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ORIGINAL PAPER

A novel sulfonamide derivative as a strong and selective apototic agent against hematological malignancies

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Abstract

Currently available chemotherapeutic drugs against hematological malignancies have several adverse effects and are associated with high mortality rates. Thus, in this study we evaluated the cytotoxic effect of 26 new sulfonamide derivatives on acute leukemia and multiple myeloma cells in order to try to discover a new selective and safe compound that might be used as a prototype for new chemotherapeutic agents. The most cytotoxic compound, **DFS16**, reduced the cell viability of K562, Jurkat and MM.1S cells in a concentration- and time- dependent manner and it was significantly less cytotoxic to non-tumor cells. On acute leukemia cells, sulfonamide **DFS16** activated intrinsic and extrinsic apoptosis with Bax/Bcl-2 inversion, increased FasR expression and $\Delta\Psi$m loss. In K562, **DFS16** induced apoptosis by caspase-3 activation, while in Jurkat, it induced AIF release and caspase-3 independent apoptosis. In multiple myeloma, **DFS16** induced cell cycle arrest at the G2/M phase and apoptosis with $\Delta\Psi$m loss. Altogether, the results suggest that the new sulfonamide derivative **DFS16** induces apoptotic-like cell death in acute leukemia and multiple myeloma cells. **DFS16** is a promising new molecule that could be used as a prototype for the development of chemotherapeutics against hematological malignancies.

Keywords Sulfonamide · Acute leukemia · Multiple myeloma · Cytotoxicity · Cell cycle · Apoptosis

Introduction

Hematological malignancies are abnormal and uncontrolled proliferations of neoplastic hematopoietic cells and occupy the fifth place among new diagnosed cases of cancer. They are considered as heterogeneous diseases as they have several sub-classifications and differences regarding the disease characterization, diagnosis and prognosis. In this group of malignancies are included acute leukemias (AL) and multiple myeloma (MM). AL are characterized by the halting of normal hematopoietic differentiation and by the accumulation of immature cells (blast cells) in the bone marrow (BM) and/or in the peripheral blood (PB) (Allart-Vorelli et al. 2015; Swerdlow et al. 2016), while MM is a malignant clonal B cell neoplasm that usually result in the excessive production of a monoclonal immunoglobulin (protein M) by the plasma cells (Choudhry et al. 2018; Touzeau et al. 2018). Despite the different protocols available today against these diseases, the treatment options for AL and MM are not effective, especially for MM, which is considered as an incurable disease. Besides the strong adverse effects associated with currently available chemotherapeutics, many patients develop drug resistance, thus the relapse and mortality rates remain very high (Kadia et al. 2016; Vasekar et al. 2016).

Considering the poor prognosis of hematological malignancies and the morbidity associated with chemotherapeutic drugs (Choudhry et al. 2018; Vasekar et al. 2016), the search for new synthetic molecules with potential cytotoxic effect against AL and MM is an urgent

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necessity. The aim is to discover a new compound that could be used as a prototype for the development of new and easily administered drugs with higher efficiency and specificity in inducing tumor cell death and little or negligible side effects to normal cells. In this context, sulfonamides are synthetic compounds widely used as antibiotics since 1935 due to its low cost, low toxicity and excellent activity against bacterial infections. Over time, the use of sulfonamides has also been extended as anticancer agents (Parasca et al. 2013). Although sulfonamides have a common chemical skeleton, there are a variety of structural alterations that justify the different mechanisms of action related to their antitumor activity. Among them, the inhibition of carbonic anhydrase, the induction of microtubule impairment, the cell cycle blockage at G1 phase (by pRb phosphorylation and by decreasing the expression of cyclin A and B1 and proteins CDK2 and CDC2) and the inhibition of angiogenesis (Sabt et al. 2018). Methanesulfonamide derivatives, for instance, are capable of binding to cell DNA and induce cytostatic effects. Amsacrine is an example of methanesulfonamide that is currently used as a chemotherapeutic in the treatment of lymphomas and leukemias (Lee et al. 2017).

Taking into advantage the promising data about sulfonamides as anticancer agents, this study aimed to evaluate the cytotoxic effect of 26 new sulfonamide derivatives on hematological malignancies and to investigate the main mechanisms involved in cell death induced by the most effective compounds on AL and MM cell lines.

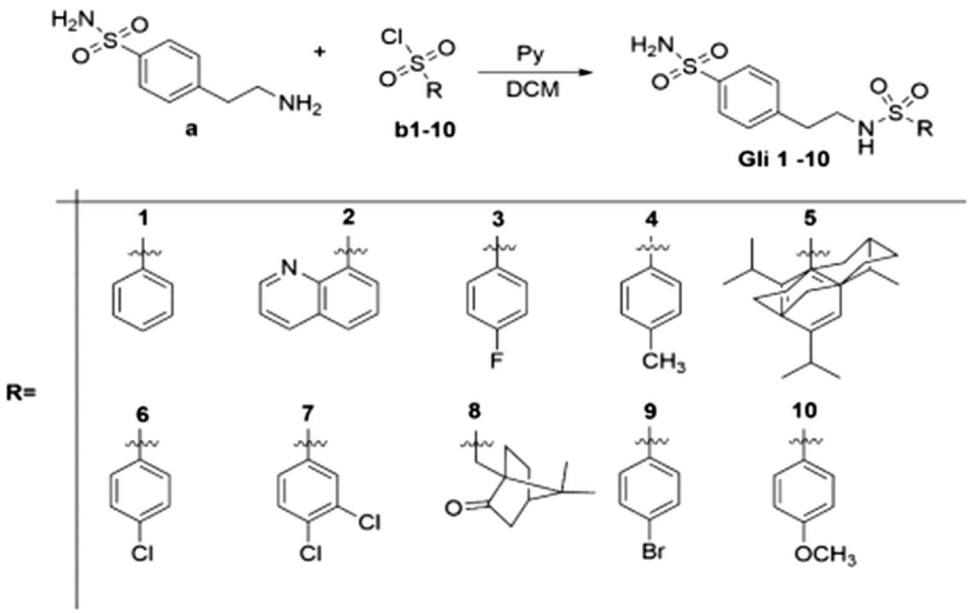
Experimental

Chemical synthesis

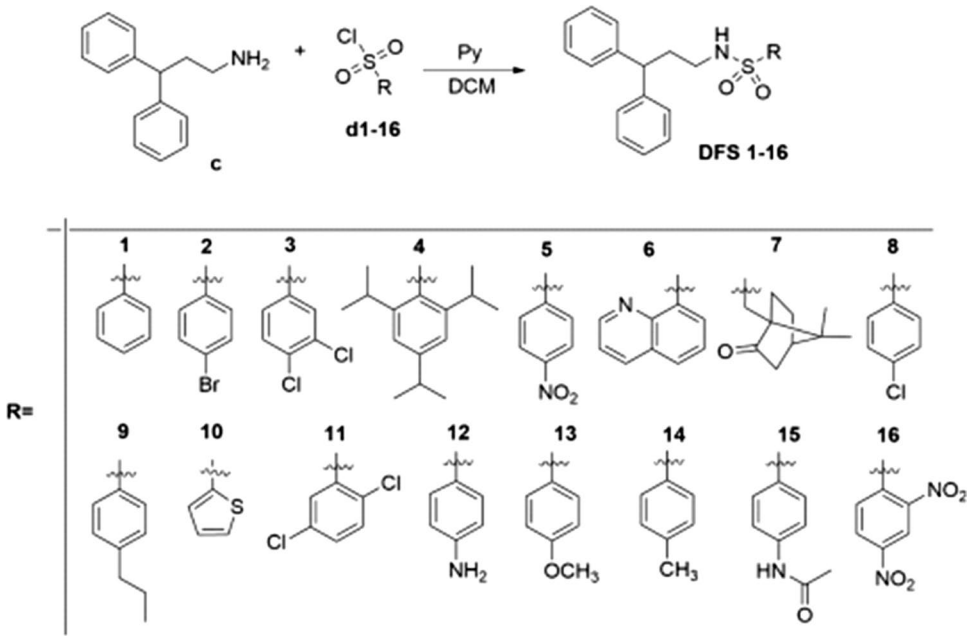
The 26 new synthetic sulfonamide derivatives included in this study were kindly provided by Dr. Alessandra Mascarello and Dr. Louise Domeneghini Chiaradia-Delatore under the supersvision of Dr. Ricardo José Nunes and Dr. Rosendo Augusto Yunes, from the Structure and Activity Laboratory, Chemistry Department, Federal University of Santa Catarina. Of them, 10 were derived from the 4-(2-aminoethyl) benzenesulfonamide (**Gly Series**) (Scheme 1) and 16 were derived from the 3,3-diphenylpropylamine (**DFS Series**) (Scheme 2). Briefly, the compounds were synthesized by 1 mm of the corresponding methyl ester (a or c), 1 mmol of sulfonyl b1-10 or d1-16, respectively, and 1 mmol of pyridine in dichloromethane (DCM), at room temperature and under magnetic stirring. Thereafter, the DCM was evaporated; the crude product was solubilized in methanol and poured on crushed ice and, finally, the final products were vacuum filtered and recrystallized with the appropriate solvent when necessary (Mascarello, 2012). The structures were characterized by nuclear magnetic resonance spectroscopy (1H), 13 NMR spectra, infrared spectra and spectrometry of mass. All new sulfonamides are soluble in dimethylsulfoxide (DMSO).

Cell culture

K562 acute myeloid leukemia (AML) and Jurkat T cell acute lymphoblastic leukemia (ALL) cell lines were originally purchased from the American Type Culture Collection (ATCC,



Scheme 1 Synthetic route of sulfonamides derived from the 4-(2-aminoethyl) benzenesulfonamide (Gly Series)



Scheme 2 Synthetic route of sulfonamides derived from from the 3,3-diphenylpropylamine (DFS Series)

USA). MM cell line (MM.1S) was kindly provided by Dr. Anamika Dhyani (University of Campinas, Brazil). L929 murine fibroblasts were purchased from the Rio de Janeiro Cell Bank (BCRJ, Brazil). Cells were cultured in Roswell Park Memorial Institute Medium (RPMI) (GIBCO®, Brazil) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 10 mM HEPES under 5% CO_2 humidified atmosphere, at 37 °C in 25 cm^2 and 75 cm^2 culture flasks.

Viability assays

For the screening, Jurkat (1×10^5 cells/well), K562 and MM.1S cells (5×10^4 cells/well) were incubated for 24 h with 100 µM of sulfonamides **Gli 1-10** and **DFS 1-16** (dissolved in DMSO, Merck Millipore®). The same volume of DMSO was added to control wells and cell viability was assessed by the methylthiazoletetrazolium (MTT) assay (Sigma Chemical Co., USA) (Mosman, 1983). Optical density of control groups (untreated cells) was considered as 100% of viable cells. Concentration and time-response curves were carried out with the most cytotoxic compound, **DFS16**. K562, Jurkat, MM.1S and L-929 cell lines were treated with increasing concentrations (1-100 µM) of **DFS16** for 24, 48 and 72 h. The IC_{50} values (half-maximal inhibitory concentrations) were calculated using GraphPad Prism 6 (GraphPad Software®, USA). The selectivity index (SI) was calculated by dividing the IC_{50} obtained on L929 cell line by the IC_{50} obtained on AL (K562 and Jurkat) and MM (MM.1S) cell lines. A high selectivity was considered as $SI \geq 5$, a moderate selectivity as $2 \leq SI < 5$ and a low selectivity as $IS < 2$ (Dahham et al. 2015).

Effect on peripheral blood (PB) cells

PB samples from six non-smoking healthy volunteers were collected at the Polydoro Ernani de São Thiago University Hospital (Florianópolis, Brazil) after approval by the University Human Research Ethics Committee–CEPSH Nº746.486/2014). Mononuclear cells (MC) were isolated by Ficoll-Hypaque (density = 1.070 g/mL). PBMC (1×10^6 cells/well) were incubated for 24 h with DMSO, paclitaxel (positive control) or sulfonamide **DFS16** and cell viability was assessed by the MTT assay as previously described. For the hemolysis test, the red blood cells (RBC) were isolated by centrifugation and then incubated with saline (negative control, 0% lysis), distilled water (positive control, 100% lysis) and increasing concentrations of **DFS16**. The % hemolysis was calculated using the following formula (Boyum, 1968):

$$\%\ \text{Hemolysis} = \frac{[(\text{sample absorbance} - \text{negative control absorbance})}{(\text{positive control absorbance} - \text{negative control absorbance})]} \times 100.$$

Cell cycle analysis

K562, Jurkat and MM.1S cells (5×10^5 cells/well) were incubated with **DFS16** at their respective 24 h IC_{50}. After 12 h, cells were harvested and cell cycle analysis was assessed according to the kit protocol (PI/RNAse Solution Kit, Immunostep®). Analysis was performed by flow cytometry (FACSCantoIITM, Becton–Dickinson Immunocytometry Systems) using the PE channel (496-578 nm)

and the data were analyzed using Infinicyt software version 1.7 (Cytognos®, Spain).

Apoptosis assays

Apoptotic cell death was first observed by the ethidium bromide (EB) and acridine orange (AO) method. K562, Jurkat and MM.1S cells (1×10^6 cells/well) were incubated for 12 h with sulfonamide **DFS16** (24 h IC_{50}). Then, cells were resuspended with a solution of EB 1% and AO 1% (1:1), morphological changes were observed in a fluorescence microscope (Olympus BX-FLA, Olympus®, Japan) at 590 nm (emission) and representative fields were photographed. The DNA fragmentation assay was performed in K562, Jurkat and MM.1S cells (6×10^6 cells/well) treated for 24 h with **DFS16** (24 h IC_{50}) or paclitaxel (10 µM, used as a positive control). DNA extraction was performed using a commercial kit (QIAGEN®, USA) and DNA samples were separated by electrophoresis in 2% agarose gel and observed using a transilluminator. Apoptotic cell death was also assessed by the Annexin V-FITC Apoptosis Detection kit (Immunostep®) according to the manufacturer's instructions. Analysis was performed by flow cytometry as previously described.

Evaluation of apoptotic factors

K562, Jurkat and MM.1S cells (5×10^5 cells/well) were incubated for 12 h with sulfonamide **DFS16** (24 h IC_{50}). For the evaluation of the mitochondrial membrane potential ($\Delta\Psi m$), cells were incubated with a MitoView 633 ™ (Biotium®, USA) solution (diluted 1:10.000) and analyzed by flow cytometry as previously described. For protein expression, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences®, USA) (except cells used in FasR analysis). Subsequently, cells were stained with anti-Bcl-2-FITC (nº 1F-668-T100, Exbio®, USA), anti-Bax-PerCP (nº SC-7480 PercP, Santa Cruz Biotechnology®, USA), anti-activated-caspase-3-V450 (nº 560627, BD Biosciences®, USA), anti-AIF-FITC (nº SC-13116 FITC, Santa Cruz Biotechnology®, USA), anti-Ki-67-FITC (nº 11-5699-41, eBioscience®, USA), anti-survivin-PE (nº SC-17779 PE, Santa Cruz Biotechnology®, USA) and anti-FasR-PE (nº SC-8009 PE, Santa Cruz Biotechnology®, USA). After staining, cells were washed and resuspended in PBS for cytometer analysis as previously described.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and each experiment was repeated at least three times independently. Statistical analysis was conducted using the paired *t* test or ANOVA one-way and two-way, complemented by Bonferroni or Tukey post hoc tests. Statistical analyses were performed using GraphPad Prism 6 software.

Results and discussion

New sulfonamide derivatives are cytotoxic against hematological malignant cells

Current therapeutic strategies against hematologic malignancies include a variety of chemotherapeutic drugs and hormonal agents that aim to disturb cellular homeostasis and induce neoplastic cells to apoptosis (Sahu et al. 2017). Although initial remission is often achieved, most patients progress to relapse as a result of drugs resistance (Hojjat-Farsangi, 2015, Foo and Michor, 2014). Thereby, the search for new therapeutic options, including the synthesis of new molecules as new bioactive compounds, may improve the prognosis and the long term survival of cancer patients. In the present study, the initial screening aimed to identify the cytotoxic effectiveness of 26 new sulfonamide derivatives on K562, Jurkat and MM.1S cells (Fig. 1a, b, c). The compounds that significantly reduced the cell viability when compared to the control groups (non-treated cells) were **DFS 1-5, DFS 7-16, Gli 1, Gli 3-8** and **Gli 10** in K562 cell line, **DFS 1-2**, **DFS 4-6**, **DFS 8-16** and **Gli 1-10** in Jurkat cell line and **DFS 1-5**, **DFS 7-13**, **DFS 15-16** and **Gli 5** in MM cell.

Considering these results, the five compounds that provided the best cytotoxic effect on these three cell lines (**DFS8, DFS12, DFS15, DFS16** and **Gli5)** were chosen to be evaluated under different concentrations (1–100 µM) for 24 h. As shown in Fig. 1d, e, f, all the first selected sulfonamides reduced the cell viability in a concentration-dependent manner. The analysis of their chemical structures (Schemes 1 and 2) demonstrates that the four sulfonamides from the DFS series (**DFS8**, **DFS12**, **DFS15** and **DFS16**) have an electronegative group at the 4-position of the aromatic ring connected to the sulfur portion of the molecule, which seems to be an important point for the creation of a pharmacophore. However, the comparison between the molecular structures of sulfonamides **DFS5** (not selected) and **DFS16** (selected), both having a NO_2 group at the 4-position of the aromatic ring, reveals that the presence of this electronegative group only is not enough to improve their cytotoxic activity. This difference seems to be associated with the presence of a NO_2 group at the 2-position of the aromatic ring, which gives more activity to the structure.

According to the literature, compounds with IC_{50} higher than 30 µM are considered inactive (Burger and Fiebig, 2014). Therefore, based on this criterion and on the fact that **DFS16** was significantly more cytotoxic than the other compounds, this sulfonamide was the only derivative selected

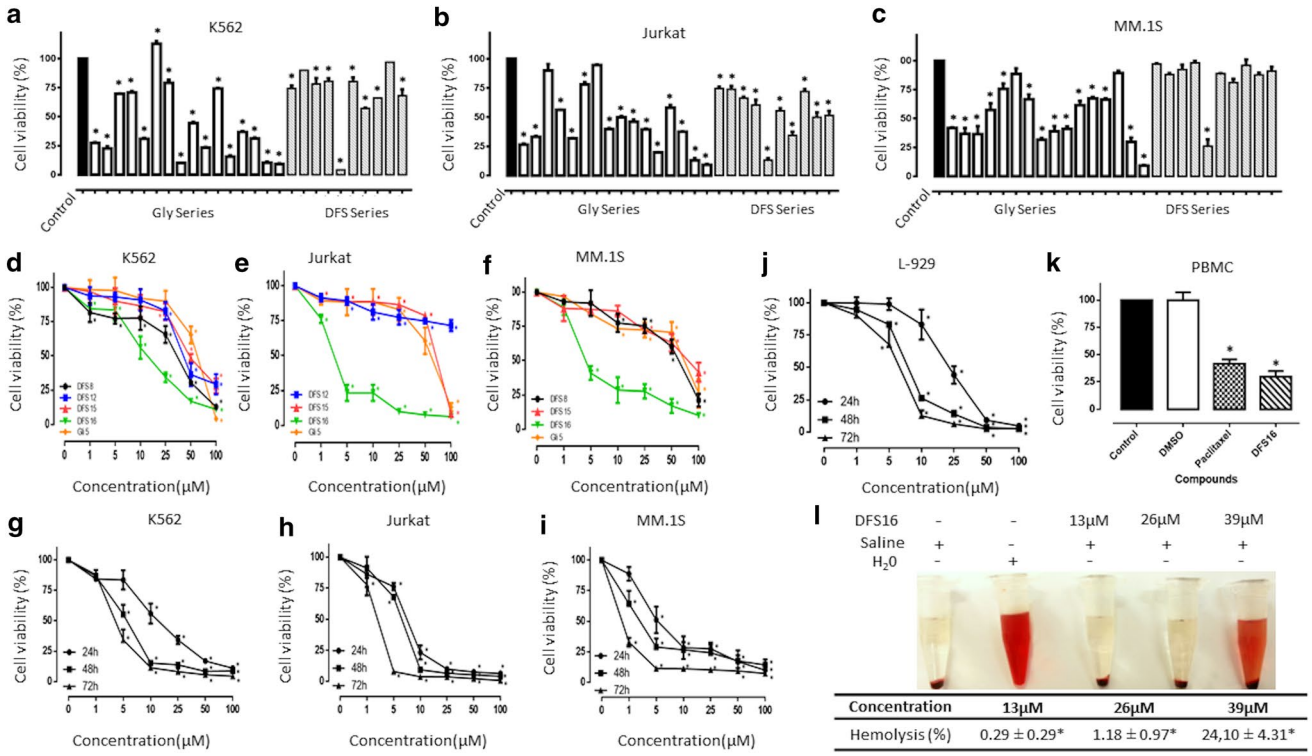


Fig. 1 Effect of new sulfonamide derivatives on hematological tumor cells and non-tumor cells. **a–c** Screening of the 26 new synthetic sulfonamides on K562 (**a**), Jurkat (**b**) and MM.1S (**c**) cells. **d–f** Cytotoxic effect of first selected sulfonamides **DFS8**, **DFS12**, **DFS15**, **DFS16** and **Gli5** on K562 (**d**), Jurkat (**e**) and MM.1S (**f**) cells. **g–i** Concentration- and time-response curves of sulfonamide **DFS16** on K562 (**g**), Jurkat (**h**) and MM.1S (**i**) cells. **j**, **k** Cytotoxic effect of sulfonamide **DFS16** on L-929 fibroblasts (**j**) and PBMC (**k**). **l** Hemolytic effect of **DFS16** on RBC. Each figure represents the mean ± SEM of at least three independent experiments. *Difference when compared to the control groups. #Statistical similarity with the highest cytotoxic compound in each series. $p < 0.05$, one-way ANOVA followed by Bonferroni or Tukey post hoc tests or t-test

Table 1 IC_{50} values calculated for DFS16. IC_{50} on AL cells K562 and Jurkat, on MM cells MM-1.S and on normal fibroblasts L929

IC_{50} (μM)	K562	Jurkat	MM-1.S	L-929
24 h	13.0 ± 0.3	6.1 ± 0.5	5.9 ± 0.3	20.9 ± 1.4
48 h	4.3 ± 0.4	4.6 ± 0.6	2.9 ± 0.6	7.2 ± 0.7
72 h	2.7 ± 0.4	1.4 ± 0.9	0.6 ± 0.2	4.7 ± 0.6

for further experiments. **DFS16** reduced the cell viability of K562, Jurkat and MM.1S cells in a concentration and time-dependent manner (Fig. 1g–i), with IC_{50} values lower than 15 μM after 24 h treatment and lower than 3 μM after 72 h (Table 1). The strong cytotoxicity of **DFS16** seems to be associated with the presence of a NO_2 group at the 2-position of the aromatic ring, as it was the only compound with an electronegative group at this position. The cytotoxic effect of sulfonamides has been extensively investigated by the scientific community (Hu, 2006; Doungsoongnuen et al. 2011; Pogorzelska et al. 2018). A study conducted by Liu et al. (2012) investigated the effect of MPSP-001, a benzenesulfonamide capable of destabilizing the microtubules, on K562 cells, and found an IC_{50} of 6.9 μM after 48 h incubation, which was higher than **DFS16** at the same conditions (IC_{50} of 4.3 μM). The divergences in cytotoxicity between the compounds investigated and those described in the literature are directly related to their structures, as it is well established that variations in the aromatic rings size and modifications of certain substituents are able to modify the compounds activity.

Sulfonamide DFS16 is not cytotoxic to non-tumor cells

Currently available chemotherapeutics are associated with low specificity and high morbidity and mortality rates as they are also cytotoxic to normal cells (Kadia et al. 2016, Vasekar et al. 2016). Thus, the search for new bioactive compounds must include their effect on non-neoplastic cells. Our results revealed that sulfonamide **DFS16** reduced the cell viability of L929 fibroblasts at a lower range when compared to hematological malignancies cells (Fig. 1j). The choice of this cell line was to mimic the medullar microenvironment which has fibroblasts in its

composition. The IC_{50} values (Table 1) in L929 cells were significantly higher when compared to tumor cells. The SI comparing non-tumor cells (L929) to hematological tumor cells showed a moderate selectivity over K562 (SI = 1.61) cells and a high selectivity over Jurkat and MM.1S cells (SI = 3.41 and 3.62, respectively).

In addition, when **DFS16** was evaluated in PBMC (Fig. 1k), the results demonstrated a 41.5% reduction in cell viability, which was similar to paclitaxel, a chemotherapeutic drug already used in clinics. Finally, the impact of **DFS16** on healthy RBC was evaluated to verify whether its systemic use would be compromised. According to the literature, a chemotherapeutic that induces hemolysis, however potent, has its therapeutic use limited due to the adverse effects to the patients (Mocan, 2010). The results showed that only treatment with 39 µM of **DFS16**, a concentration three times higher than the higher IC_{50} calculated in hematological malignancies (13.0 ± 0.3 µM in K562) was able to induce significant hemolysis on normal RBC (Fig. 1L). Thus, as the selected sulfonamide did not cause significant hemolysis on RBC even at concentrations much higher than its IC_{50}, this suggests that it could be safely administered intravenously.

Sulfonamide DFS16 induced cell cycle arrest in MM cells

Multiple cell signaling pathways can be influenced by chemical compounds, such as the induction of DNA damage, the disruption of cell cycle progression and the induction of apoptosis (Galluzzi et al. 2018). Several studies have reported the interaction of sulfonamides with the microtubule dynamics by preventing the mitotic spindle formation and resulting in cell cycle blockage (Liu et al. 2012; Fortin et al. 2011; Pogorzelska et al. 2018; Sabt et al. 2018). In the present study, sulfonamide **DFS16** increased the proportion of cells at the sub-G0/G1 phase (dead cells) in K562, Jurkat and MM.1S cell lines when compared with the control groups (Fig. 2a, b, c). This result confirms the previously discussed data that demonstrates that this new compound induced potent cell death to hematological neoplastic lineages. In addition, **DFS16** blocked the G2/M phase of MM.1S cells, as reflected by a significant 10% increase in the percentage of cells at this phase when compared with the control group. As the literature reports the capacity of sulfonamide derivatives to modulate the microtubules dynamics and, therefore, prevent chromosome segregation during cell division, an event related to the G2/M phase (Pogorzelska

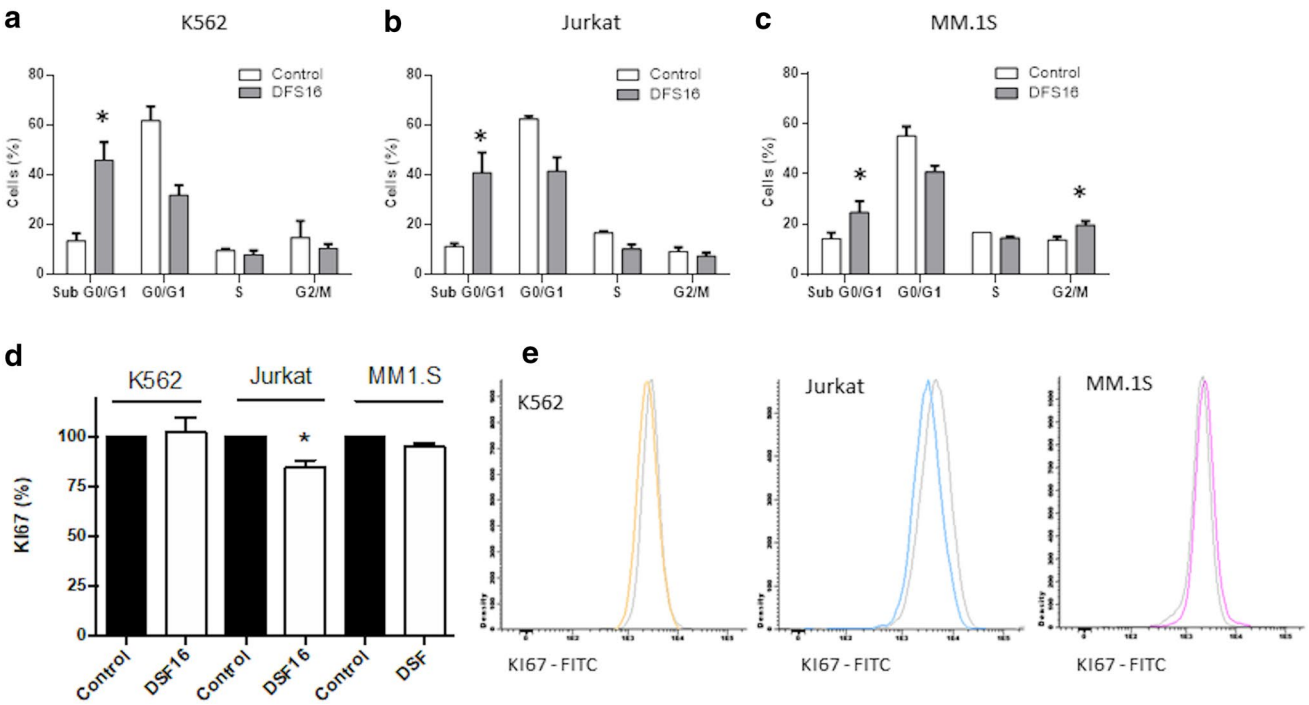


Fig. 2 Effect of sulfonamide DFS16 on the cell cycle of hematological tumor cells. **a**–**c** The cell cycle of K562 (**a**), Jurkat (**b**) and MM.1S cells (**c**) treated with **DFS16** (IC_{50}) was evaluated after PI staining by flow cytometry. **d** K562, Jurkat and MM.1S cells were incubated for 12 h in the absence (control groups) and in the presence of **DFS16** (IC_{50}) and the medium fluorescence intensity (MFI) was determined by flow cytometry after incubation with anti-KI67. Histograms (**e**) illustrate one experiment, grey line represents control groups and colored lines represent DSF16 groups. Each point and/or figure represents the mean ± SEM of at least three independent experiments. *$p < 0.05$ when compared to the control groups, *t*-test or one-way ANOVA followed by Tukey post hoc test

et al. 2018; Sabt et al. 2018), this might explain MM.1S cycle blockage by **DSF16**. Still, further studies should be carried out to elucidate whether this compound is inducing disturbances in the cell cytoskeleton or modulating cyclines and CDKs involved in cell cycle progression. Additionally, as after only 12 h incubation, no statistical difference was observed in the other two cell lines, further experiments should be conducted at different incubation periods to clarify if **DSF16** would then be able to block K562 and Jurkat cells cycles or if the heterogeneity of hematological malignancies play a role in the different responses to this sulfonamide derivative.

Interestingly, as shown in Fig. 2d, **DFS16** reduced the expression of the cell proliferation marker KI67 in Jurkat cells (16%), but not in K562 or MM.1S. Uncontrolled cell proliferation is one of the main characteristics of neoplastic cells. KI67 nuclear protein is an important marker of cycling cells, whether they are healthy or tumoral, and it is used in clinics to evaluate cancer prognosis (Jaafari-Ashkavandi et al. 2018). To date, no published articles have been found demonstrating the influence of sulfonamides on KI67 expression. However, because this protein is strongly associated with cell proliferation, tumor progression and a poor prognosis especially in patients with diffuse large B-cell lymphoma (Koff et al. 2015), its decreased expression in Jurkat cells after **DFS16** treatment which may indicate that this compound also has some cytostatic activity. Nevertheless, the non-modulation of KI67 observed in K562 and MM.1S cells could be a resistance mechanism against **DFS16** treatment as, according to the literature, malignant cells try to replicate more in response to cytotoxic agents (Galluzzi et al. 2018). Especially in MM.1S cells, as **DFS16** induced cell cycle arrest, therefore, a decreased KI67 expression was expected, an attempt to escape cell death induced by **DFS16** is very likely as this is known to be a very resistant lineage and, in clinics, a very aggressive malignancy.

Sulfonamide DFS16 induced apoptotic-like cell death in malignant cells

As previously mentioned, disruptions in cell proliferation and cell cycle arrest are mechanisms desired by new chemotherapeutic compounds because if the cell fails to repair these damages, signaling pathways are initiated so that the cell can be destroyed (Galluzzi et al. 2018). Morphological changes resulting from cell death induced by sulfonamide **DFS16** were first observed by fluorescence microscopy. Figure 3a shows that K562, Jurkat and MM.1S cells exhibited late apoptosis characteristics (formation of apoptotic bodies and loss of membrane integrity) after treatment with **DFS16**. K562 cells also exhibited characteristics of cells in initial apoptosis such as chromatin condensation and pycnosis. According to the literature, apoptosis is a regulated type of cell death that maintains the morphology of cellular organizations and whose main biochemical characteristics include phosphatidylserine exposure, caspase activation and mitochondrial depolarization. One of the last apoptotic steps is the cellular DNA fragmentation in multiple fragments of 180 to 200 base pairs by endonucleases, which form a breaking pattern called "ladder pattern" when separated on agarose gel. Finally, there is the formation of prolongations in the plasma membrane called blebs which lead to cell fragments called apoptotic bodies. These structures are recognized as phagocytic targets and phagocytosed by immune cells (Zhang et al. 2015, Goldar et al. 2015, Galluzzi et al. 2018). As apoptosis is a regulated type of cell death that results in low inflammatory events, it is a very desirable target in the search for new compounds with chemotherapeutic potential.

In our study, apoptosis was confirmed by the DNA fragmentation assay, as the "ladder pattern" was observed in **DFS16**-treated K562, Jurkat and MM.1S cells (Fig. 3b). Finally, the percentage of apoptotic cells was determined by flow cytometry by detecting phosphatidylserine exposure after **DFS16** treatment. As expected, the results showed an increase of 53.7%, 79.8% and 121.6% in Annexin V-positive K562, Jurkat and MM.1S cells, respectively, after treatment with the sulfonamide, when compared with the respective controls (Fig. 3c). As we have discussed, the activation of pathways that result in apoptosis is very advantageous for new bioactive compounds as apoptosis is a regulated type of cell death, therefore resulting in fewer adverse effects to adjacent cells and, consequently, to less side effects in clinics (Galluzzi et al. 2018). In the present study, we have demonstrated by three different methodologies that **DFS16** induced an apoptotic-like cell death on hematological malignancies cells, which corroborates other studies that reported the induction of apoptosis in different neoplastic cells after treatment with sulfonamides (Fortin et al. 2011; Lee et al. 2017; Liu et al. 2012; Pogorzelska et al. 2018; Sabt et al. 2018).

Sulfonamide DFS16 activates intrinsic and extrinsic apoptosis

It is well established that the processes responsible for the regulation of apoptosis involve two main pathways: extrinsic apoptosis or the death receptor pathway, and intrinsic or mitochondrial apoptosis (Zhang et al. 2015). Intrinsic apoptosis involves mitochondrial disruption in response to internal and/or external stimuli. When a signal stimulates mechanisms that interfere with cellular homeostasis, such as DNA damage, members of the Bcl-2 family are inhibited or activated. The inhibition of antiapoptotic protein Bcl-2 and the Bax-facilitated opening of the mitochondrial permeability transition pore result in a disruption of $\Delta\Psi m$ and in the release of pro-apoptotic proteins to the cytosol

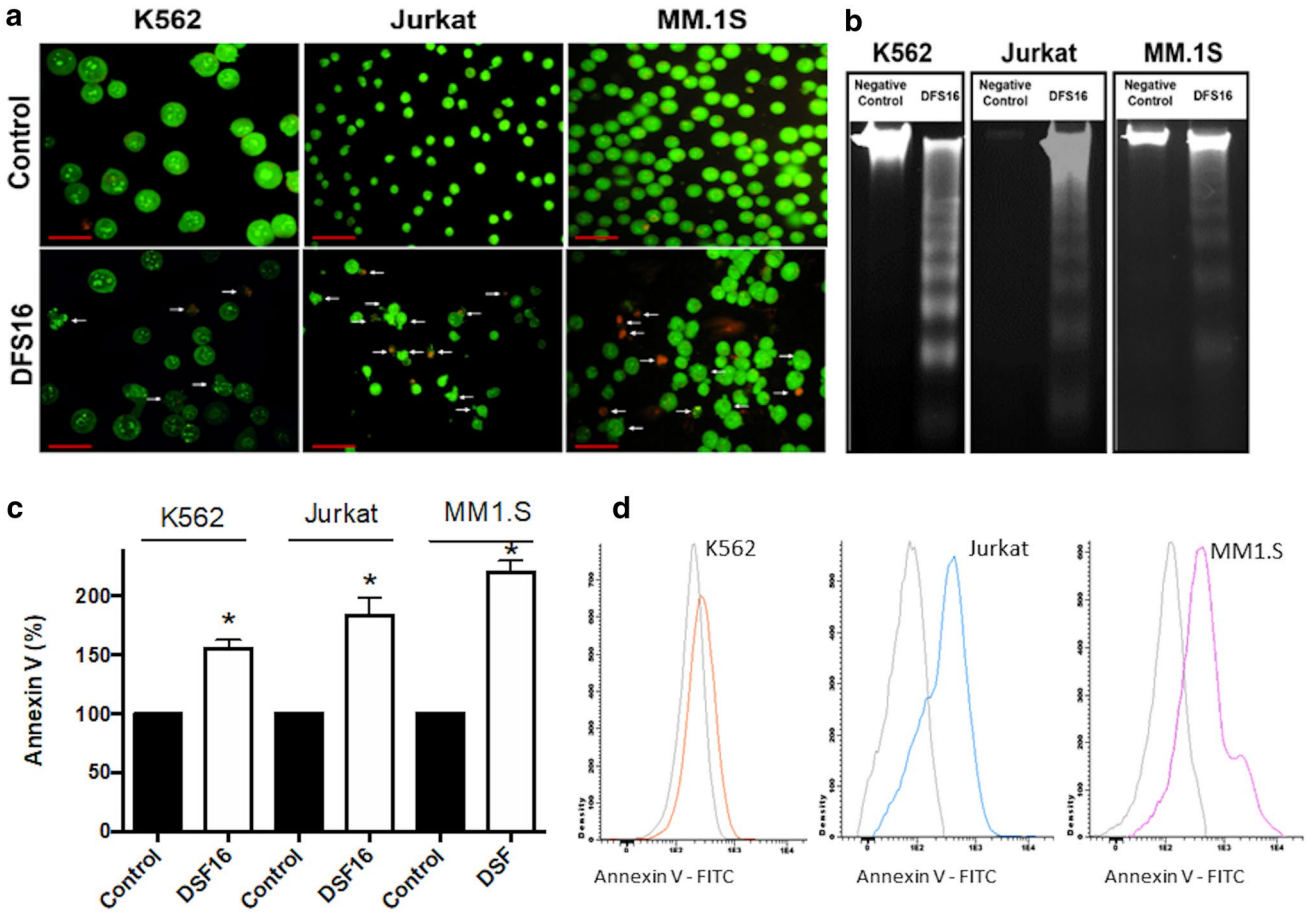


Fig. 3 Apoptosis induced by sulfonamide DFS16 on hematological tumor cells. **a** K562, Jurkat and MM.1S cells treated with DFS16 (C_{50}) for 12 h were stained with EB/AO and observed in a fluorescence microscope. Green cells stained with AO indicate intact cell membranes, while red cells stained with EB indicate loss of membrane integrity. White arrows indicate cells with morphological changes suggestive of apoptosis. Scale bars (red lines) represent 100 µM. **b** Cells were incubated with DFS16 (IC_{50}) for 24 h and DNA fragmentation was evaluated. **c** Cells were incubated for 12 h in the absence (control groups) and in the presence of DFS16 (IC_{50}) and Annexin V-positive cells were quantified by flow cytometry. Histograms (**d**) illustrate one experiment, grey line represents control groups and colored lines represent DSF16 groups. Each point and/or figure represents the mean ± SEM of at least three independent experiments. $^{*}p < 0.05$ when compared to the control groups, *t*-test or one-way ANOVA followed by Tukey post hoc test

(Galluzzi et al. 2018). Sulfonamide **DFS16** decreased Bcl-2 expression in MM.1S cells by 14% (Fig. 4a) and increased Bax expression in both K562 (30%) and Jurkat (25%) cell lines (Fig. 4b). According to the literature, a decrease of Bcl-2 antiapoptotic protein or an elevation of Bax pro-apoptotic protein is, by itself, sufficient to invert the Bax/Bcl-2 ratio, which favors the pro-apoptotic signal and results in the activation of apoptosis (Galluzzi et al. 2018, Sabt et al. 2018). **DFS16** was able to increase the Bax/Bcl-2 ratio in all three cell lines (Fig. 4c, d), demonstrating the predominance of Bax in relation to Bcl-2 (K562 = 1.16, Jurkat = 1.30, MM1.S = 1.25). Possibly as a result of the imbalance between Bcl-2 family members, treatment with **DFS16** resulted in the reduction of ΔΨm in all three hematological malignancies cell lines: 8.4% in K562, 44.4% in Jurkat and 37.8% in MM.1S (Fig. 4e, f). The ΔΨm loss by **DFS16** resulted in AIF release on Jurkat cells (24% increase in AIF expression) (Fig. 4g, h). After being released, AIF migrates directly to the nucleus, where it induces DNA condensation and fragmentation independently of caspase activation (Sabt et al. 2018). AIF expression was not increased in K562 and MM.1S cells after **DFS16** treatment, which suggests that other mitochondrial proteins such as cytochrome c might be involved in cell death induced by this compound on these two cell lines. Cytochrome c, the main mitochondrial protein involved in apoptosis, is located in the space between inner and outer mitochondrial membranes where it acts as an electron transporter in the transport chain. When released through the mitochondria it decreases ATP production and is associated with oxidative stress. Then, cytochrome c induces the formation of apoptosome, a multimeric complex

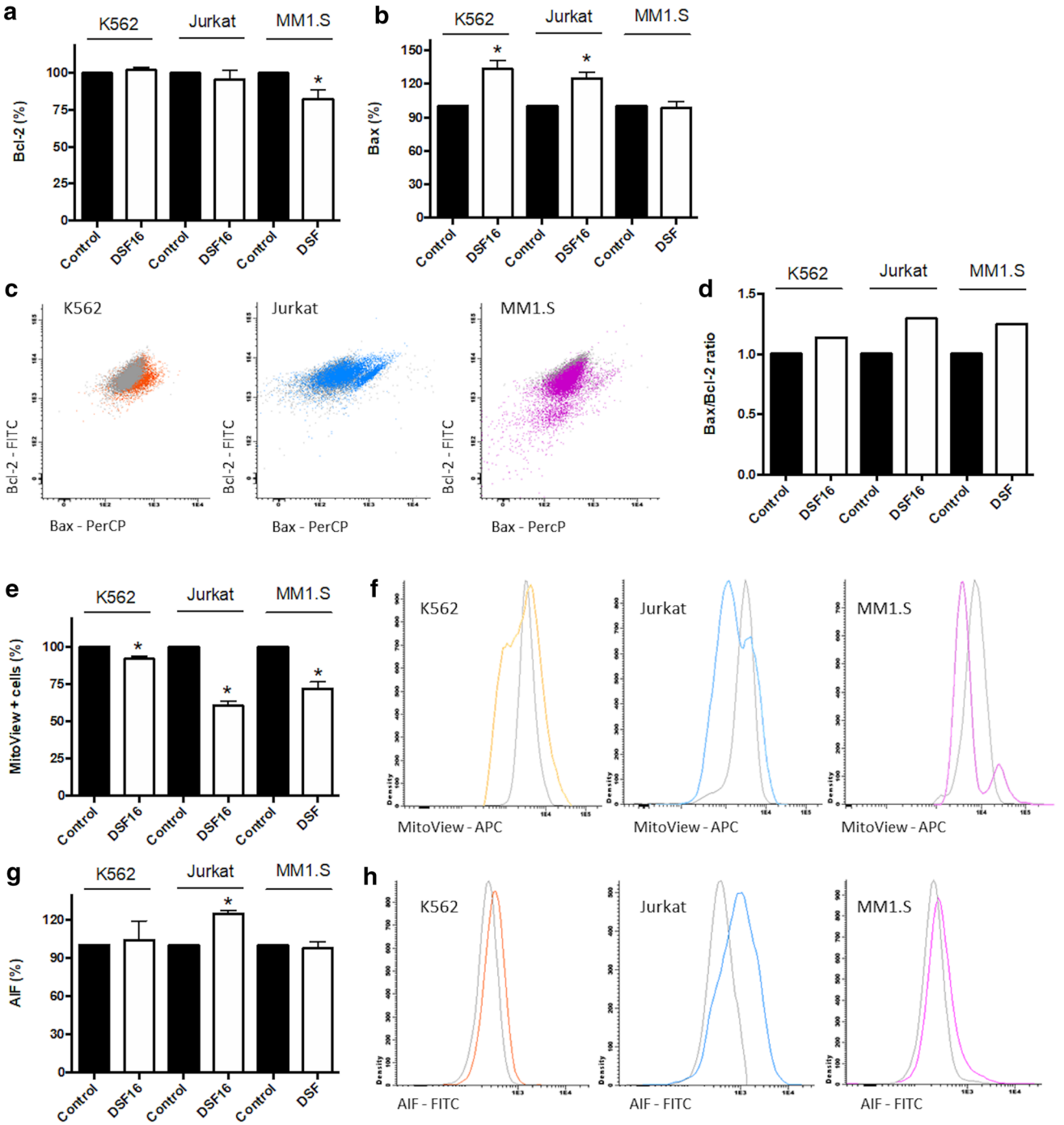


Fig. 4 Effect of DFS16 on intrinsic apoptosis. K562, Jurkat and MM.1S cells were incubated for 12 h in the absence or in the presence of **DFS16** (IC_{50}) and the MFI was determined by flow cytometry. Treated and untreated cells were stained with anti-Bcl-2 and anti-Bax (**a–d**) or anti-AIF (**g, h**), while the ΔΨm was determined using the MitoView 633 ™ kit (**e, f**). Dotplots and histograms (**c**, **f**, **h**) illustrate one experiment, grey line represents control groups and colored lines represent DSF16 groups. Bax/Bcl-2 ratio (**d**) was determined by dividing the MFI_{Bax} by the MFI_{Bcl-2} and compared with the control group. Each point and/or figure represents the mean ± SEM of at least three independent experiments. $^*p < 0.05$ when compared to the control groups, *t*-test or one-way ANOVA followed by Tukey post hoc test

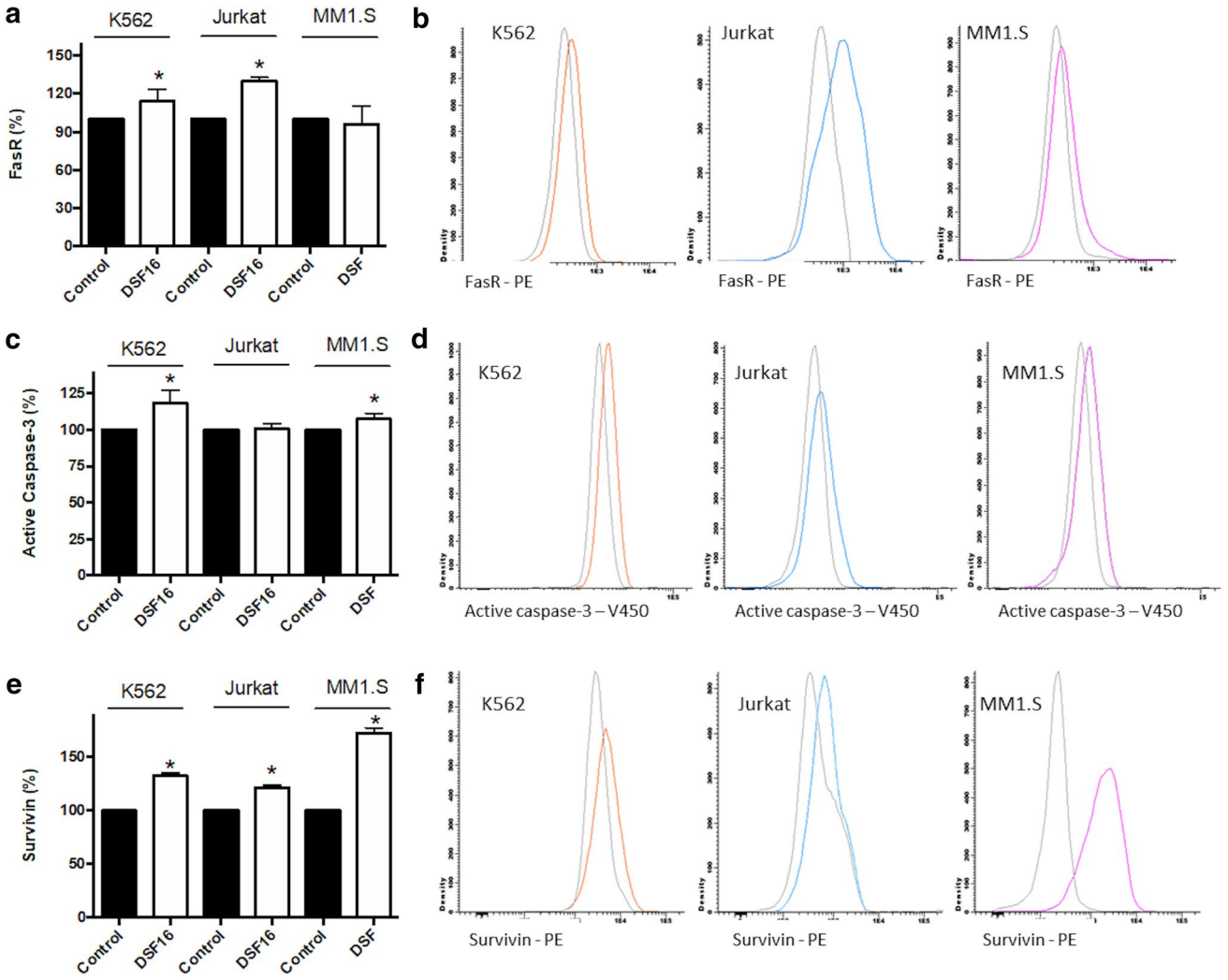


Fig. 5 Effect of DFS16 on extrinsic and common apoptosis. K562, Jurkat and MM.1S cells were incubated for 12 h in the absence or in the presence of **DFS16** (IC_{50}) and the MFI was determined by flow cytometry. Treated and untreated cells were stained with anti-FasR (**a**, **b**), anti-activated caspase-3 (**c**, **d**) or anti-survivin (**e, f**). Histograms (**b, d, f**) illustrate one experiment, grey line represents control groups and colored lines represent DSF16 groups. Each point and/or figure represents the mean ± SEM of at least three independent experiments. *$p < 0.05$ when compared to the control groups, *t* test or one-way ANOVA followed by Tukey post hoc test

ATP-dependent that results in the activation of caspase 9 and caspase-3 (Goldar et al. 2015).

Extrinsic apoptosis is activated by receptors present on the plasma membrane, such as FasR and TRAIL-R. These receptors are capable of triggering an intracellular signaling cascade through the cleavage of pro-caspase-8 and -10 and the formation of the death-inducing complex (DISC) in the cytoplasm, consequently activating caspase-3 (Sabt et al. 2018). **DFS16** increased FasR expression in both K562 (12%) and Jurkat (30%) cells Fig. 5a, b), which indicates that this sulfonamide derivative is also capable of activate the extrinsic apoptosis in AL cells. In MM.1S cells, though FasR modulation was not observed, extrinsic apoptosis may not be excluded, as other membrane receptors might be involved in cell death.

As previously discussed, the activation of both the intrinsic and the extrinsic apoptosis converge to the activation of effector caspases, such as caspase-3, -6 and -7 (Galluzzi et al. 2018). We have demonstrated that sulfonamide **DFS16** significantly increased active-caspase-3 expression in K562 (18%) and MM1.S (7%) cells (Fig. 5c, d), confirming the execution of apoptosis and suggesting that cytochrome c might be involved in these cells' death mechanisms. While **DFS16** seems to induce very classical intrinsic and extrinsic apoptosis in K562 cells, in MM.1S cell line, this sulfonamide seems to activate different pathways than those observed on AL cells. In MM.1S cells, **DFS16** induced cell cycle arrest at G2/M phase and an apoptotic-like cell death with $\Delta\Psi m$ loss, DNA fragmentation and a slight, however significant caspase-3 activation. These differences may be explained by the

fact that AL is originated from immature cells, while MM originates from a differentiated disease and, considering the heterogeneity of hematological malignancies, suggest that other pathways, including other types of cell death should be investigated to elucidate the sulfonamide mechanisms of cell death in MM. In Jurkat cells, even though **DFS16** appears to activate apoptosis, as demonstrated by ΔΨm loss, increased expression of Fas, Bax and AIF and DNA fragmentation, no significant change in caspase-3 was observed. Nevertheless, Jurkat was the only investigated cell line in which a significant AIF release was observed after **DFS16** treatment. As this protein is known to trigger a caspase-independent cell death, this might explain the non-activation of caspase-3 by **DFS16** in Jurkat cells and might be one of the mechanisms of sulfonamides in lymphoblastic malignancies. Additionally, several other proteins released by the mitochondria have pro-apoptotic activities independently of caspases. Endonuclease G, for instance, is involved in DNA fragmentation and ΔΨm loss and, after release into the cytoplasm, translocates to the nucleus to execute the apoptotic signal. SMAC protein also promotes apoptosis by inhibiting the endogenous inhibitors of caspases, such as survivin, which is considered an antiapoptotic protein because of its ability to inhibit caspase-9, -3 and -7 (Galluzzi et al. 2018). Interestingly, survivin was overexpressed in all three cell lines treated with sulfonamide **DFS16** (Fig. 5e, f). According to the literature, this protein is overexpressed in most human neoplasms and is related to cell resistance, therefore, it is associated with a worse prognosis. Our result indicate that K562, Jurkat and MM1.S cells are resisting against the cytotoxic effects of **DFS16**, however, despite the efforts to escape cell death, this new sulfonamide is still able to induce these hematological malignancies cells to die, as we have demonstrated in this paper, which is a very promising and interesting result.

It is important to highlight that several other possible routes should be investigated in regard of sulfonamide **DFS16** mechanisms on hematological malignancies, such as the activation of endoplasmic reticulum stress, the participation of ROS and the activation of MAP kinases and NFkB pathways (Galluzzi et al. 2018, Sabt et al. 2018).

Conclusions

The set of results presented in this study suggests that the novel sulfonamide derivative **DFS16** induced apoptotic-like cell death on different hematological malignancies, yet by the activation of different pathways. On ALL cells, sulfonamide **DFS16** decreased the cell proliferation marker Ki67 and activated extrinsic and intrinsic apoptosis by decreasing FasR and by inverting Bax/Bcl-2 ratio. This led to ΔΨm loss and AIF release with no caspase-3 modulation, which suggests a caspase-3-independent apoptosis. On AML, **DFS16** activated both intrinsic and extrinsic apoptosis with caspase-3 activation, which indicates a more classical mechanism. In MM, **DFS16** induced cell cycle arrest at the G2/M phase and apoptosis with ΔΨm loss and DNA fragmentation, which might hypostatize that cell cycle mechanisms are first involved in cell death induced by this compound on MM cells. Finally, **DFS16** seems to be selective for malignant cells and might be administered intravenously. The superiority of sulfonamide **DFS16** when compared with the other 25 compounds investigated in this paper, as well as its strong and selective cytotoxic activity against hematological malignancies, might be explained by its interesting chemical structure, specially by the presence of an electronegative group at the 4-position of the aromatic ring connected to the sulfur portion of the molecule, which seems to be an important point for the creation of a pharmacophore. Additionally, the presence of a NO2 group at the 2-position of the aromatic ring seems to give even more activity to **DFS16** structure. Altogether, the results presented in this paper suggest that **DFS16** is a promising molecule that could be used as a prototype for the development of new chemotherapeutics to treat hematological malignancies and further experiments should be conducted to elucidate its cell death mechanisms.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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