilized β-catenin or mutations in the associated protein adenomatous polyposis coli (APC) results in the activation of target genes implicated in cell proliferation and transformation [21]. We therefore investigated the effect of SF002-96-1 on canonical Wnt signaling using a synthetic TCF-responsive reporter (TOPFLASH) in Colo 320 cells which contain a truncated APC protein [22] and therefore display high constitutive TCF/β-catenin transcription. Interestingly, SF002-96-1 showed no significant inhibition of the TCF/β-catenin dependent luciferase expression up to the highest concentration tested (8 µM).

To investigate the effect of the compound on the transcription of the survivin gene, quantitative real-time PCR experiments were performed with total RNA isolated from Colo 320 cells treated with different concentrations of the test compound for 8 h as described in the experimental section. As illustrated in Figure 4B, application of 18.42 µM (7 µg/mL) SF002-96-1 reduced the survivin mRNA level in Colo 320 cells by around
50%. To confirm the data obtained from the qRT-PCR experiments, Western blot experiments were performed for survivin protein expression. As shown in Figure 4C, SF002-96-1 significantly reduced endogenous survivin protein level starting at 13.16 µM (5 µg/mL) after 8 h treatment which suggests that the suppression of survivin by SF002-96-1 is through the transcriptional inhibition of the survivin gene promoter.

To further investigate whether the fungal compound could affect the binding of Stat3 and NF-κB to the survivin promoter in living cells, we performed ChIP assays with primers covering suggested Stat3 and NF-κB (p65) binding sites [23,24]. Q-PCR of the −1231/−1009 (primers Sat3_1), −131/+46 (primers Stat3_2) and −920/−773 (primers Stat3_3) regions of the survivin promoter with Stat3-immunoprecipitated and IL-6 treated samples resulted in a strong induction of Stat3 binding to the two distal as well as to the proximal binding sites of the survivin promoter (Figure 5).

Pretreatment of the cells with 18.42 µM SF002-96-1 resulted in a strong reduction of Stat3 binding to all binding sites in the survivin promoter. ChIP experiments with primers comprising the suggested proximal NF-κB p65 binding site surrounding the transcriptional start site revealed a reduction of p65 binding after stimulation of NF-κB activity with 10 ng/mL TNF-α, 5 ng/mL IL-1β and treatment of the cells with the fungal compound (Figure 5), corroborating the results obtained in the reporter gene assays. As a control, we investigated the influence of the compound on the binding of lysine9 acetylated histone H3 (H3K9Ac) to the constitutive gapdh promoter as a marker for accessible chromatin that is transcriptionally active. SF002-96-1 did not significantly affect the levels of H3K9Ac in the constitutive gapdh promoter (Figure 5). These results indicate that the compound inhibits survivin expression by preventing the DNA binding of Stat3 and NF-κB transcription factors.

To determine the induction of cell death by SF002-96-1 in Colo 320 cells, the cells were treated with the compound for 48 h, after which the cell viability was assessed by measuring the reduction of the tetrazolium compound 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium (XTT) into a colored formazan. SF002-96-1, at concentrations from 10.5 µM (4 µg/mL) significantly decreased cell viability of Colo 320 cells in a dose-dependent manner (Figure 6A). In our studies, the compound proved to be a strong inducer of apoptosis in Colo-320 cells showing the typically biochemical characteristic of apoptosis, like cleavage of the chromosomal DNA at internucleosomal sites into fragments of approximately 200 bp and the fragmented morphology of the nuclear bodies (Figure 6B,C) after 5 h of treatment. Exposure of the cells with different concentrations of SF002-96-1 showed a concomitant increase in caspase-3 activity (Figure 6D), indicating that the compound triggers the apoptotic cascade.

Although several agents including natural products (e.g. curcumin, cucurbitacin, resveratrol) have been described to
inhibit survivin expression by interfering with oncogenic signaling pathways [25-27], only two survivin transcriptional inhibitors (e. g. YM155, terameprocol) are in development or in clinical trials [28,29].

Recently, FL188, a camptothecin analog, has been described to inhibit survivin promoter activity and inhibits the expression of cancer-associated survival genes (Mcl-1, XIAP, cAP2) in p53 status independent manner at nanomolar concentrations [30]. We have identified a new drimane sesquiterpene lactone, SF002-96-1, which inhibits survivin expression by interfering with critical signaling cascades (JAK/Stat, NF-κB) involved in transcriptional activation of the survivin promoter and subsequently triggering apoptosis in Colo 320 cells. Drimane sesquiterpenes are widespread in plants, fungi and marine organisms such as algae, sponges and corals and have attracted some attention for their potent antibacterial, antifungal, cytotoxic, antifeedant, phytotoxic, piscicidal and molluscicidal activities [31-33].

**Conclusion**

In summary, we identified a new fungal drimane sesquiterpene lactone, SF002-96-1, which inhibits survivin expression by blocking the binding of critical transcription factors (Stat3, NF-κB) to the promoter of the survivin gene and triggers apoptosis in the colon carcinoma cell line Colo 320. Due to the lack of a larger portfolio of survivin antagonists, SF002-96-1 may serve as lead structure for the development of novel cancer therapeutics. Further investigations on the cellular targets and the mode of action of the compound are now under way.
Experimental

General procedures

1D and 2D NMR data were recorded with a Bruker AVANCE III 600 MHz spectrometer equipped with a 5 mm inverse TCI cryoprobe using standard pulse sequences. APCI–MS spectra were measured from a solution of the analyte in MeCN/H$_2$O with a Hewlett Packard MSD 1100 using an evaporator temperature of 400 °C, a drying gas temperature of 350 °C at a flow of 6 L/min (N$_2$). In positive ionization mode, the capillary voltage amounted to 3.5 kV, the corona discharge current was 4 μA. In negative ionization mode, the capillary voltage amounted to 2.2 kV, the corona discharge current was 6 μA. HRESI–MS data were measured from a solution of the analyte in acetonitrile with a Waters Q-TOF-Ultima 3 equipped with a LockSpray interface (tri-n-octylamine as external reference). IR and UV spectra were measured with a Bruker IFS48 FTIR spectrometer and a Perkin-Elmer Lambda-16 spectrophotometer, respectively. The optical rotation was measured on a Perkin-Elmer 241 polarimeter at 578 nm and 546 nm and extrapolated to 589 nm using Drude’s equation [34].

Producing organism, fermentation and isolation of compound SF002-96-1

Aspergillus sp. strain IBWF002-96 was obtained from the culture collection of the Institute of Biotechnology and Drug Research (IBWF e.V.), Kaiserslautern, Germany. The strain IBWF002-96 showed all characteristics of the genus Aspergillus, the species however could not be unequivocally determined. ITS sequence analysis of the ITS1-5.8S rDNA-ITS2 region of nuclear DNA [35] showed high similarity to an uncultured soil fungus (100% in 554 bp, Genbank accession no. GQ921753.1) and to Aspergillus janus (98% in 575 bp, Genbank accession no. EU021598).

For maintenance, the fungus was grown on HMG agar slants (98% in 575 bp, Genbank accession no. EU021598).

The purity of the isolated compound was analyzed with a Hewlett-Packard Series 1100LC-MSD instrument fitted with a LiChroCART Superspher 100 RP-18 column (125 × 2 mm, 4 mm particle size; Merck). The chromatographic conditions consisted of a gradient from 1% to 100% acetonitrile in 20 min, and an isocratic step at 100% acetonitrile for 1 min at 40 °C and 10 μL injection volume was used. The flow rate was 0.45 mL/min. The fragmentor voltage was set to 140 V in the positive and negative APCI modes. The compound showed the highly characteristic fragmentation pattern in the APCI-positive mass spectrum revealing the pseudo-molecular ion [M + H]+ with m/z of 281.2. The purity of SF002-96-1 as estimated by HPLC-DAD/MS analysis was greater than 98.5% (Figure 7).

Evaporated in vacuo and the crude extract (3.2 g) was separated by chromatography on silica gel (Merck 60). Elution with cyclohexane/EtOAc (50:50 v/v) resulted in 580 mg of an enriched fraction which was further purified by preparative HPLC (Macherey-Nagel, Düren, Germany; Nucleosil RP18; column 21 × 250 mm, flow 20 mL/min) with MeCN/H$_2$O (60:40) as eluent to yield 28 mg of SF002-96-1 (tR: 13 min). During bioactivity-guided fractionation and isolation of compound SF002-96-1, no other compounds have been detected inhibiting survivin promoter activity.

Cell culture

Colo 320 (DSMZ ACC 144) cells were maintained in RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-gluta-
mine, supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C and 5% CO₂.

**Reporter gene assays**

The 1092 bp human survivin promoter (region between nucleotide 1821 and nucleotide 2912 within the human survivin gene; GenBank™ accession number U75285) was amplified by polymerase chain reaction from genomic DNA isolated from MonoMac6 cells using oligonucleotides derived from published sequences [36]. The PCR product was cloned into the XhoI-HindIII site of the promoterless luciferase vector pGL3-basic (Promega, Mannheim, Germany) to generate the hSurvivin-promoter-driven luciferase reporter plasmid (pGL3-hsurvpro). The plasmid pRL-CMV for normalizing transfection efficiency was obtained from Promega (Dual-Luciferase-Reporter-Assay). The NF-κB driven reporter plasmid pNF-xB-Luc was obtained from Clontech (Saint-Germain-en-Laye, France). The IL-6 responsive STAT3-dependent reporter vector pMW-IRF7 has been described previously [37]. TOPFLASH and FOPFLASH luciferase reporters were obtained from Upstate Biotechnology, Inc. (Lake Placid, USA).

Transient transfections of Colo 320 cells were performed by electroporation (BioRad, Gene Pulser) of 1 × 10⁷ cells/mL in 0.4 mL RPMI 1640 medium containing 10% FCS together with 50 μg of the indicated plasmids at 290 V and 975 m. After electroporation, the cells were seeded at 1 × 10⁶ cells/mL in RPMI medium containing 10% FCS in a 24 well plate and allowed to recover for 24 h. For induction of reporter gene expression, the cells were treated with 10 ng/mL TNF-α and 5 ng/mL IL-1β for pNF-xB-Luc and 10 ng/mL IL-6 for pMW-IRF7 with or without test compounds. Luciferase activity was measured 24 h after induction using the Dual-Glo luciferase assay system (Promega, Mannheim, Germany) according to the manufacturer’s instructions with a luminometer.

**Cell viability testing**

The cytotoxicity of the compound was determined after 48 h using a XTT-based cell viability assay as previously described by Roehm et al. [38].

**Quantitative real-time polymerase chain reaction analysis (qRT-PCR analysis)**

The mRNA expression in human Colo 320 cells was analyzed by two-step real-time RT-PCR as described before [39] with gene-specific primers for human survivin (Genbank Accession NM001168) forward: 5’-ACCAGGTGAAAGTGAGGGA-3’ and reverse: 5’-AACAGTAGAGGAGGGAGGGA-3’ (size of the PCR product is 309 bp) and GAPDH (Genbank Accession M33197) forward: 5’-CCTCCGGGAAACTGTGG-3’ and reverse: 5’-AGTGAGGACACGGAGAAG-3’ (size of the PCR product is 140 bp). Relative mRNA amounts were determined using the mathematical model for relative quantification in real-time PCR proposed by Pfaffl [40].

**Chromatin immunoprecipitation (ChIP) assay**

Colo 320 cells were seeded out in 75 cm² tissue culture flasks at a density of 5 × 10⁵ cells per mL. After 24 h of cultivation, cells were starved for additional 16 h in RPMI 1640 medium with 0.5% FCS. After that, cells were pretreated with SF002-96-1 for 1 h. Stimulation of the NF-xB signaling pathway was performed for 30 min with a cytokine mixture consisting of 10 ng/mL...
TNF-α and 5 ng/mL IL-1β. Stimulation of the Stat3 signaling pathway was performed for 30 min with 10 ng/mL IL-6. Chromatin immunoprecipitation (ChIP) assays were performed as described by Carey et al. [41], using specific anti-Stat3 (79D7, New England Biolabs, Frankfurt/M), anti-NF-κB p65 (SC-109X, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-lysine9 acetylated histone H3 antibody (9671, New England Biolabs, Frankfurt/M) at dilutions recommended by the manufacturers (1:50). The immunoprecipitates were pelleted and incubated at 65 °C overnight to reverse cross-links. The DNA was extracted by phenol–chloroform extraction and cross-links were reversed with ethanol. The precipitated DNA was resuspended in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and used for PCR amplification. Three putative Stat3-binding sites within the survivin promoter were analyzed. The sequences of the PCR primers used are as follows: Stat3_1: forward primer, 5′-CAGTGAGCTGATCGATCAGCC-3′; reverse primer (fragment size: 223 bp), Stat3_2: forward primer, 5′-CGCCCTCTACTCCAGAG-3′; reverse primer (fragment size: 195 bp), Stat3_3: forward primer, 5′-CATGACAGACAGACACAC-3′; reverse primer (fragment size: 165 bp). The primer sequences used for the NF-κB binding site within the survivin promoter were: NF-κB: forward primer, 5′-CTGCACCGTTCTTTGA-3′; reverse primer (fragment size: 165 bp). The gapdh primer mixture was obtained from New England Biolabs, Frankfurt/M (SimpleChIP® Human GAPDH Exon 1 primers).

**Western immunoblotting**

Colo 320 cells were seeded into a 6-well plate at cell density of 5 × 10^5 cells/mL and allowed to grow for 24 h. The cells were then treated for an additional 8 h with and without different concentrations of test compound and total cell extracts were prepared by lysing the cells with Totex buffer (20 mM HEPES, pH 7.4, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na₃VO₄, 1:25 complete protease-inhibitor cocktail according to the manufacturer’s recommendation (Roche Diagnostics, Mannheim, Germany)). 25 µg total cell extracts were subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with antibodies specific for human survivin (1:1000, 71G4B7, New England Biolabs Frankfurt/M) or β-actin (1:1000, 13E5, New England Biolabs Frankfurt/M) and then with the appropriate secondary antibody conjugated to horse radish peroxidase (1:2000, Anti-rabbit IgG, HRP-linked New England Biolabs, Germany). Immuno-reactive proteins were visualized by the enhanced chemoluminiscence detection system (New England Biolabs, Germany).

**Caspase-3 activity assay**

Colo 320 cells were seeded at a density of 5 × 10^5 cells/mL in 96 well plates and treated with test compound for 5 h. After incubation, the cells were centrifuged at 1000g for 10 min at 4 °C, washed with PBS and lysed with 50 µL cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA, pH 7.4). After a freezing step at −80 °C and rethawing, the cell lysate was centrifuged at 1000g for 10 min at 4 °C. 25 µL of the supernatants were transferred into a new 96-well plate and incubated with 75 µL assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol, pH: 7.4) containing 20 µM Ac-DEVD-amino-4-methylcoumarin (AC-DEVD-AMC; Calbiochem, Bad Soden, Germany) for 2 h at 37 °C. AMC released from the cleavage of AC-DEVD-AMC was measured by a spectrophotometer with excitation and emission wavelengths of 355 nm and 460 nm respectively.

**Measurement of DNA fragmentation**

Colo 320 cells were resuspended at 5 × 10^5−1 × 10^7 cells/mL and incubated with the test compound for 5 h. After incubation, the cells were centrifuged (1000g, 10 min), washed once with ice cold PBS and lysed with 600 µL lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.2% Triton X-100, pH 7.5) on ice for 10 min. The lysate was centrifuged at 14 000 rpm for 10 min at 4 °C and the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was precipitated with two volumes of ethanol (100%) and 0.1 volume of sodium acetate (3 M, pH 5) overnight. The DNA pellet was rinsed with ethanol (70%) and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH: 7.4) and incubated with RNase (0.1 mg/mL) for 30 min at 37 °C. The samples were separated on a 1.5% agarose gel and stained with ethidium bromide.

**DAPI staining**

Colo 320 cells were incubated for 5 h with test compound and washed once with ice cold PBS. After centrifugation at 1000g for 10 min at 4 °C, the cell pellet was resuspended in 5% PBS buffered paraformaldehyde solution containing 10 µg/mL DAPI (4′,6-diamidino-2-phenylindole) and incubated for 10 min on ice. A portion (10 µL) of the cell suspension was then placed on a glass-slide, covered with a coverslip and the morphology of the cell nuclei was observed by using a fluorescence microscope (Zeiss, Axioskop).

**Supporting Information**

Supporting Information File 1
1D and 2D NMR spectra of compound SF002-96-1.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-9-323-S1.pdf]
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References

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