

# Selected nitrostyrene compounds demonstrate potent activity in chronic lymphocytic leukaemia cells, including those with poor prognostic markers

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**Abstract.** The nitrostyrene scaffold was previously identified as a lead target structure for the development of effective compounds targeting Burkitt's lymphoma. The present study aimed to develop these compounds further in haematological malignancies, including chronic lymphocytic leukaemia (CLL). Cellular viability, flow cytometry and lactate dehydrogenase assays, amongst others, were used to examine the effects of nitrostyrene compounds on CLL cells, including a cell line representing disease with poor prognosis (HG-3) and in *ex vivo* CLL patient samples (n=14). The results demonstrated that two representative nitrostyrene compounds potently induced apoptosis in CLL cells. The pro-apoptotic effects of the compounds were found to be reactive oxygen species and caspase-dependent, and had minimal effects on the viability of

normal donor peripheral blood mononuclear cells. Nitrostyrene compounds exhibited synergistic augmentation of apoptosis when combined with the phosphatidylinositol 3-kinase inhibitor idelalisib and demonstrated potent toxicity in *ex vivo* CLL cells, including those co-cultured with bone marrow stromal cells, making them more resistant to apoptosis (n=8). These compounds also demonstrated activity in samples from patients with poor prognostic indicators; unmutated immunoglobulin heavy chain genes, expression of CD38 and deletions in chromosomes 17p and 11q. These results suggest that this class of pharmaceutically active compounds offer potential in the treatment of CLL.

## Introduction

Chronic lymphocytic leukaemia (CLL) is the most common form of leukaemia and involves the clonal expansion of abnormal B cells in the lymph nodes and/or blood. B cells, part of the adaptive immune response, function to produce antibodies against antigens; however in CLL, B cells fail to mature and differentiate correctly, eventually crowding out healthy blood cells (1). CLL is a disease which mainly affects adults and can clinically be subdivided into two major types depending on the mutational status of the immunoglobulin heavy chain gene (IGHV): Patients with CLL cells harbouring unmutated IGHV (UM-IGHV) have an average survival of 8 years, whereas patients with CLL with mutated IGHV (M-IGHV) have an average survival of >25 years. In addition, acquired chromosomal abnormalities involving deletions in chromosomes 11q, 13q and 17p and trisomy 12 are common in CLL, and these abnormalities predict the time to first treatment and CLL-specific survival rate. The genes principally responsible for an adverse prognosis, associated with deletions (del) in 17p13 and del11q22, have been recognised as *TP53* and *ATM*, respectively (2). Other poor prognostic markers have been identified, including the lack of expression of CD38, expression of cytoplasmic marker z-associated protein 70 and, more recently, mutations in p53, Notch-1, splicing factor 3B subunit 1 and baculoviral IAP repeat-containing protein 3 (2).

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**Abbreviations:** Btk, Bruton's tyrosine kinase; CAP, cyclophosphamide/doxorubicin/prednisone; CHOP, cyclophosphamide/hydroxydaunorubicin (doxorubicin)/oncovin (vincristine)/prednisone; CLL, chronic lymphocytic leukaemia; EBV, Epstein Barr virus; EMA, European Medicines Agency; FBS, foetal bovine serum; FDA, US Food and Drug Administration; LDH, lactate dehydrogenase; M-IGHV, mutated immunoglobulin heavy chain gene; 4-MTA, 4-methylthioamphetamine; Nac, N-acetyl-cysteine; PBMCs, peripheral blood mononuclear cells; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; SAR, structure activity relationship; SEM, standard error of the mean; SERT, serotonin reuptake transporter; UM-IGHV, unmutated immunoglobulin heavy chain gene; ZAP-70, serum marker z-associated protein 70

**Key words:** nitrostyrene, chronic lymphocytic leukaemia, B cell, idelalisib

Current treatment paradigms for CLL involve use of fludarabine phosphate, cyclophosphamide and monoclonal antibodies, including rituximab (known as the FCR regime) (3). Fludarabine phosphate, the gold standard treatment for CLL, was originally synthesised in 1969 (4), and received US Food and Drug Administration (FDA)-approval for the treatment of CLL in 1991. It is a synthetic adenine nucleoside pro-drug, which is activated to a triphosphate form, F-ara-ATP. This activated form of fludarabine phosphate interferes with DNA synthesis by inhibiting ribonucleotide reductase, DNA polymerases, DNA ligase I and DNA primase. It is indicated for the first- and second-line treatment of CLL, and leads to more complete remissions (CR; 7-40%) than other conventional chemotherapies, including cyclophosphamide/doxorubicin/prednisone (CAP) and cyclophosphamide/hydroxydaunorubicin (doxorubicin)/oncovin (vincristine)/prednisone treatment (CHOP) regimens (5). Fludarabine phosphate is also successfully used in combination with several other chemotherapeutics.

A number of more recent agents have also received European Medicines Agency (EMA) and FDA approval for the treatment of CLL: Ibrutinib, as a frontline treatment, which targets B-cell receptor signalling through the inhibition of Bruton's tyrosine kinase (Btk) (6); obinutuzumab approved in combination with chlorambucil, which is a type-2 monoclonal anti-CD20 antibody that results in direct and antibody-dependent cell-mediated cytotoxicity of leukaemia cells (7); and idelalisib approved in combination with rituximab for relapsed CLL, which inhibits B-cell receptor signalling through the inhibition of phosphatidylinositol 3-kinase (PI3K)  $\delta$  molecules (8). The most recently approved CLL drug, venetoclax, is a B-cell lymphoma 2 (Bcl-2) inhibitor. However, there are significant issues associated with these drugs, including the development of resistance, and there is an unmet need for alternative drug therapies for these diseases.

Antidepressants are a class of compounds which mimic the effects of naturally occurring neurotransmitters. They inhibit reuptake transporters, maintaining high concentrations of neurotransmitters in the synapse, and are used to treat the symptoms of depression, anxiety and several other psychological illnesses (9,10).

The finding of the serotonin reuptake transporter (SERT) in B cell malignancies (11) led to the investigation of antidepressants and their analogues as potential anti-lymphoma/leukaemic agents. Activity has been demonstrated by tricyclic antidepressants citalopram, imipramine and clomipramine in HL-60 human acute myeloid leukemia cells and normal lymphocytes (12-14); and fluoxetine (11,15,16), MDMA and its analogues (11,17), fenfluramine (11), clomipramine (11), and maprotiline and its analogues (15,16,18) in Burkitt's lymphoma cell lines. In addition, our previous studies reported on the synthesis and SERT inhibitory activity of a number of structurally related 1,3-bis(aryl)-2-nitro-1-propene and 1,3-bis(aryl)-2-propanamine compounds and identified their antiproliferative activity against Burkitt's lymphoma (19,20). However, antidepressants have been shown to exhibit toxicity against a wide range of cancer cell lines (21-24), other than those of lymphatic origin, and toxicity has been reported to be independent of SERT and other transporters (15).

The drug 4-methylthioamphetamine (4-MTA) is an illegal analogue of the antidepressant amphetamine. Nitrostyrenes

[(2-nitrovinyl)benzenes] are a family of compounds based on the scaffold structure of 4-MTA. Nitrostyrene derivatives have also been reported to induce potent anticancer effects in a range of cancer cell lines; for example, nitrostyrenes have demonstrated pronounced anticancer activity in oral (25) and colon cancer (26), osteosarcoma (27) and Erlich ascetic tumour cell lines (28). Nitrostyrene derivatives have also been shown to possess a diverse range of other biological activities, including antimicrobial (29), anti-inflammatory (30) and immunosuppressive properties (31). Various mechanisms of action have been identified for nitrostyrene analogues, including tubulin inhibition (29,32), telomerase inhibition (33), protein tyrosine phosphatase (PTP) inhibition (34) and phospholipase A<sub>2</sub> inhibition (35).

Our previous study reported on the synthesis of a library of novel analogues and structural derivatives of 4-MTA; 1,3-bis(aryl)-2-nitro-1-propene derivatives ( $n=193$ ), which contain a classic nitrostyrene structure, and evaluated their potential activity as antiproliferative agents against Burkitt's lymphoma (36). This screen allowed for the development of a structure-activity relationship (SAR) and an optimised nitrostyrene structure for predicted antiproliferative activity. The aim of the present study was to evaluate representative nitrostyrene compounds (Fig. 1) in a range of cancer types, with a focus on CLL.

## Materials and methods

**Materials.** Alamar blue was obtained from BioSource Europe S.A (Nivelles, Belgium) and foetal bovine serum (FBS) was from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). RPMI-1640 and DMEM were sourced from Biosciences, Ltd. (Dublin, Ireland) and Lymphoprep from Axis-Shield (Oslo, Norway). Cell culture consumables were from Greiner Bio-One, Ltd. (Stonehouse, UK), and all other reagents used were from Sigma; Merck KGaA (Darmstadt, Germany). Compounds 1, 2 and 3 were prepared as previously reported (36).

**Cell culture.** The DG-75, BJAB and Ramos Burkitt's lymphoma cell lines were provided by Dr Dermot Walls (School of Biotechnology, Dublin City University, Dublin, Ireland). The MUTU-I (c179) cell line was provided by Professor Martin Rowe (Division of Cancer Studies, The University of Birmingham, Birmingham, UK). The EBV-transformed CLL PGA-1, I83 (M-IGVH, good prognosis), HG-3 and CII (UM-IGVH, poor prognosis) cell lines were provided by Professor Anders Rosén (Linköping University, Linköping, Sweden) (37). Other cell lines were sourced as follows: HL-60 promyelocytic leukaemia cells (cat. no. 98070106; ECACC, Salisbury, UK), HeLa cervical cancer cells (cat. no. 93021013; ECACC), MCF-7 breast cancers (cat. no. 86012803; ECACC) and HS-5 bone marrow stromal cells [cat. no. CRL-11882; American Type Culture Collection (ATCC) Manassas, VA, USA].

The CLL and HL-60 Burkitt's lymphoma cell lines were grown in RPMI-1640 (Glutamax; Thermo Fisher Scientific, Inc.) medium supplemented with 10% (v/v) FBS and 50  $\mu$ g/ml penicillin/streptomycin and seeded at a density of  $2 \times 10^5$  cells/ml. The MUTU-I cell line also

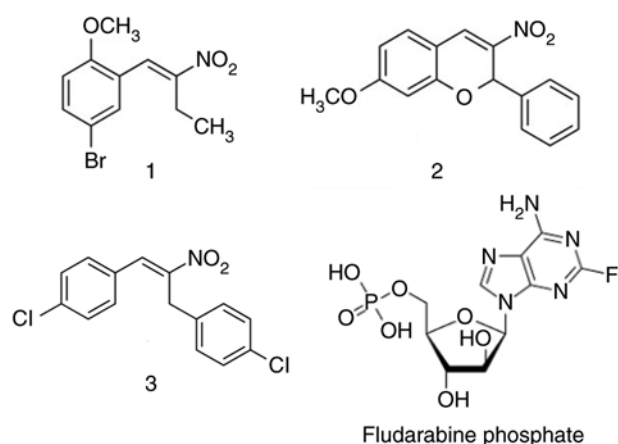


Figure 1. Structure of compounds 1, 2 and 3 and fludarabine phosphate.

required the additional supplements of  $\alpha$ -thioglycerol [5 mM in phosphate-buffered saline (PBS) with 20  $\mu$ M bathocuprione disulfonic acid], sodium pyruvate (100 mM) and HEPES (1 mM). The HS-5 bone marrow stromal, HeLa cervical cancer and MCF-7 breast cancer cell lines were grown in DMEM supplemented with 10% (v/v) FBS and 50  $\mu$ g/ml penicillin/streptomycin and seeded at a density of  $5 \times 10^4$  cells/ml. All cells were grown in a humidified environment of 37°C maintained at 95% O<sub>2</sub> and 5% CO<sub>2</sub> and passaged at least twice weekly depending on their levels of confluency.

**Alamar blue viability assay.** The cells were seeded in 200  $\mu$ l medium in a 96-well plate and were treated and incubated as required (up to 72 h). Alamar blue (20  $\mu$ l) was then added to each well and incubated at 37°C in the dark for 4 h. The plates were then read on a fluorescence plate reader (SpectraMax Gemini; Molecular Devices LLC, Sunnyvale, CA, USA) with excitement and emission wavelengths of 544 and 590 nm, respectively. The experiments were performed in triplicate. A sample containing only reagent and medium (without any cells) was used as a blank control. The vehicle samples were set as 100% viability, from which any decrease in viability was calculated. To determine the IC<sub>50</sub> values, the cells were treated with seven concentrations of nitrostyrene compounds over a range of 0.5–20  $\mu$ M (0.5, 1, 2.5, 5, 7.5, 10 and 20  $\mu$ M) or five concentrations of fludarabine phosphate over a range of 1–50  $\mu$ M (1, 5, 10, 20 and 50  $\mu$ M).

**Flow cytometry.** The CLL cells ( $5 \times 10^4$  cells/ml) were treated 37°C with the nitrostyrene compounds for 48 h, harvested by centrifugation at 400  $\times$  g and rinsed with 0.5 ml of Ca<sup>2+</sup> Annexin V-binding buffer (0.1 M HEPES, pH 7.4; 0.14 M NaCl; 25 mM CaCl<sub>2</sub>). The samples were resuspended in 50  $\mu$ l FITC Annexin V (diluted 1:33 in Ca<sup>2+</sup> Annexin V-binding buffer), incubated on ice for 10 min, washed with Annexin V-binding buffer and resuspended in 500  $\mu$ l of propidium iodide (PI) solution (0.5  $\mu$ g/ml). The samples were analysed within 1 h using a CyAn ADP flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) counting 10,000 cells and analysed using the FlowJo software (FlowJo LLC, Ashland, OR, USA) package. Early apoptotic cells were Annexin V-positive and PI-negative, and late apoptotic cells were Annexin V-positive and PI-positive.

**LDH assay.** The CLL cells were seeded in 200  $\mu$ l medium in a 96-well plate, following treatment with 5  $\mu$ M nitrostyrene compounds for 48 h, 50  $\mu$ l of cell supernatants were placed in a fresh 96 well plate and 100  $\mu$ l lactate dehydrogenase (LDH) assay mixture (Promega Corp., Madison, WI, USA) was added to each sample. The plate was covered with tin foil and incubated at room temperature for 30 min. The reaction was terminated by the addition of 15  $\mu$ l of 1 N HCl to each well. The absorbances of each sample were then obtained at 490 and 690 nm. The lysis solution wells were set at 100% lysis, from which the LDH release of the treated samples was calculated.

**Inhibitor experiments.** The cells ( $5 \times 10^4$  cells/ml) were pretreated at 37°C with either 5 mM N-acetyl-cysteine (Nac) for 1 h or 40  $\mu$ M caspase inhibitor (Z-VAD; MBL International, Co., Woburn, MA, USA) for 4 h prior to 5  $\mu$ M compound treatment for 48 h.

**Co-treatment analysis.** Drug interactions were determined by median dose effect analysis using CalcuSyn version 2.0 software (BioSoft, Cambridge, UK). This method is based on the drug effect equation of Chou and Talalay and can determine the degree of synergism or antagonism between two compounds by generating a combination index (CI) value. CI values of <1, =1 and >1 indicate synergism, an additive effect and antagonism, respectively (38).

**Ex vivo CLL patient samples.** Fresh peripheral blood (10 ml) was collected with written informed consent from patients with CLL (n=14) in EDTA-anticoagulant tubes. Ethical approval for all procedures performed on *ex vivo* CLL patient samples was obtained from the St. James's Hospital and Adelaide and Meath incorporating the National Children's Hospital Joint Ethics Committee (Dublin, Ireland). Informed consent for the use of tissues and publication of patient data was obtained from each subject in line with the Declaration of Helsinki. Patients were included if they were diagnosed with CLL, consented to the trial and were >18 years of age. Patients were excluded if the spontaneous background levels of apoptosis were  $\geq 70\%$ . Patients were recruited between July 2013 and August 2014. The peripheral blood was diluted with an equal volume of RPMI medium and peripheral blood mononuclear cells (PBMCs) were isolated via lymphoprep ficoll gradient centrifugation at 600  $\times$  g for 30 min at 4°C. The pelleted cells were resuspended in freezing medium (10% DMSO, 90% FBS) and frozen until required. When required, the frozen stocks were resuspended in RPMI-1640 (Glutamax) medium supplemented with 10% (v/v) FBS and 50  $\mu$ g/ml penicillin/streptomycin, and seeded at a density of  $1 \times 10^6$  cells/ml. The cells were then treated for 48 h with the compounds or the required vehicles and positive controls.

**Statistical analysis.** GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis on all samples. For comparisons in datasets containing multiple groups, one way analysis of variance and Tukey's post hoc test was used, and P<0.05 was considered to indicate a statistically significant difference. For comparisons in datasets containing two groups, Student's paired t-test was used.

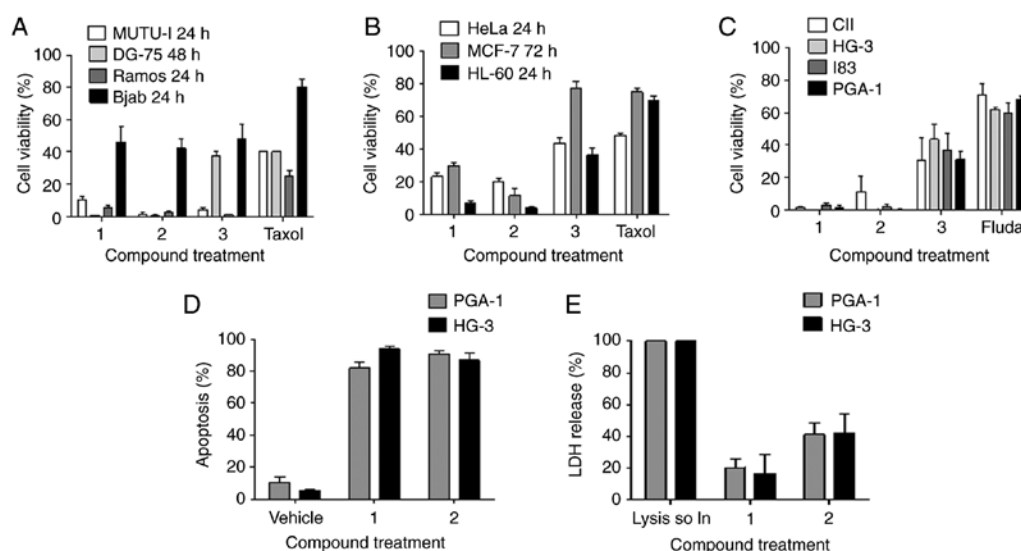


Figure 2. Nitrostyrene compounds reduce cellular viability in a range of cancer cell lines and induce apoptosis in CLL cell lines. Viability results following 10  $\mu$ M treatment with the indicated nitrostyrene compounds or a comparative control (Fluda or taxol) in (A) Burkitt's lymphoma, (B) solid tumour and (C) CLL cell lines. (D) Induction of early and late apoptosis in CLL cells following treatment for 48 h with 5  $\mu$ M of the indicated compounds, as determined by Annexin V and propidium iodide staining. (E) Induction of necrotic cell death in CLL cells following treatment for 48 h with 5  $\mu$ M of the indicated compounds, as determined by measuring LDH release. Values are presented as the mean  $\pm$  standard error of the mean of three independent experiments. CLL, chronic lymphocytic leukaemia; Fluda, fludarabine phosphate, LDH, lactate dehydrogenase.

## Results and Discussion

*Lead nitrostyrene compounds potently reduce cellular viability and induce apoptosis in a range of cancer cell lines.* Representative nitrostyrene compounds, 1 (previously named 145) (36) and lead compound 2 (previously named 66) (36), together with the previous lead compound 3 (previously named 85) (20) were evaluated for their ability to reduce the viability of a range of cancerous cell lines via Alamar blue viability assays. Fludarabine phosphate was used as a comparative control for CLL cell lines, whereas taxol was used as a comparative control for all other cancer cell lines. The results demonstrated that compounds 1 and 2 potently reduced the viability of a range of Burkitt's lymphoma (Fig. 2A); HeLa, MCF-7 and HL-60 (Fig. 2B) and CLL cell lines (Fig. 2C) at a concentration of 10  $\mu$ M. The results in Fig. 2A are in agreement with previously published results (36). Of note, compounds 1 and 2 reduced the viability of all cell lines to a greater extent than either comparator compound, fludarabine phosphate or taxol.

In the CLL cell lines, the  $IC_{50}$  values obtained were in the low micromolar range for compounds 1 and 2 (2-5  $\mu$ M) and were  $\sim$ 2-3-fold lower than the  $IC_{50}$  values obtained for compound 3. Fludarabine phosphate, the current frontline treatment for CLL, was significantly less active than the nitrostyrene compounds, with  $IC_{50}$  values of between 20 and 50  $\mu$ M (4-15-fold higher than for 1 and 2). The  $IC_{50}$  values for the nitrostyrene compounds were similar across all four cell lines, irrespective of IGVH mutational status (I83 and PGA-1: M-IGVH; HG-3 and CII: UM-IGVH) (Table I).

The PGA-1 and HG-3 cell lines (39) were selected for subsequent experiments to investigate the ability of the compounds to induce apoptosis in these two cell lines. The cells underwent a significant increase in apoptosis, as determined using Annexin V and PI staining (Fig. 2D). In agreement

Table I.  $IC_{50}$  values following treatment with the indicated compounds for 48 h in chronic lymphocytic leukaemia cell lines.

Compound	$IC_{50}$ ( $\mu$ M)			
	CII	HG-3	I83	PGA-1
Fluda	49.9	28.1	20.7	32
1	3.46	2.54	4.74	2.9
2	3.46	3.08	4.13	2.17
3	8.05	8.69	8.43	6.74

Fluda, fludarabine phosphate.

with these results, compound 1 induced only a low percentage of LDH release, indicative of necrosis, an alternative but less desirable form of cell death from a clinical point of view due to its inflammatory effects. Compound 2 induced a higher, but moderate percentage of LDH release (Fig. 2E).

*Lead nitrostyrene-induced cell death is reactive oxygen species (ROS)- and caspase-dependent.* Pretreatment of the cells with N-acetyl-cysteine (Nac), a ROS inhibitor, caused a significant reduction in nitrostyrene-induced apoptosis compared with that in cells treated with the nitrostyrene compounds alone (Fig. 3A and B). ROS are normally produced in low number as a toxic by-product following oxidative phosphorylation in the mitochondria; the results suggested that the target of nitrostyrene compounds 1 and 2 may be the mitochondria. Further experiments revealed that the inhibition of caspases, which are intracellular cysteine proteases commonly associated

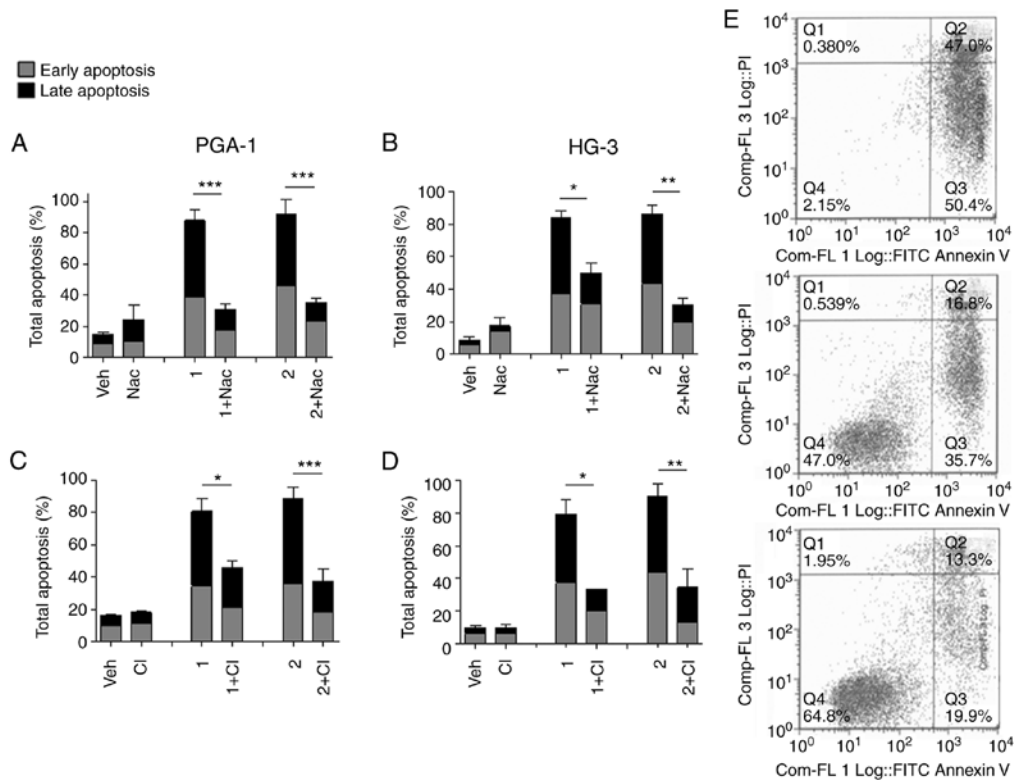


Figure 3. Nitrostyrene analogue-induced apoptosis is caspase- and ROS-dependent. CLL cells were pretreated with the ROS inhibitor (5 mM Nac) for 1 h or the CI (40  $\mu$ M Z-VAD) for 4 h, followed by treatment for a further 48 h with 5  $\mu$ M of the indicated nitrostyrene compounds. Cells were subsequently analysed for apoptosis by Annexin V and PI flow cytometry. Early apoptotic cells were Annexin V-positive, PI-negative, late apoptotic cells were Annexin V- and PI-positive. (A) PGA-1 cells, ROS inhibition, (B) HG-3 cells, ROS inhibition, (C) PGA-1 cells, caspase inhibition and (D) HG-3 cells, caspase inhibition. Values are presented as the mean  $\pm$  standard error of the mean of three independent experiments. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001. (E) Representative dot plots of HG-3 cells treated with (top) 5  $\mu$ M compound 1, (middle) Nac + 5  $\mu$ M compound 1 and (bottom) CI + 5  $\mu$ M compound 1. CLL, chronic lymphocytic leukaemia; ROS, reactive oxygen species; CI, caspase inhibitor; PI, propidium iodide; Veh, vehicle; Nac, N-acetyl-cysteine.

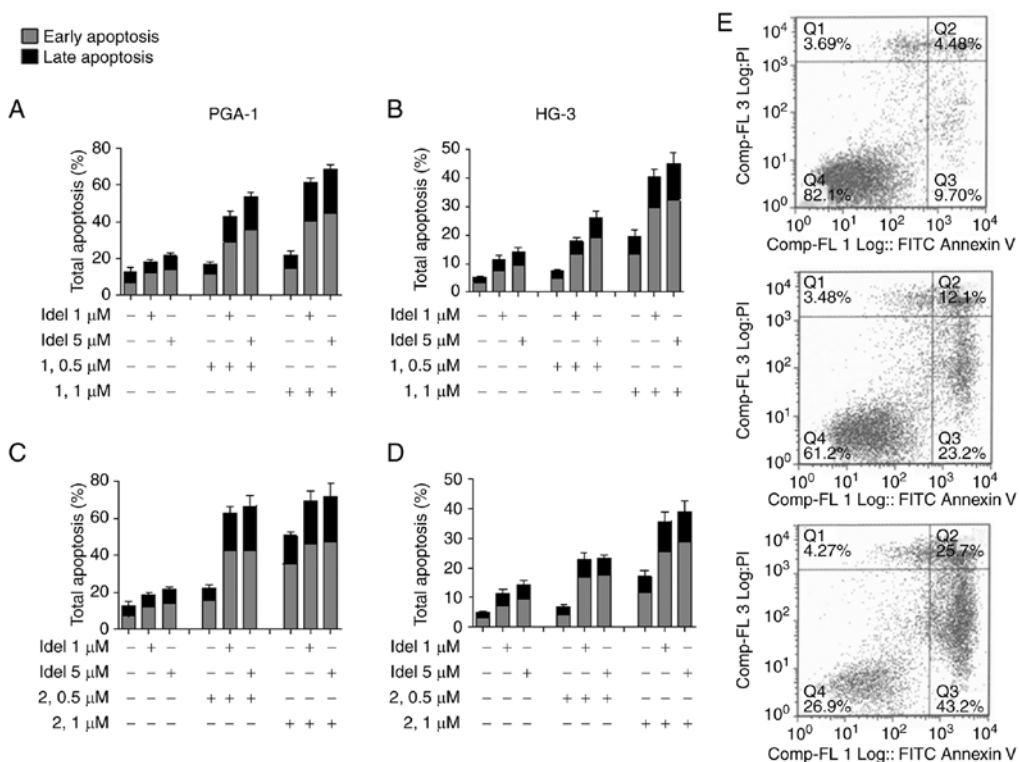


Figure 4. Idel synergistically enhances the apoptotic effect of lead nitrostyrene compounds in CLL cell lines. CLL cells were treated for 48 h with the indicated compounds and subsequently analysed for apoptosis by Annexin V and propidium iodide flow cytometry. (A) PGA-1 cells, compound 1; (B) HG-3 cells, compound 1; (C) PGA-1 cells, compound 2; and (D) HG-3 cells, compound 2. Values are presented as the mean  $\pm$  standard error of the mean of three independent experiments. (E) Representative dot plots of PGA-1 cells treated with (top) 1  $\mu$ M Idel, (middle) 1  $\mu$ M compound 1 and (bottom) 1  $\mu$ M Idel + 1  $\mu$ M compound 1 + CI. CLL, chronic lymphocytic leukaemia; Idel, idelalisib.

with apoptosis, significantly reduced the percentage of cells undergoing apoptosis following treatment with nitrostyrene compounds 1 and 2, suggesting a caspase-dependent form of cell death may be occurring (Fig. 3C and D). Representative dot plots are shown for illustrative purposes (Fig. 3E). These results are in agreement with those of previous studies on nitrostyrene analogues and the involvement of caspases. For example, caspase activation was observed with *trans*- $\beta$ -nitrostyrene in LoVo adenocarcinoma cells (40), two nitrostyrene derivative compounds (NTS1 and NTS2) in Ehrlich ascitic tumour cells (28) and 2-aryl-3-nitro-2*H*-chromenes in breast cancer cells (41).

*Lead nitrostyrene compounds synergistically enhance the effect of idelalisib.* Few cancer treatments exist as a stand-alone therapy, so it is important to develop small molecule inhibitors that can potentiate the effects of other treatments. Idelalisib, a PI3K-inhibitor, has recently been FDA-approved for the treatment of relapsed CLL in combination with rituximab and EMA-approved in combination with rituximab or ofatumumab (8). Therefore, the present study aimed to determine whether idelalisib augments the apoptotic effect of the lead nitrostyrene compounds, or vice versa. The results demonstrated that treatment with 1  $\mu$ M of compound 1 alone caused 22.0 $\pm$ 7.5% of cells to undergo apoptosis, however, following co-treatment with 5  $\mu$ M idelalisib, increased this apoptosis to a mean of 68.4 $\pm$ 8.0% in the PGA-1 cells (Fig. 4A). Similar increases in apoptosis were also obtained in the HG-3 cells treated with compound 1 (Fig. 4B) and in the PGA-1 and HG-3 cells treated with compound 2, respectively (Fig. 4C and D). Representative dot plots are shown for illustrative purposes (Fig. 4E). The median dose analysis showed that, for all combinations assessed in both CLL cell lines, a CI value of <1 was obtained, indicating synergism between the compounds (Table II). These results are in agreement with a variety of other anticancer agents that have been shown to synergise with idelalisib, including the Syk inhibitor GS-9973 (42), histone deacetylase inhibitor panobinostat (LBH589) and suberoylanilide hydroxamic acid (43) and ibrutinib (44). Reports of nitrostyrenes in combination with other anticancer agents are limited; however, nitrostyrenes have previously been documented to synergise with another anticancer agent, 5-fluorouracil, in colon cancer cells *in vitro* (40).

*Lead nitrostyrene compounds potently induce apoptosis in ex vivo CLL patient samples, including those with poor prognostic indicators.* The present study investigated whether these novel nitrostyrene compounds were also capable of inducing apoptosis in cancer cells isolated from patients with CLL. For this, informed consent was obtained from a cohort of patients with CLL (n=14); the details of these patients are outlined in Table III. Of these 14 patients, three were women and 11 were men, and the cohort ranged in age between 49 and 78 years.

The PBMCs were isolated from the blood samples of patients with CLL by Ficoll gradient and stored in liquid nitrogen in biobanking facilities until required. Primary CLL cells are known to die rapidly in culture and as a result underwent a high and varied background level of spontaneous

Table II. Median dose effect analysis of CLL cell lines.

Treatment	Idelalisib ( $\mu$ M)	Nitrostyrene ( $\mu$ M)	Fa	CI
HG-3				
Idelalisib-1 combination	1	0.5	0.179	0.774
	1	1	0.403	0.759
	5	0.5	0.259	0.588
	5	1	0.452	0.680
Idelalisib-2 combination	1	0.5	0.228	0.482
	1	1	0.357	0.639
	5	0.5	0.231	0.545
	5	1	0.391	0.589
PGA-1				
Idelalisib-1 combination	1	0.5	0.429	0.416
	1	1	0.616	0.475
	5	0.5	0.537	0.301
	5	1	0.684	0.380
Idelalisib-2 combination	1	0.5	0.627	0.284
	1	1	0.693	0.445
	5	0.5	0.667	0.246
	5	1	0.720	0.400

Analysis was performed using Calcsyn software. CI values of >1, =1 and <1 indicates antagonism, an additive effect and synergism, respectively. Fa, fraction of cells affected by apoptosis; CI, combination index.

apoptosis in the present study. Therefore, the results are presented as the increase in apoptosis observed following treatment with compound 1 or 2 over vehicle-treated cells. The cells were incubated with a range of concentrations of compounds 1 and 2 for 48 h. The results demonstrated CLL cells underwent a significant dose-dependent increase in apoptosis when treated with representative lead nitrostyrene compounds. Apoptosis increased, on average by 15.4 $\pm$ 2.6% when treated with 250 nM compound 1 (range, -2.6-29.8%). This increased to 44.9 $\pm$ 5.7% apoptosis when treated with 10  $\mu$ M compound 1 (range, 23.2-81.9%) (Fig. 5A). Similarly, cells underwent an average increase in apoptosis of 15.8 $\pm$ 3.6% (range, 0.3-43.6%) and 43.7 $\pm$ 6.0% (range, 23.2-80.3%) following treatment with 250 and 10  $\mu$ M compound 2, respectively (Fig. 5B). By contrast, patient samples treated with fludarabine phosphate underwent a lower increase in apoptosis of 24.6 $\pm$ 4.0% (range, 4.0-49.0%) at the highest concentration of 50  $\mu$ M (Fig. 5C).

The samples treated with compound 1 demonstrated similar levels of apoptosis when harbouring poor clinical markers (UM-IGVH, CD38-, del17p and del11q) compared with samples with favourable clinical markers (M-IGVH, CD38+) (Fig. 6A-C). Similar results were obtained for compound 2 (Fig. 6D-F). These chromosomal abnormalities are associated with clinically progressive disease, shorter remission durations and shorter overall survival rates following standard chemotherapy (2).

Table III. List of patient details.

Patient no.	Age (years)	Sex	Binet stage	Karyotype	CD38	IGVH
1	78	M	A	ND	Negative	UM
2	60	M	C	Del13q, Del 11q	Positive	UM
3	68	M	A	ND	ND	ND
4	66	M	A	Del13q, Del17p	Negative	ND
5	72	M	B	Del11q	Negative	UM
6	78	F	A	Normal	Positive	UM
7	49	F	A	Del13q	Negative	M
8	78	M	A	ND	Negative	ND
9	72	M	A	ND	Negative	ND
10	61	F	A	Del17p	Negative	UM
11	59	M	C	Normal	Negative	UM
12	57	M	B	Del13q	Negative	M
13	69	M	A	ND	Negative	ND
14	73	M	A	Normal	ND	

ND Positive patients have  $\geq 30\%$  leukaemic cells positive for CD38. IGVH, immunoglobulin heavy chain gene; M, mutated; UM, unmutated; Del, chromosomal deletion; ND, not determined.

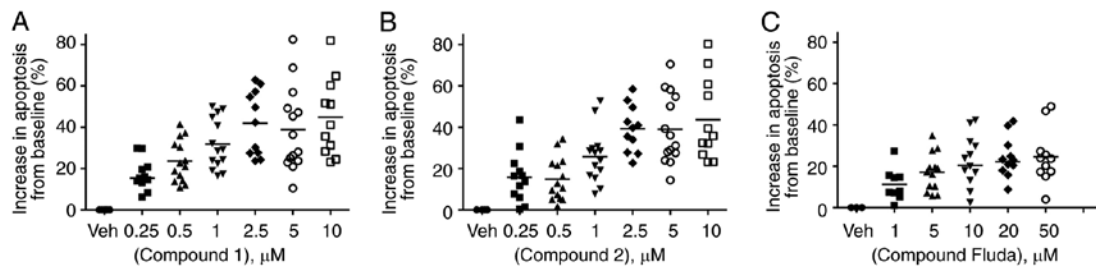


Figure 5. Nitrostyrene analogues induce more potent apoptosis in *ex vivo* CLL patient samples than Fluda. Peripheral blood mononuclear cells were isolated with informed consent from 14 patients with CLL, incubated with the indicated concentration of compounds 1 and 2 for 48 h and analysed for apoptotic cell death. Fluda was used as a comparable control. (A) Compound 1, (B) compound 2 and (C) Fluda. Graphs represent the increase in early and late apoptosis from baseline. CLL, chronic lymphocytic leukaemia; Veh, vehicle; Fluda, fludarabine phosphate.

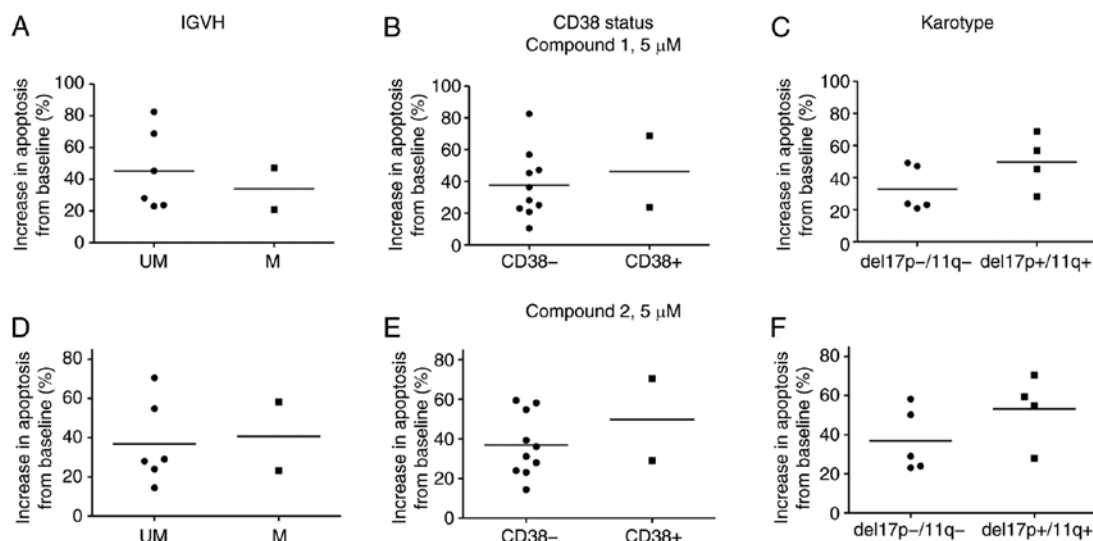


Figure 6. Nitrostyrene analogues potently induce apoptosis in poor prognosis *ex vivo* CLL patient samples. Peripheral blood mononuclear cells were isolated with informed consent from CLL patients and incubated with 5  $\mu\text{M}$  of either compounds 1 or 2 for 48 h and analysed for apoptotic cell death. Apoptosis results are presented for compound 1 by (A) IGVH status, (B) CD38 status and (C) karyotype, and for compound 2 by (D) IGVH status, (E) CD38 status and (F) karyotype. Poor prognostic factors include UM-IGVH, CD38<sup>-</sup>, del17p<sup>+</sup> or del11q<sup>+</sup>. Graphs represent the increase in early and late apoptosis from baseline. CLL, chronic lymphocytic leukaemia; IGVH, immunoglobulin heavy chain gene; M, mutated; UM, unmutated.

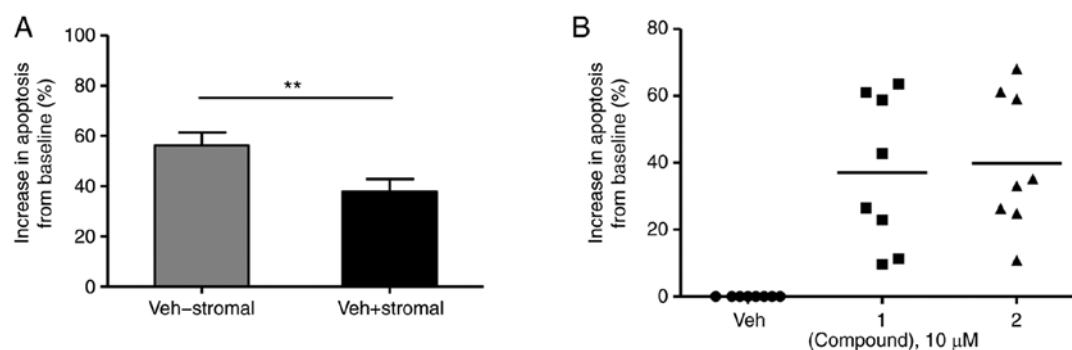


Figure 7. Nitrostyrene analogues overcome resistant *ex vivo* CLL cells. Peripheral blood mononuclear cells from eight patients with CLL were cultured with stromal H5S cells to mimic their microenvironment. Cells were subsequently treated with 10  $\mu$ M of the indicated compounds for 48 h and analysed for apoptotic cell death. (A) Microenvironment increases survival of *ex vivo* CLL cells. (B) Resistant CLL cells underwent apoptosis when treated with nitrostyrenes. Graphs represent the increase in early and late apoptosis from baseline. \*\* $P < 0.01$ . CLL, chronic lymphocytic leukaemia; Veh, vehicle.

CLL cells *in vivo* are known to be resistant to apoptosis, assisted by interactions with lymph node and bone marrow microenvironments. CLL cells adhering to bone marrow stromal cells through integrins show decreased protein levels of the anti-apoptotic Bcl-2 and are influenced by stromal cell secretion of interleukin IL-4 and interferon- $\alpha/\lambda$  (45,46). In order to mimic this microenvironment-mediated resistance of cancer cells to apoptosis, primary CLL cells ( $n=8$ ) were co-cultured with bone marrow-derived stromal HS5 cells. As a result, primary untreated CLL cells developed a greater ability to survive in culture, with spontaneous background apoptotic levels dropping significantly by almost 20% from  $56.2 \pm 5.1$  to  $37.9 \pm 5.0\%$  (Fig. 7A). The co-cultured *ex vivo* primary CLL cells also underwent a significant increase in apoptosis following co-treatment with 10  $\mu$ M compound 1 and 2 of  $37.0 \pm 7.9$  (range, 9.6–63.5%) and  $39.9 \pm 7.2\%$  (range, 10.8–68.1%), respectively (Fig. 7B). This suggests the ability of nitrostyrene compounds to overcome the drug resistance afforded to primary CLL cells by their *in vivo* microenvironment.

Although nitrostyrene derivatives have been confirmed to be potent at inducing apoptosis in a wide variety of cell types, reports on clinical development are limited; however, Bartels *et al* demonstrated the successful *in vivo* application of two nitrostyrene derivatives in a BALB/c myelopoiesis model mouse model (47). The results outlined above suggest the clinical potential for the development of novel nitrostyrene compounds as a treatment for CLL, and the study by Bartels *et al* suggests that nitrostyrene compounds are well tolerated *in vivo*.

Drugs used to treat CLL include fludarabine phosphate, rituximab, the PI3K inhibitor idelalisib and the Btk inhibitor ibrutinib (1). However, there are significant issues associated with these drugs, including the development of resistance, and an unmet requirement is emerging for alternative drug therapies for this disease. In the present study, it was demonstrated that lead nitrostyrene compounds offer potential for the treatment of CLL (compounds 1, 2 and 3). Although the original target of nitrostyrene compounds was considered to be SERT, which is overexpressed in a number of cancer types, it is now known that SERT is not connected with the compound mechanism of action. As a result, the molecular target of these compounds remains to be elucidated. However, numerous studies on structurally similar compounds have pointed to a

diverse range of potential targets, including tubulin (29,32), telomerases (33), protein tyrosine phosphatases (34) and phospholipase A<sub>2</sub> (35). Follow-up investigations into these target/s are ongoing.

In the present study, two lead nitrostyrene compounds with aryl substitutions, compounds 1 and 2, potently reduced cell viability in a broad range of cancer cell lines, including CLL cells, associated with a poor prognosis. These lead compounds demonstrated significantly higher potent antiproliferative activity than the conventional drug treatment fludarabine phosphate and demonstrated IC<sub>50</sub> values of  $<10 \mu$ M for all CLL cells. The apoptotic cell death was caspase and ROS-dependent and was significantly and synergistically enhanced by idelalisib. The compounds also successfully induced apoptosis in primary cells from patients with CLL, including those with poor prognostic markers. The compounds also overcame stromal cell-induced chemoresistance in CLL cells. These results demonstrate the potential of nitrostyrene analogues for the treatment of CLL.

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## Availability of data and materials

The corresponding author can be contacted for more information.

## Authors' contributions

SAB performed the majority of the experiments and wrote the manuscript; AJB performed some experiments and synthesized the compounds; EV provided access to the patient samples; EV, PVB, AMM, MJM and DCW contributed to the design of the study and supervised the project. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.



## Ethics approval and consent to participate

Ethical approval for all procedures performed on *ex vivo* CLL patient samples was obtained from the St. James's Hospital and Adelaide and Meath incorporating the National Children's Hospital Joint Ethics Committee (Dublin, Ireland). Informed consent for the use of tissues was obtained from each subject in line with the Declaration of Helsinki.

## Patient consent for publication

Informed consent for publication of patient data was obtained from each subject in line with the Declaration of Helsinki.

## Competing interests

The authors declare that they have no competing interests.

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