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Synthesis and serotonin transporter activity of 1,3-bis(aryl)-2-nitro-1-propenes as a new class of anticancer agents

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ABSTRACT

Structural derivatives of 4-MTA, an illegal amphetamine analogue have been previously shown to have anticancer effects in vitro. In this study we report the synthesis of a series of novel 1,3-bis(aryl)-2-nitro-1-propene derivatives related in structure to 4-MTA. A number of these compounds containing a classic nitrostyrene structure are shown to have antiproliferative activities in vitro in a range of malignant cell lines, particularly against Burkitt's lymphoma derived cell lines, whilst having no effect on 'normal' peripheral blood mononuclear cells. Such effects appear to be independent of the serotonin transporter, a high affinity target for amphetamines and independent of protein tyrosine phosphatases and tubulin dynamics both of which have been previously associated with nitrostyrene-induced cell death. We demonstrate that a number of these compounds induce caspase activation, PARP cleavage, chromatin condensation and membrane blebbing in a Burkitt's lymphoma derived cell line, consistent with these compounds inducing apoptosis in vitro. Although no specific target has yet been identified for the action of these compounds, the cell death elicited is potent, selective and worthy of further investigation.

1. Introduction

The serotonin transporter (SERT) transports 5-hydroxytryptamine (5-HT) from central and enteric nervous system synapses back into pre-synaptic neurons determining the duration and magnitude of 5-HT responses. It is an important high affinity target in vivo for antidepressants and a non-selective target for stimulants including cocaine and amphetamines. Despite its abundance in the nervous system, SERT is also expressed in a wide range of specialised non-neuronal cells as well as being expressed in a number of B cell malignancies including diffuse large B cell lymphoma, multiple myeloma and Burkitt's lymphoma (BL).[1] Recently, a number of SERT-targeting ligands have been shown to induce apoptosis in a number of malignant cell lines including Burkitt's lymphoma.[2–6] SERT has also been implicated in serotonin-mediated apoptosis in Burkitt's lymphoma[7] and in the mechanism of cytotoxicity associated with the amphetamine analogues, fenfluramine[8] and 3,4-methylenedioxymethamphetamine (MDMA).[9,10] Although these ligands can target the serotonin transporter, their anticancer effects are thought to occur independently of SERT[1,2,11] suggesting that (a) molecular target(s) other than SERT may exist on the lymphoma cell with the possibility of these ligands preferentially targeting the proliferating malignant cell.[1]

Amphetamine analogues substituted at the 4-position with an alkylthio group are a pharmacologically important class of compounds which demonstrate potent biochemical activity in the central nervous system. The parent member of the series, 4-MTA (4-methylthioamphetamine) (Fig. 1) is recognised as a

Abbreviations: 4-MTA, 4-methylthioamphetamine; 5-HT, 5-hydroxytryptamine; BL, Burkitt's lymphoma; BSA, bovine serum albumin; CML, chronic myelogenous leukaemia; DAT, dopamine transporter; DMEM, Dulbecco's modified Eagle's medium; ECACC, European collection of cell cultures; ER, estrogen receptor; ESI-MS, electrospray-ionisation mass spectrometry; FACS, fluorescence activated cell sorter; FBS, foetal bovine serum; HBSS, Hanks balanced salt solution; HRMS, high resolution mass spectrometry; IR, infra-red; LRMS, low resolution mass spectrometry; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MOE, molecular operating environment; NAT, noradrenaline transporter; NMR, nuclear magnetic resonance; NR, neutral red; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PCA, *para*-chloroamphetamine; PCD, programmed cell death; PI, propidium iodide; PMA, *para*-methoxyamphetamine; PMK, 1-(3,4-methylenedioxyphenyl)-2-propanone; PTP, Protein tyrosine phosphatase; SAR, structure–activity relationship; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; TBNS, *trans*-β-nitrostyrene; TCA, tricyclic antidepressant; TMS, tetramethylsilane; TLC, thin layer chromatography.

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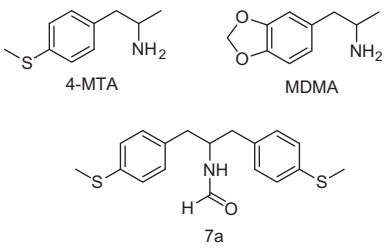


Figure 1. Structure of 4-MTA (4-methylthioamphetamine), MDMA (3,4-methylenedioxymethamphetamine) and 1,3-bis(aryl)-2-propanamine compound **7a**.

MDMA-like drug of abuse and is classified as a Schedule-1 controlled substance.[12] 4-MTA is a ligand for SERT, is a potent inhibitor of serotonin reuptake in the rat brain, a serotonin releasing agent in rat brain synaptosomes and has been shown to have inhibitory effects on 5-HT-mediated vascular contraction in isolated rat aortas.[13] Recently, our laboratory reported the isolation and study of route-specific byproducts associated with the clandestine synthesis of 4-MTA.[12] In our study, a *N*-formyl-1,3-bis(4-methylthiophenyl)-2-propanamine compound (**7a**) (Fig. 1) was shown to have a cytotoxic effect on a malignant neuroblastoma cell line, SHSY-5Y (EC_{50} of 32 μM), and was found to be selectively toxic to SERT-overexpressing human embryonic kidney cells (HEK).[12]

As compounds of type **7a** are structurally related derivatives of 4-MTA and MDMA, it was proposed that these derivatives could behave like amphetamines by binding to SERT and therefore may have potential as antiproliferative agents against lymphomas, in particular, Burkitt's lymphoma. The initial objective of this study is to therefore synthesize a number of structurally related analogues of **7a** and investigate their SERT-binding activity and antiproliferative potential against Burkitt's lymphoma derived cell lines. Burkitt's lymphoma accounts for 30–50% of lymphomas in children and remains a serious health problem in those areas where it is endemic: namely the malarial belts of equatorial Africa, north-eastern Brazil, and Papua New Guinea.[7] In addition, because of its growing association with HIV infection, Burkitt's lymphoma is becoming a more common malignancy in adults, making up the largest group of HIV-associated non-Hodgkin lymphomas (35–50% of these neoplasms).[14] A variety of chemotherapeutic drugs are used in the treatment of BL tumours including DNA intercalating agents and topoisomerase inhibitors. Such drugs are usually successfully supplemented with the monoclonal antibody rituximab which sensitises B cell lymphomas and leukaemias to chemotherapy[15] allowing for survival rates of up to 60% in children affected with the disease. However, for older adults and HIV infected patients the long term survival rates in response to chemotherapy is only 25%[7,16] with reoccurrence and resistance common. In addition, in parts of the developing world where BL is endemic, access to chemotherapeutic regimens is limited. Consequently, there is a need to develop more selective, potent, economical alternatives for the treatment of Burkitt's lymphoma.

In this study, we wish to therefore evaluate the potential activity of a series of 1,3-bis(aryl)-2-nitro-1-propenes as novel antiproliferative agents against Burkitt's lymphoma.

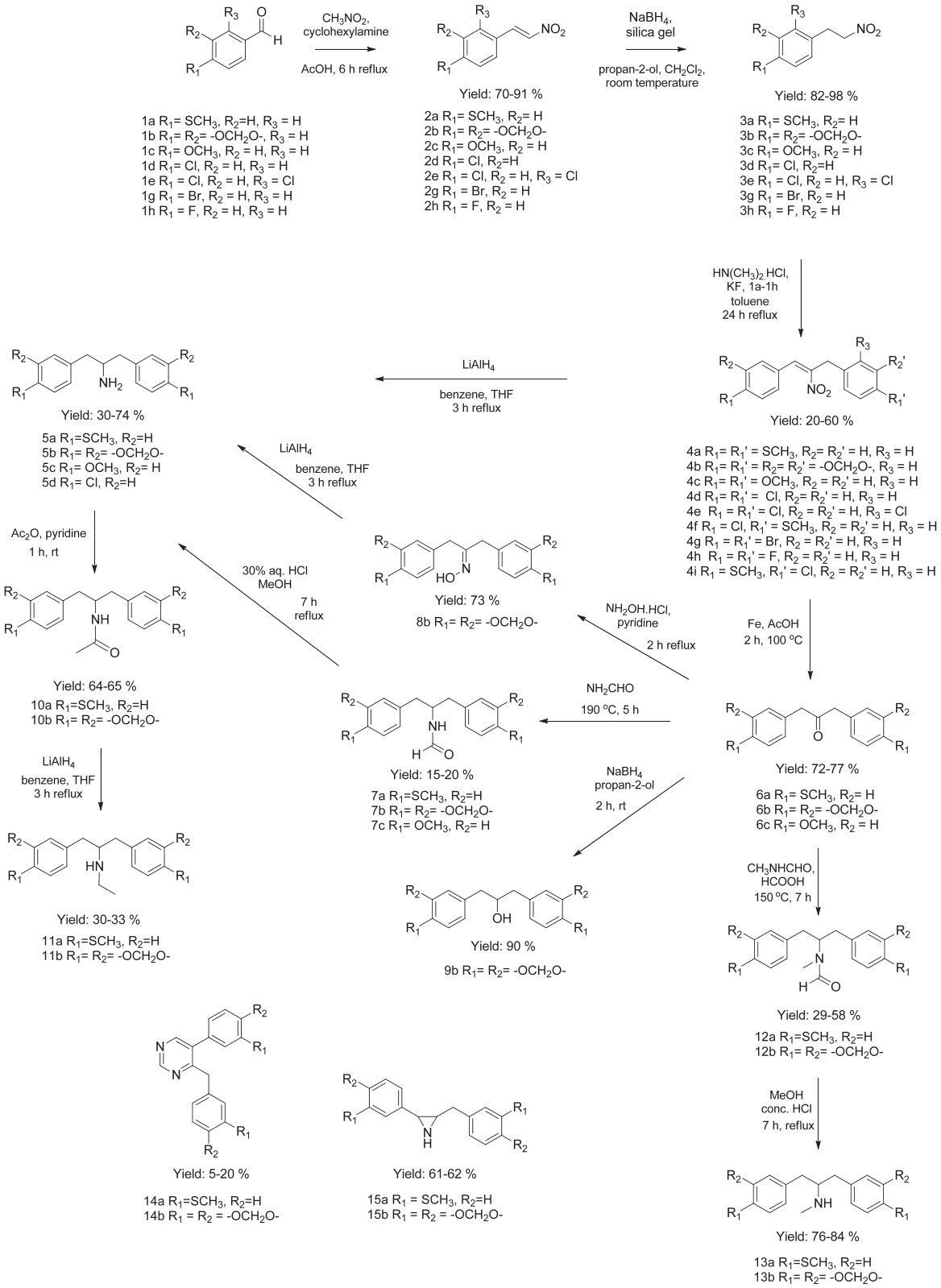
2. Results and discussion

2.1. Chemistry

Synthetic routes available for the production of amphetamines, together with their characteristic impurities have been extensively reported.[17–19] We have previously examined the synthesis of a series of 1,3-bis(aryl)-2-propanamine compounds structurally similar to **7a**, which are related to synthetic byproducts in the Leuckart–Wallach route of 4-MTA synthesis.[12] The compounds chosen for the present study were designed to have modifications to the aromatic substituents, and also include derivatives where the amino group is alkylated and acylated. The complete panel of compounds evaluated in this study are synthesized via multi-step synthesis as illustrated in Scheme 1 based on modified literature procedures.[19–21]

A Henry–Knovenagel reaction of the appropriate benzaldehyde **1a**–**1h** and nitromethane is employed to produce the 1-aryl-2-nitroethenes **2a**–**2h**. The alkenes were reduced with $NaBH_4$ and silica gel to give 1-aryl-1-nitroethanes **3a**–**3h**. This was then followed by a second Henry condensation of 1-aryl-1-nitroethane **3a**–**3h** with the appropriate benzaldehyde **1a**–**1h**, potassium fluoride and *N*,*N*-dimethylamine hydrochloride to afford a series of 1,3-bis(aryl)-2-nitro-1-propenes **4a**–**4i** with variable yields (20–60%). These nitrostyrene compounds **4a**–**4d** were then reduced with $LiAlH_4$ to give the primary amines **5a**–**5d**. 1,3-Bis(aryl)-2-nitro-1-propenes **4a**–**4c** and 1,3-bis(aryl)-2-propanamines **5a**–**5d** can be used as precursors for a variety of structurally related analogues. Compounds **4a**–**4c** were reduced using iron powder and acetic acid to give 1,3-bis(aryl)-2-propanones **6a**–**6c**. These propanone compounds (**6a**, **6b**) can then undergo the Leuckart reaction using *N*-methylformamide, to generate tertiary amides **12a** and **12b**. When reacted with formamide, propanones **6a**–**6c** afforded the secondary amides **7a**–**7c**. Pyrimidine based impurities **14a** and **14b** were isolated from this reaction in high yields (5% and 7%) compared to the yields of the *N*-formyl compounds **7a** and **7b** (15% and 20%). Numerous examples of pyrimidine impurities arising from the Leuckart reactions of ketones and formamide have been documented in the literature,[17,22] however pyrimidines derived from dibenzylketone impurities have not been documented. In the ^1H NMR spectrum for compound **14b**, a singlet at δ 4.04 integrating for two protons is characteristic of the methylene protons of a benzyl group positioned α to a nitrogen of a heteroaromatic ring. Two singlets, each integrating for two protons, at δ 5.90 and δ 6.05 correspond to the OCH_2O protons of the methylenedioxyphenyl and benzyl rings, respectively. Two 1,3,4-trisubstituted ring systems are evident from the splitting patterns of six resonances between δ 6.50 and δ 6.90, each integrating for one proton. At δ 8.54, a singlet integrating for one proton is characteristic of a proton α to one pyrimidine ring nitrogen, while a further singlet at δ 9.13 is assigned to the proton located between both pyrimidine nitrogens. Compounds **5b**, 6b and **7b** have been previously identified by Bohn et al. as impurities arising from the synthesis of MDA from 3,4-methylenedioxyphenylacetic acid by the Leuckart route.[17] In this study these compounds were synthesized as previously reported by Pifl et al..[21] We have previously reported the synthesis of compounds **4a**, **5a**, **6a**, **7a**, **10a**, **11a**, 12a and **13a**, 15a[13] and they have been included in Scheme 1 for clarity.

Hydrolysis of the *N*-formyl amines **7a** and **7b** with methanolic HCl affords the primary amine derivatives **5a** and **5b** in sufficient yields (84%), however when the same hydrolysis conditions were applied to *N*-formyl-*N*-methyl propanamines **12a** and **12b**, only trace amounts of *N*-methylamines **13a** and **13b** were formed. Reaction conditions were changed to include a concentrated HCl/methanol mixture, resulting in much higher yields of the hydrolysed products **13a** and **13b** (76% and 84%). Compounds **12b** and **13b** have previously been identified by Bohn et al., as impurities of MDMA synthesized via the Leuckart reaction from PMK (1-(3,4-methylenedioxyphenyl)-2-propanone), which itself arises as a synthetic byproduct from the acetic acid route of MDMA synthesis from 3,4-methylenedioxyphenylacetic acid.[17] Compounds **5a** and **5b**, when reacted with acetic anhydride and pyridine gives the amides **10a** and **10b**, which are then reduced with $LiAlH_4$ to the *N*-ethyl derivatives **11a** and **11b**. The ketone **6b** is reduced directly with $NaBH_4$ to give 1,3-(3,4-methylenedioxyphenyl)-2-propanol



Scheme 1. Reagents and conditions: (a) CH_3NO_2, cyclohexylamine, AcOH, 6 h reflux; (b) $NaBH_4$ silica gel, propan-2-ol, CH_2Cl_2, room temperature; (c) $HN(CH_3)_2$·HCl, KF, toluene, 24 h reflux; (d) $LiAlH_4$, benzene, THF, 3 h reflux; (e) Fe, AcOH; (f) NH_2CHO 190 °C, 5 h; (g) NH_2OH·HCl, pyridine, 2 h reflux; (h) $NaBH_4$, propan-2-ol; (i) CH_3NHCHO, HCOOH, 150 °C, 7 h; (j) Ac_2O, pyridine, 1 h, room temperature; (k) MeOH, concd HCl, 7 h reflux; (l) 30% aq HCl, MeOH, reflux, 7 h. Compounds **14a**, **14b**, **15a** and **15b** are formed as route-specific byproducts during the synthesis of compounds **7a**, **7b**, **5a** and **5b**, respectively.

9b. The oxime **8b** is obtained from **6b** via reaction with hydroxylamine hydrochloride and pyridine. Oxime **8b** was subsequently reduced with $LiAlH_4$ to afford the 1,3-bis(3,4-methylenedioxyphenyl)-2-propanamine **5b**, along with a novel aziridine based byproduct **15b**. The aziridine derivative was formed in high yields (62%) compared to the desired propanamine **5b** (27%).

2.2. SERT-dependent cytotoxicity

A number of novel pyridines, dihydropyridone and *N,N*-di(1-aryl-2-propyl) amines described as route-specific byproducts associated with clandestine synthesis of 4-MTA and related amphetamines[12] were previously evaluated for SERT-selective cytotoxicity using human embryonic kidney cells overexpressing SERT, and two malignant cell lines; the DG-75 cell line, which has been previously shown by our laboratory to express SERT[2,13] and to the SHSY-5Y neuroblastoma cell line, which does not express SERT.[23] As previously described, the compound of most interest from this study was the *N*-formyl-1,3-bis(4-methylthiophenyl)-2-propanamine (**7a**) (Fig. 1) which was found to be cytotoxic to the hSERT-overexpressing cell line and not to the HEK293 cell line implying a selective-SERT cytotoxic effect.[12]

Compound **7a** was therefore selected as a lead compound that may have potential to target SERT-expressing malignancies such as BL. Further investigations by this study found compound **7a** to be selectively antiproliferative towards two Burkitt's lymphoma cell lines (DG-75, a chemoresistant cell line and MUTU-1, a chemosensitive cell line[24]), (Fig. 2A), having little effect on a number of breast-cancer derived cell lines (Fig. 2B) and having no effect on peripheral blood mononuclear cells (Fig. 2C). It was also found to induce the formation of apoptotic bodies, as detected by propidium iodide (PI) FACS (fluorescence activated cell sorter) analysis in Burkitt's lymphoma cells (Fig. 2D). Dose–response curve analysis of the apoptotic effect of **7a** in DG-75 and MUTU-I cell lines estimated the EC_{50} values to be approximately 10 μM and 13 μM, respectively (data not shown). These results show that compound **7a** is a novel antiproliferative apoptotic agent selective for B cell malignancies.

In this study, the cytotoxic activity of the series of 1,3-bis(aryl)-2-nitro-1-propenes based on the structure of **7a** and described in Section 2.1, were evaluated in the HEK, HEK-SERT, DG-75 and SHSY-5Y cell lines (presented in Table 1). Again, compound **7a** had a potent effect on the DG-75, Burkitt's lymphoma cell line (pEC_{50} 4.5) as well as to SHSY-5Y neuroblastoma cell line (pEC_{50} 4.5) (Table 1). In general, this study found that the majority of compounds had cytotoxic effects in the low micromolar range (pEC_{50} values of between 4 and 5, correlating to an EC_{50} value of between 1 and 20 μM) to HEK cells and to hSERT-overexpressing cells consistent with these compounds having no selective cytotoxic effects towards SERT ($P<0.05$). The sulfur-substituted 4-MTA compounds **5a**, **7a**, **10a** and **11a** have previously been reported to be cytotoxic to monoamine-expressing cell lines as well as to the PC-12 neuronal cell line model.[12]

Compounds **5a** and **11a** (previously found to have cytotoxic effects to a SERT-over expressing cell line, a dopamine transporter (DAT)-over expressing cell line as well as to the SHSY-5Y cell line but having little effect on a noradrenaline transporter (NAT) over-expressing cell line[12]) were found by this study to have potent cytotoxic effects (pEC_{50} 4.6–4.9) to all of the cell lines including

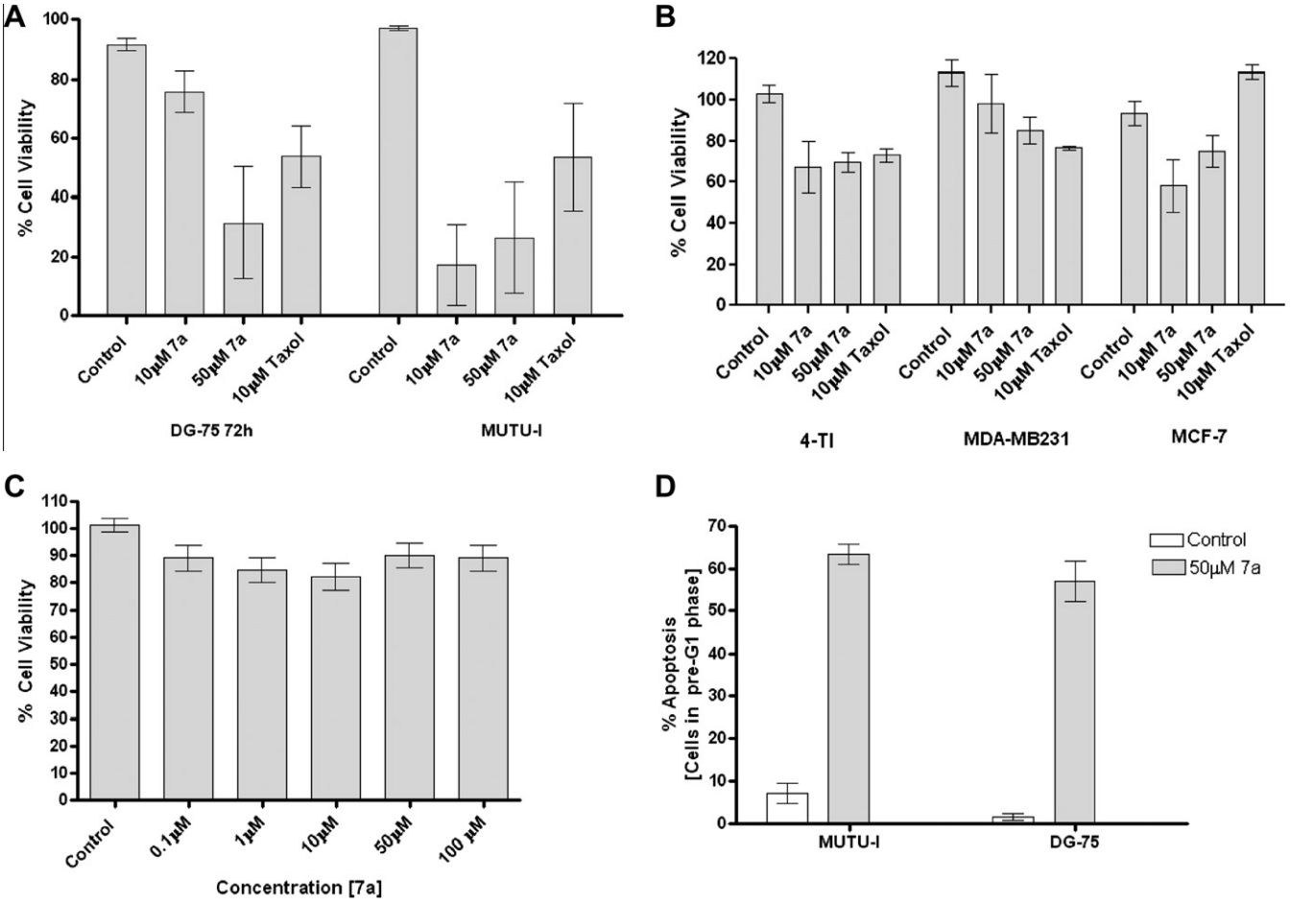


Figure 2. Compound **7a** is a potent antiproliferative pro-apoptotic agent against Burkitt's lymphoma but has no effect on PBMC's. (A, B and C) 1–5 × 10^4 cells/200 μl were seeded and treated with the 10 μM or 50 μM **7a** for 24 h (MUTU-I, 4TI, MDA-MB231 and MCF-7) or 72 h (DG-75). 10 μl of Alamar Blue reagent was added to each well and fluorescence was read at 544 nm (excitation 590 nm) and values represent the mean value ± the SEM of six data points (recording in triplicate on two independent days). Large error bars may indicate low solubility of **7a** at high concentrations. (D) 7 × 10^5 cells/5 mL Cells were treated with 50 μM 7a for 24 h (MUTU-I) and 72 h (DG-75), harvested by centrifugation and fixed overnight in 70% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNase A. 10,000 cells were counted using appropriate gates. Values represent the mean ± SEM of three independent experiments.

the Burkitt's lymphoma cell line, DG-75 (Table 1). Compounds **5a** and **11a** both contain methylthio-substituents at the 6-position of the aromatic rings, which is similar in structure to 4-MTA (also been shown to have a general cytotoxic effect in vitro[12]).

Compounds **5c** and **5b** were also found to be cytotoxic to all the cell lines used in this study. Interestingly derivatives **5b**, **4b**, **7b** and **6b** have been previously reported as synthetic byproducts of clandestine MDMA synthesis[21] and when investigated for interaction with human monoamine transporters were found to interact with SERT at a similar potency to MDMA without displaying any neurotoxic effects (carrier-mediated release experiments).[21] In this study we report that the 1,3-bis(3,4-methylenedioxyphenyl)-2-propamine (**5b**) and the 1,3-bis(3,4-methylenedioxyphenyl)-2-nitro-1-propene (**4b**) are cytotoxic to HEK cells and to HEK cells overexpressing SERT with EC_{50} values in the low micromolar range (pEC_{50} values of 4–5 correlating to an approximate EC_{50} value of 1–10 μM) (Table 1). These derivatives also had a potent cytotoxic effect on the two malignant cell lines, DG-75 and SHSY-5Y (pEC_{50} 4.7–6.5, EC_{50} values 0.5–10 μM). Compounds **7b** and **6b** were found to have little effect on the hSERT overexpressing cell line in agreement with Pifl et al.[21] and were also found to have little effect on the malignant cell lines. These results suggest that the free amine and nitroethene functional groups may be involved in the cytotoxic ability of these compounds, with no significant difference between the toxicity of compounds with 3,4-methylenedioxy- and 4-methoxy- aromatic substituents. Compounds **10a** (previously found to have a general cytotoxic effect to all monoamine transporter-expressing cell lines and to the SHSY-5Y cell line[12]), **10b** and compound **7c** (which was more cytotoxic to the HEK cell line compared to the hSERT-overexpressing HEK cell line) (Table 1) were found to have little effect to the DG-75 cell line and the SHSY-5Y cell line.

We conclude that the majority of these 1,3-bis(aryl)-2-propanamines have significant cytotoxic effects against the BL cell line DG-75 and the neuroblastoma cell line SHSY-5Y. We find that compound **7a** had a potent effect on the SHSY-5Y neuroblastoma and on the DG-75 Burkitt's lymphoma cell line, implying that SERT may not be involved in its cytotoxic mechanism of action (Table 1). By contrast, the methoxy analogue of **7a**, compound **7b** an *N*-formyl-1,3-bis(aryl)2-propanamine had little effect on the SHSY-5Y cell line but was found to be cytotoxic to the BL cell line implying that the aldehyde is possibly partly responsible for the selective activity of these derivatives against BL and the cytotoxic effects of compound **7b** may be mediated through SERT. To address this hypothesis, compounds will be evaluated for SERT inhibitory activity in Section 2.3.

Lastly, overall the EC_{50} values for the antiproliferative effect of **7a** and related 1,3-bis(aryl)-2-propanamines is high compared to other chemotherapeutic agents, such as the microtubule targeting agent, Paclitaxel (Taxol), which has been shown to have effects in BL cell lines with IC_{50} values of between 10 and 40 nM.[25] It was therefore decided to synthesize and examine a range of novel 1,3-bis(aryl)-2-nitro-1-propenes (**4a–4i**) and 1,3-bis-(aryl)-2-propanamines (**5a–5d**) related in structure to **7a** with a view to identifying a more potent range of antiproliferative compounds (Section 2.1).

2.3. Inhibition of SERT activity

To determine if these new compounds synthesized as described in Section 2.1, along with the previously described derivative **7a** could behave like other amphetamines and inhibit the activity of SERT, the effects of these derivatives on 5-HT reuptake was assessed using a novel fluorescence neurotransmitter transporter assay[26] in HEK cells stably expressing hSERT.[13]

It has been difficult to determine if amphetamines are genuine substrates of SERT, due to their lipophilic nature and to the lack of a crystal structure of human SERT. However, amphetamines in general are thought to release stores of catecholamines from nerve endings by converting the respective molecular transporters into open channels. They are thought to compete with substrate for the transporters, reversing the transport of monoamines by either binding to the transporter as a substrate or binding without being transported.[27] The amphetamine analogues, 4-MTA, MDMA, 3,4-methylenedioxyamphetamine (MDA) and *para*-chloroamphetamine (PCA) have all been previously shown to inhibit SERT activity with IC_{50} values of 74 nM (4-MTA), 425 nM (MDMA), 478 nM (MDA) and 182 nM (PCA), respectively.[13] These amphetamines also inhibit the activity of the noradrenaline transporter (2,375 nM (4-MTA), 405 nM (MDMA), 266 nM (MDA) and 207 nM (PCA)) and inhibit the activity of the dopamine transporter (3,073 nM (4-MTA), 1,442 nM (MDMA), 890 nM (MDA) and 424 nM (PCA)).[13]

In this study, a subset of the 1,3-bis(aryl)-2-nitro-1-propene and 1,3-bis(aryl)-2-propanamines were synthesized and evaluated for their ability to bind to and inhibit SERT (Table 2). The percentage reuptake for the compounds was initially determined at concentrations of 1 and 100 μM. The IC_{50} value for reuptake inhibition of the more potent compounds (>80% uptake inhibition at 100 μM) was then determined from the appropriate sigmoidal dose-dependent curves (data not shown). The selective serotonin reuptake inhibitor (SSRI antidepressant) citalopram (10 μM) was used as a positive control, resulting in >97% SERT inhibition. The IC_{50} for citalopram was determined as 19.3 nM (compared to a reported K_i value of 8.9 nM[28]).

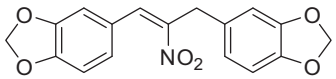
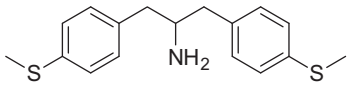
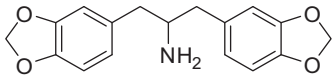
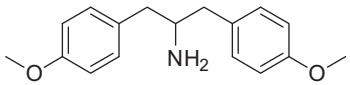
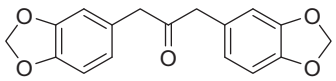
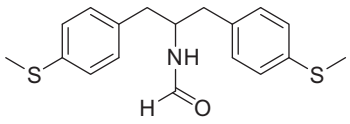
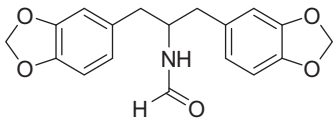
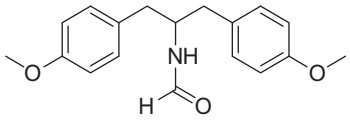
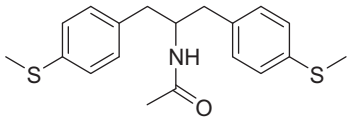
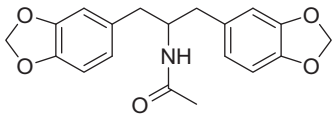
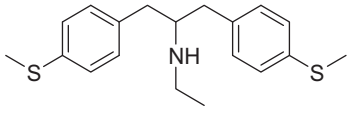
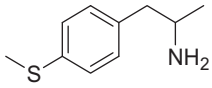
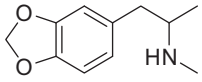
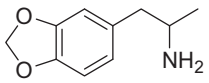
The lead compound **7a** was found to inhibit SERT with an IC_{50} of 15.7 μM, implying that the cytotoxic activity of **7a** is unlikely mediated through SERT, as the IC_{50} value for SERT binding is high compared to amphetamines 4-MTA and MDMA (74 nM and 425 nM, respectively). Compound **7b**, the methoxy analogue of **7a** also showed little SERT inhibition, again implying that that the cytotoxic activity of **7b** may not be mediated through SERT. At concentrations of 100 and 1 μM, 1,3-bis(aryl)-2-nitro-1-propenes **4a–4d** and 1,3-bis(aryl)-2-propanamines **5c**, **7c**, **10a**, did not bind to SERT with <20% inhibition observed at 1 μM. Compounds **5a**, **5b**, **5d**, **7a** and **11a** displayed SERT inhibition in a low micromolar range, with the most active compound **5b**, having an IC_{50} of 1.6 μM. Compounds **5a**, **5d** and **11a** showed a similar affinity for SERT compared to **5b**, with IC_{50} values of 2.5, 1.9 and 2.2 μM, respectively. Previous studies investigating the SERT inhibition of compounds **5b** and **7b** found that while a similar SERT inhibition activities were found for compound **5b** (IC_{50} of 7.7 ± 1.5 μM compared to 2.2 μM in our study), contrasting activities were found for compound **7b**, as no SERT inhibition was observed in this study (compared to 8.0 ± 2.0 μM).

Compared to SERT inhibition values for amphetamines 4-MTA (74 nM),[29] MDMA (425 nM)[30] and PCA (173 nM),[31] which are in the low nanomolar range, observed IC_{50} values for the compounds in this study are high, indicating that they most likely do not bind strongly to SERT. This is probably due to the presence of the second aromatic group, which may hinder the binding of the free amine to the appropriate residues in the 5-HT binding site. We can therefore hypothesise that while displaying low micromolar binding to SERT, compounds, **5a**, **5b**, **5d**, **7a** and **11a**, may not be interacting in the same way as amphetamines at SERT. Although these compounds may not be directly binding to SERT in the same manner as amphetamines, it is possible that there may be some other indirect interaction with the protein, however; further binding studies would be required to determine this.

2.4. Molecular modelling

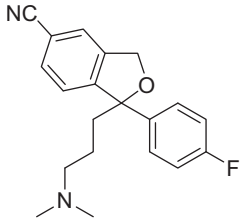
A molecular modelling study was carried out to investigate possible binding orientations for compounds **4a**, **5a**, **5b**, **7a**, 5-HT and

Table 1
The effects of 1,3-bis(aryl)-2-propanamine compounds on SERT-expressing HEK cells and on the malignant cell lines, DG-75 and SHSY-5Y.

Compound structure	Compound number	HEK293 pEC_{50}[a] ± SE	HEK293 hSERT pEC_{50}[a] ± SE	DG-75 pEC_{50}[a] ± SE	SHSY-5Y pEC_{50}[a] ± SE
	**4b**	5.1 ± 0.2	4.9 ± 0.1	6.0 ± 0.1	4.8 ± 0.1
	**5a**[b]	4.7 ± 0.2	4.5 ± 0.2	4.6 ± 0.1	4.7 ± 0.1
	**5b**	4.6 ± 0.2	4.5 ± 0.2	4.8 ± 0.2	4.5 ± 0.2
	**5c**	4.5 ± 0.2	4.6 ± 0.2	4.7 ± 0.1	4.7 ± 0.1
	**6b**	2.9 ± 0.2	2.3 ± 0.3	2.5 ± 0.2	<2
	**7a**	<2	4.0 ± 0.3*	4.5 ± 0.2	4.5 ± 0.1
	**7b**	<2	2.8 ± 0.2	4.8 ± 0.2	<2
	**7c**	4.5 ± 0.1	2.4 ± 0.3*	2.8 ± 0.3	<2
	**10a**	4.3 ± 0.2	4.4 ± 0.3	2.4 ± 0.3	2.4 ± 0.5
	**10b**	2.4 ± 0.4	<2	2.9 ± 0.2	<2
	**11a**	2.9 ± 0.4	4.6 ± 0.2	4.9 ± 0.1	4.8 ± 0.1
	4-MTA	4.7 ± 0.1	4.6 ± 0.1	3.5 ± 0.7	3.2 ± 0.5
	MDMA	3.6 ± 0.1	3.3 ± 0.2	4.5 ± 0.2	<2
	MDA	3.5 ± 0.2	3.2 ± 0.3	2.7 ± 0.2	<2

(*continued on next page*)

Table 1 (*continued*)

Compound structure	Compound number	HEK293 pEC_{50}[a] ± SE	HEK293 hSERT pEC_{50}[a] ± SE	DG-75 pEC_{50}[a] ± SE	SHSY-5Y pEC_{50}[a] ± SE
	Citalopram	4.8 ± 0.1	468 ± 0.1	4.9 ± 0.1	4.6 ± 0.2

5×10^4 cells/well were seeded and treated for 48 h. Cells were incubated for 3 ± 1 h with neutral red dye solution. Absorbance was read at 540 nm (690 nm background). Relative cell viability was expressed as percent of vehicle treated cells. The cytotoxic potency of each compound was quantified by a pEC_{50} value determined by non-linear regression analysis of sigmoidal log concentration dependence curves whereby pEC_{50} is $-[-\log EC_{50}] \pm SE$ ($\log EC_{50}$ is the log [Dose] when response is equal to 50% cell viability).

The effect of each compound on the HEK293 cell line was compared to the effect of the compound on each of the transporter-overexpressing cell lines using Two-way ANOVA statistical analysis, where $P < 0.05$ (*) represented a significant difference.

Sodium azide (30 mM) and Triton-X (2%) acted as positive controls for cytotoxicity resulting in 80–90% cytotoxicity to all cells.

[a] pEC_{50} ($-(-\log EC_{50}$ is the log [Dose] when response = 50%)) values were calculated from % cell viability versus −log concentration curves, using four concentrations in triplicate on two independent days. Data was subjected to non-linear regression analysis using a sigmoidal dose–response (Hill slope = 1) using GRAPHPAD Prism4 software (Graphpad software Inc., San Diego, CA).

[b] Compound evaluated as the hydrochloride salt.

* $P < 0.05$. hSERT cells were compared to HEK293 wildtype cells using a two-way ANOVA test (GRAPHPAD Prism 4) with no matching followed by a Bonferroni Post Test. ND: not determined.

4-MTA within the 5-HT binding site of SERT to allow us to rationalise results of SERT uptake experiments. Currently, there is no existing crystal structure of SERT; however, a number of homology models of hSERT are available which were constructed using the bacterial leucine transporter (LeuT) as a template.[32,33] For this study the homology model of hSERT constructed by Jørgensen et al. was used.[33] A number of previous studies have identified key binding residues involved in the binding of SERT ligands.[33–36] 5-HT, the natural substrate of SERT, and amphetamines such as 4-MTA interact with residues Ala96, Asp98 and Phe335 via binding of the protonated amine (Fig. 3A), while the tricyclic antidepressant imipramine is also thought to interact strongly with the Asp98 residue.[13,32,37] A study by Koldsø et al. discovered that the two enantiomers of the SSRI citalopram bind to a central binding site of the hSERT homology model with reversed orientations.[38] It has also been previously shown that the protonated amine of a series of sulfur-substituted α-alkyl phenethylamines is essential for protein–ligand binding. Other commonly annotated significant binding residues include Ile172 and Tyr95, which allow for hydrophobic and aromatic interactions, respectively.[34]

In this study, firstly as validation, both 5-HT and 4-MTA were docked with both receptor and ligand flexibility using Molegro Virtual Docker (http://www.molegro.com). Figure 3A and B clearly illustrate the key interactions (H-bonding to Ala96, Asp98 and Phe335 are common) made by both compounds via the protonated primary amine. 5-HT makes additional H-bonding interactions with Ser336 and also Thr439 (Fig. 3A). Subsequent docking of a representative series **4a**, **5a** and **7a** was undertaken.

The nitrostyrene compound **4a** was found to have no significant protein–ligand binding interactions with the 5-HT binding site of SERT (Fig. 3C). This result is consistent with our SERT uptake experiments, in which none of the nitrostyrene compounds **4a–4d** showed SERT inhibition.

As previously discussed, derivatives **5a**, **5b** and **5d** were found to have a moderate inhibitory effect on SERT (EC_{50} approx. 1.6–2.5 μM). Amine **5a** is shown to have similar critical H-bonding interactions between the protonated amine group and residues, Ala96, Asp98 and Phe335, as observed with both 5-HT and 4-MTA (Fig. 3C). Additional H-bonding with Leu337 is also observed as well as π-stacking with Phe341 to stabilise the compound in the binding site.

Compound **7a**, illustrated in Figure 3E, is shown to make a single π-stack with Phe341 without any added H-bonding interactions. Significantly, **7a** contains an amide which does not become protonated like the amine groups of compounds **5a**–**5d**, preventing strong acid/base interactions and therefore explains the reduction in SERT binding, as indicated by the high IC_{50} determined from SERT uptake experiments (Section 2.3).

Figure 4 highlights the key interactions made by compound **5b**, which was selected for docking analysis based on having the lowest determined IC_{50} of 1.6 μM. Parallel displaced π-stacking is observed with Phe341 and from visual analysis, possible T-shaped π-stacking with Tyr176 may occur in addition to H-bonding with Tyr95, Ala96, Asp98 and Leu337. Overall it is clear from the molecular modelling studies that for minimal SERT binding within the 5-HT site, H-bonding interactions to Ala96 and Asp98 are necessary.

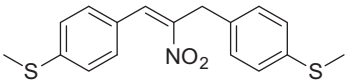
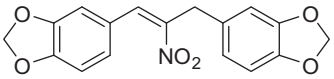
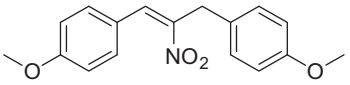
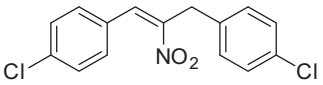
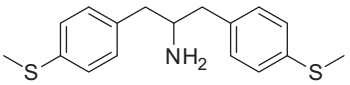
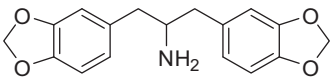
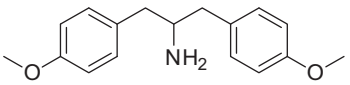
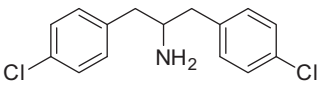
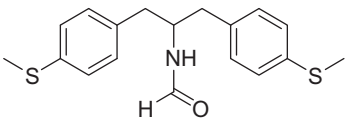
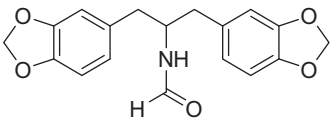
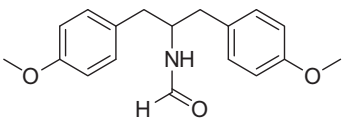
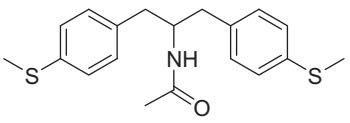
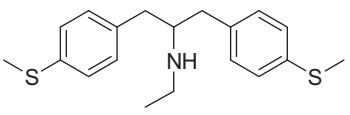
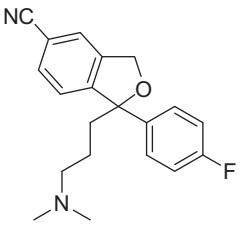
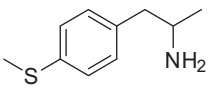
2.5. Biological activity

2.5.1. Antiproliferative activity

To assess if the observed cytotoxicity of each compound was due to an antiproliferative effect, each compound (**4a–4i**, **5a–5d**, **6b**, **7a–7c**, **8b**, **9b**, **10a**) was evaluated using the Alamar Blue assay in a range of malignant cell lines; two Burkitt's lymphoma cell lines (MUTU-I and DG-75) and the human dopaminergic neuroblastoma cell line, (SHSY-5Y). EC_{50} values for antiproliferative activity were estimated from log concentration sigmoidal dose–response curves where the cytotoxic potency of each compound was quantified by a pEC_{50} value, where pEC_{50} is $-[-\log EC_{50}] \pm SE$ ($\log EC_{50}$ is the log [Dose] when response is equal to 50% cell viability) (Table 3 and Fig. 5).

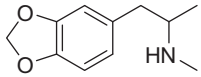
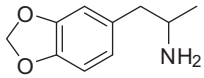
Compound **5d** was found to have the most potent antiproliferative effect on the MUTU-1 BL cell line (pEC_{50} of 6.8 ± 0.4), with an approximate EC_{50} value of 160 nM. The slightly more lipophilic nitrostyrene analogue of **5d**, compound **4d** ($c \log P$ values of 4.5 and 5.3, respectively, as shown in Table 3) also displayed a potent effect against the MUTU-1 cells (pEC_{50} of 6.0 ± 0.1), with an approximate EC_{50} value of 1 μM. The other 1,3-bis(aryl)-2-propanamine compounds **5a–5c** exhibited only moderate antiproliferative effects on this cell line (pEC_{50} range of 3.1–4.9). This is in contrast to the other 1,3-bis(aryl)-2-nitro-1-propenes (**4a–4f**), which all showed similarly potent effects on the MUTU-1 cell line (pEC_{50} range of 5.5–5.6) (Table 3 and Fig. 5A). This suggests that the presence of the slightly more lipophilic nitropropene structure may be crucial for a potent antiproliferative effect in MUTU-1 cells.

Table 2
Inhibition of SERT by 1,3-bis(aryl)-2-nitro-1-propenes and related compounds

Compound structure	Compound number	% SERT inhibition (100 μM)	% SERT inhibition (1 μM)	IC_{50} (μM)
	**4a**	18	25	ND
	**4b**	36	15	ND
	**4c**	10	25	ND
	**4d**	23	0	ND
	**5a**[a]	98	50	2.5
	**5b**	77	44	1.6
	**5c**	65	11	ND
	**5d**	94	39	1.9
	**7a**	85	5	15.7
	**7b**	55	18	ND
	**7c**	18	8	ND
	**10a**	28	0	ND
	**11a**	92	31	2.2
	Citalopram	n/a	89	0.019
	4-MTA	n/a	n/a	0.2[*]

(*continued on next page*)

Table 2 (*continued*)

Compound structure	Compound number	% SERT inhibition (100 μM)	% SERT inhibition (1 μM)	IC_{50} (μM)
	MDMA	n/a	n/a	1.1*
	MDA	n/a	n/a	0.9*

Cells were seeded at a density of 2×10^4 in black, clear bottomed, poly-L-lysine coated plates. After 24 h, the media was aspirated cells were washed with 100 μl of 1× HBSS. Cells were incubated for 15–20 min with 100 μl of compound dissolved in 1× HBSS + 0.1% BSA, before 100 μl of the fluorescent dye was added. Plates were read at 520 nm (excitation 440 nm). The background fluorescence of the cells and 1× HBSS buffer was taken away from each group. Untreated cells represented 100% fluorescence/0% inhibition. Citalopram (10 μM) was used as a positive control resulting in >98% inhibition. Fluorescence for each compound was calculated as a percent of untreated cell response. This value was then subtracted from 100% to determine the percent inhibition. IC_{50} values were calculated for compounds having >80% inhibition at 100 μM. IC_{50} for citalopram was calculated as 19.7 nM.

Citalopram (10 μM) was used as a positive control for SERT inhibition, resulting in >98% inhibition. IC_{50} values were calculated for compounds having >80% inhibition at 100 μM.

[a] Compound evaluated as free base. ND: not determined.

* Evaluated using [^{3}H] 5-HT as previously described by Cloonan et al.[13]

1,3-Bis(aryl)-2-propanamines **5a–5d** did not show any significant antiproliferative effect on the resistant BL cell line, DG-75 (pEC_{50} of 3.4–4.6) compared to 1,3-bis(aryl)-2-nitro-1-propene compounds **4a–4d**, which displayed a moderate antiproliferative effect in this cell line (pEC_{50} range of 4.3–4.9) with an approximate EC_{50} range of 11–37 μM (Table 3 and Fig. 5A). Interestingly, halogenated compounds **4e** and **4f** were found to have a much more potent antiproliferative effect on the resistant DG-75 cell line (pEC_{50} of 5.5 ± 0.1 and 5.1 ± 0.1, respectively), with approximate EC_{50} values of 3.3 μM and 9.3 μM, respectively (Table 3 and Fig. 5A). Other halogenated analogues **4g** and **4h**, displayed potent effects on both the MUTU-1 and DG-75 cell lines along with compound **4i**, a structural isomer of compound **4f** which has approximate EC_{50} values of 2.6 μM and 1.5 μM in MUTU-1 and DG-75 cell lines, respectively (data not shown). When evaluated for their antiproliferative effect on the SHSY-5Y cell line, compounds **4d** and **5d** showed a potent effect (pEC_{50} of 5.6 ± 0.1 and 5.3 ± 0.0, respectively) with approximate EC_{50} values of 2.5 μM and 4.6 μM, respectively. Other propanamine and nitropropene compounds **4a–4c** and **5a–5c** showed only moderate effects against the SHSY-5Y cell line (pEC_{50} of 4.5–4.9) with an approximate EC_{50} range of 11–33 μM (Table 3 and Fig. 5A).

All other compounds were found not to possess a significant antiproliferative effect on each of the cell lines (Table 3 and Fig. 5A). Compounds were also evaluated for their antiproliferative activity on the K562 cell line, a chemoresistant chronic myelogenous leukaemia (CML) cell line. Only one compound, compound **5a** (pEC_{50} of 4.4, approximate EC_{50} value of 50.1 μM) was found to have significant activity on this cell line (data not shown). From these results it appears that these 1,3-bis(aryl)-2-nitro-1-propenes (**4a**–**4i**) appear to be selective to Burkitt's lymphoma derived cell lines.

In order to assess the selectivity of these derivatives towards proliferating malignant cells over the 'normal' cells of the body, the cytotoxic effects of a representative group of these derivatives were assessed in peripheral blood mononuclear cells (PBMCs). After 24 h, it was found that at 10 μM, most of the derivatives had little effect on the viability of PBMCs compared to their effect on a range of malignant cells lines (Fig. 6B). Such results are consistent with these agents selectively targeting cells of malignant origin over 'normal cells' of the body. However, 1,3-bis(aryl)-2-propanamines **5a** and **5d** were found to have a potent toxic effect in PBMCs, implying a non-specific toxicity toward malignant cell lines as well as 'normal' PBMCs. From the results of the cell viability studies, it appears that the nitrostyrene compounds (**4a–4i**), in general, have a more potent, selective antiproliferative activity than their propanamine analogues (**5a–5d**), suggesting further evidence that the antiproliferative abilities of these compounds are not related to their SERT binding ability, as the nitrostyrene compounds do not bind to SERT (Table 2).

2.5.2. Potential anticancer activity

Designing drugs that can induce programmed cell death (PCD), namely apoptosis, of a cancer cell, whilst ignoring the 'normal cells' of the body is imperative to the future development of safe effective anticancer agents. A drug that can induce PCD in vitro in malignant cell lines has the potential to become an anticancer agent. Propidium iodide FACS analysis was carried out on a representative subset of compounds in the MUTU-I cell line, initially using 1 and 10 μM for potent compounds (EC_{50} <20 μM in MUTU-1 cells) in the Alamar Blue assay. Taxol (10 μM and 1 μM) was used as positive control, showing 58–77% cell death at both concentrations (Fig. 6A).

At 10 μM, compounds **4a**, **4b**, **4c** and **4d** showed 50%, 30%, 32% and 41% cells in pre-G1 phase of the cell cycle, respectively; indicating the formation of apoptotic bodies in MUTU-1 cell line (Fig. 5A). These compounds were also able to activate caspases 3 and 7 (Fig. 6B), induce PARP cleavage (Fig. 6C) and elicit morphological features of apoptosis such as chromatin condensation and membrane blebbing (Fig. 6D) in the MUTU-I cell line all conclusive with these compounds inducing Type-I programmed cell death (apoptosis) in Burkitt's lymphoma cells. It is also worth noting that after 4 h of treatment with these representative compounds, the majority of MUTU-I cells displayed all of the features of apoptotic PCD, whereas Taxol at the same concentration (10 μM) did not display all of features (just morphological features associated with G2/M arrest). While compound **5d** displayed the most potent activity in MUTU-1 cells as shown in the cell viability assay, no apoptotic bodies were observed by FACS analysis and there was no evidence of PARP cleavage (Fig. 6C), perhaps indicative of this compound eliciting a non-specific toxic effect or inducing an alternative form of programmed cell death such as Type-II PCD. Compound **5d** was also found to have a potent toxic effect in the PBMC cell line, further suggesting a non-selective cytotoxicity. These results indicate that the nitroethene structure may be essential for induction of apoptosis. Further studies on the structure–activity relationship of the nitroethene functional group are currently ongoing to identify structural requirements important for inducing the antiproliferative and pro-apoptotic effects observed in this study.

As a number of compounds (**4e**, **4g**, **4h**, **4i**) in this screen also had an antiproliferative effect in chemoresistant BL cell line DG-75 (which cannot die by classical apoptosis[24] but dies by Type-II

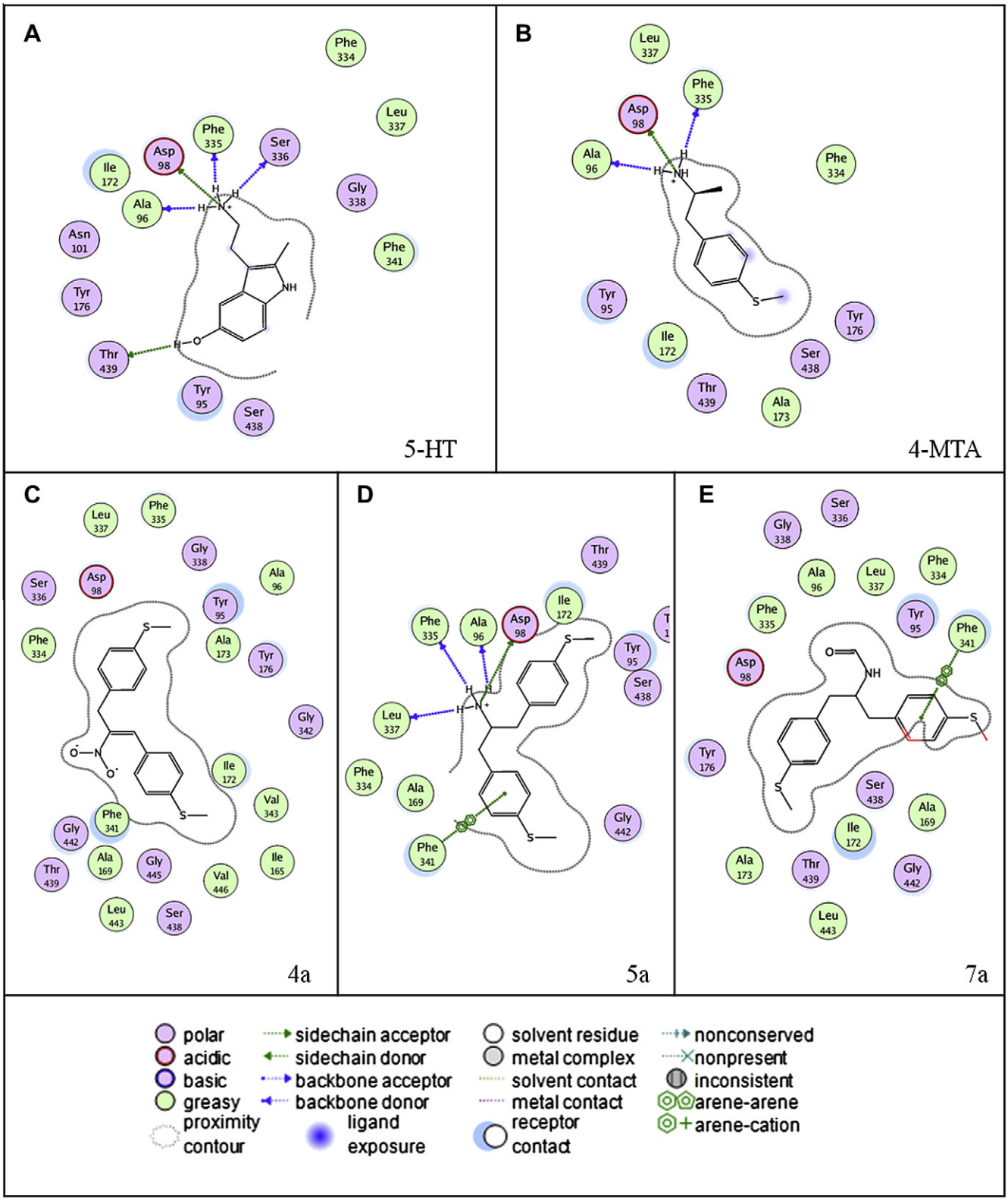


Figure 3. 2D representation of the interactions produced by 5-HT, 4-MTA, **4a**, **5a** and **7a** in the 5-HT SERT binding site. It has been previously shown that the binding of the protonated amine of a series of sulfur-substituted α-alkyl phenethylamines and amphetamines to Asp98 is essential for protein-ligand binding.[13] Nitrostyrene compound **4a** shows no binding interactions with the 5-HT binding site of SERT (Fig. 3C). Compound **5a** is shown to have similar critical H-bonding interactions between the protonated amine group and residues, Ala96, Asp98 and Phe335, as observed with both 5-HT and 4-MTA (Fig. 3A, B and D). Compound **7a** is shown to make a single π-stack with Phe341 without any added H-bonding interactions (Fig. 3E). Docking was carried out using Molegro Virtual Docker (www.molegro.com), using a recent homology model of hSERT, constructed by Jørgensen et al.[33] containing 5-HT as a bound ligand.[13]

autophagic PCD), investigations are currently underway to evaluate if these compounds can act as pro-autophagic agents in the treatment of chemoresistant cancers (cancer cells that cannot die by the archetypical Type-I apoptotic response). In addition, since these active compounds were also found from an initial screen, with little information regarding their suspected target-based

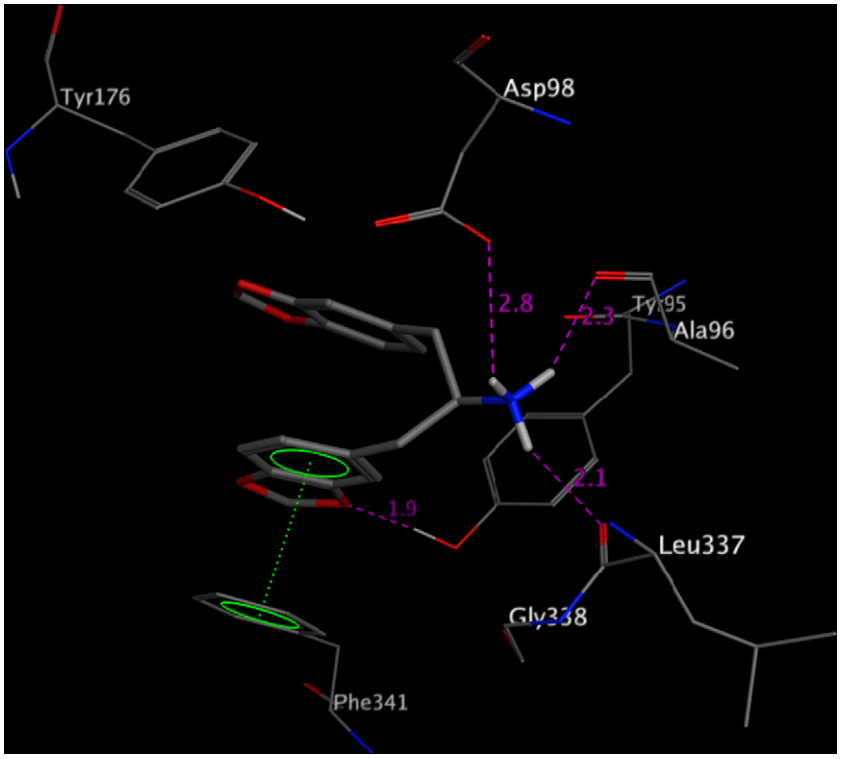


Figure 4. 3D image of the docked pose of compound **5b** in the 5-HT binding site of SERT with isolated important residues for binding. Key H-bonding interactions are highlighted (purple) with distances annotated. π-Stacking between Phe341 and **5b** is also highlighted (green). Docking was carried out using Molegro Virtual Docker (www.molegro.com), using a recent homology model of hSERT, constructed by Jørgensen et al.[33] containing 5-HT as a bound ligand.[13]

mechanism of action, extensive further investigations are required to develop more potent analogues and to identify their mechanism of action.

Interestingly, a number of these compounds (**4a–4i**) are structurally related to simple nitrostyrene compounds which have been previously shown to have a variety of biological effects including anti-tumour and pro-apoptotic effects.[39–41] In recent years, there have been many studies investigating the biological effects and functions of these compounds, with nitrostyrenes having been shown to have antimicrobial[39,41] and insecticidal[42] properties. Some have even been shown to affect the down regulation of interleukins, suggesting an immunological role.[43] Some β-nitrostyrenes and related compounds have been found to possess anti-tumour capabilities via the inhibition of tubulin polymerisation[44] and protein tyrosine phosphatases (PTP). *trans*-β-Nitrostyrene (TBNS) is a byproduct in the synthesis of methamphetamine and there is evidence to suggest that it induces apoptosis in cancer cell lines via inhibition of PTP.[40,45]

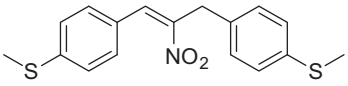
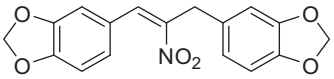
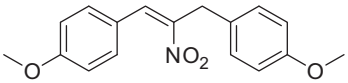
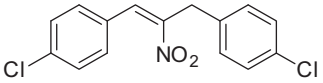
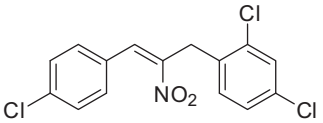
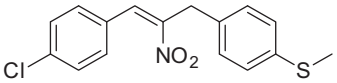
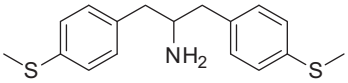
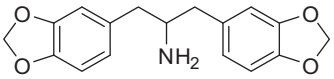
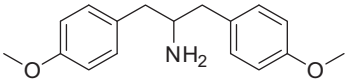
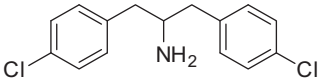
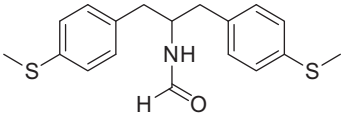
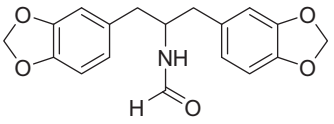
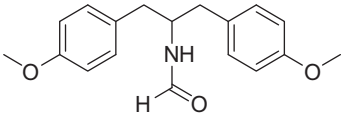
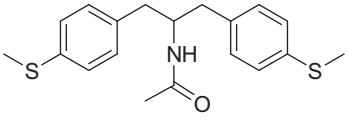
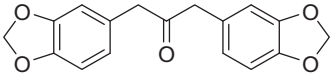
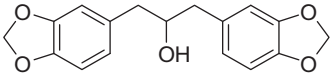
Work carried out by Park and Pei, found that TBNS and a 4-methoxy-TBNS derivative inhibited three PTPs (Yop, PTP1B, SHP-1) at concentrations in the low micromolar range (2.5 and 4.5 μM, respectively),[40] which is similar to the concentration at which our 1,3-bis-(4-methoxyphenyl)-2-nitro-propene **4c** induces cell death in the MUTU-1 cell line. Due to the structural similarity between TBNS and the 1,3-bis(aryl)-2-nitro-1-propenes synthesized in this study, we thought it possible that they are targeting PTP in order to cause an apoptotic effect. However, further investigation into the ability of these compounds to inhibit protein tyrosine phosphatases (PTP1B) revealed that compared to the known PTP1B inhibitor Suramin, compounds **4a**, **4c** and **4d** had no effect on the activity of PTP1B (Fig. 7A). Investigations into the ability of these compounds to affect microtubules was investigated using PI FACS analysis, assessing the amount of cells in the G2/M phase (characteristic of microtubule disrupting drugs) of the cell cycle (Fig. 7B) revealed that at 2, 4, 6 and 8 h compounds **4a–4d** had little effect on the G2/M phase of the cell cycle compared to Taxol. Interestingly, the morphology of cell death elicited by compounds **4a–4d** after 4 h of treatment shows some evidence of these compounds inducing apoptotic cell death (Fig. 7C) and affecting cell cycle dynamics as indicated by the apparent arrest of chromosomal alignment in the nucleus (Fig. 6D). This maybe an indirect effect of the action of these drugs or may be as a result of these compounds targeting a yet undefined cell cycle target. Further investigations are required to evaluate such possibilities.

3. Conclusion

The initial rationale for this study was to create a library of novel potential SERT ligands and assess their antiproliferative activities against Burkitt's lymphoma. This was based on previous literature documenting the ability of SERT ligands to induce potent selective programmed cell death in Burkitt's lymphoma[1,5,7] and from preliminary work arising from a screen of synthesis byproducts associated with the clandestine synthesis of 4-MTA which identified one lead compound **7a**[12] as having SERT-selective toxicity and antiproliferative and pro-apoptotic activities (Fig. 2) against Burkitt's lymphoma cell lines.

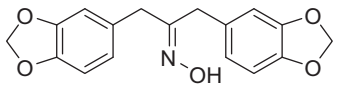
In the current study, we report the synthesis and SERT-inhibitory activity of a number of structurally related 1,3-bis(aryl)-2-nitro-1-propene and 1,3-bis(aryl)-2-propanamine compounds. We find that despite the lack of SERT binding activity of the majority of these derivatives, a number of compounds showed antiproliferative activity against malignant cell lines DG-75, MUTU-I and SHSY-5Y showing apparent selectivity for BL-derived cell lines. Nitrostyrene-like derivatives had more potent antiproliferative activity than their propanamine equivalents with the nitrostyrene derivatives **4a–4d** inducing the formation of apoptotic bodies, (Fig. 6A), activating caspases (3 and 7) (Fig. 6B), inducing chroma-

Table 3
In vitro, antiproliferative effects (pEC_{50}) of 1,3-bis(aryl)-2-nitro-1-propenes and related compounds in various cell lines

Compound structure	Compound number	pEC_{50}[a] ± SE cell viability			*c* log *P*[c]
		MUTU-1	DG-75	SHSY-5Y	
	**4a**	5.5 ± 0.1	4.4 ± 0.0	4.7 ± 0.1	5.0
	**4b**	5.5 ± 0.1	4.3 ± 0.0	4.5 ± 0.1	4.0
	**4c**	5.6 ± 0.1	4.9 ± 0.0	4.9 ± 0.1	4.3
	**4d**	6.0 ± 0.1	4.9 ± 0.0	5.6 ± 0.1	5.3
	**4e**	5.5 ± 0.1	5.5 ± 0.1	4.6 ± 0.1	6.8
	**4f**	5.5 ± 0.1	5.1 ± 0.1	4.7 ± 0.1	5.3
	**5a**[b]	4.9 ± 0.0	4.6 ± 0.0	4.9 ± 0.1	4.9
	**5b**	4.3 ± 0.1	3.6 ± 0.1	4.5 ± 0.2[d]	3.8
	**5c**	3.1 ± 0.6	3.4 ± 0.1	4.7 ± 0.1[d]	4.1
	**5d**	6.8 ± 0.4	4.6 ± 0.0	5.3 ± 0.0	4.5
	**7a**	0.9 ± 1.8	2.3 ± 1.1	4.5 ± 0.1[d]	3.9
	**7b**	4.2 ± 0.1	3.8 ± 0.1	3.65 ± 0.2	2.7
	**7c**	3.7 ± 0.2	3.7 ± 0.1	3.5 ± 0.1	2.6
	**10a**	3.8 ± 0.1	3.4 ± 0.1	1.8 ± 1.4	3.8
	**6b**	4.0 ± 0.1	3.8 ± 0.1	3.6 ± 0.1	3.1
	**9b**	4.4 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	2.9

(*continued on next page*)

Table 3 (*continued*)

Compound structure	Compound number	pEC_{50}[a] ± SE cell viability			*c* log *P*[c]
		MUTU-1	DG-75	SHSY-5Y	
	**8b**	4.6 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	3.5

5×10^4 cells/well were treated with the respective drug for 24 h. Each well was treated with 20 μl of Alamar Blue (pre-warmed to 37 °C) and left to incubate at 37 °C in the dark for 4–6 h. Fluorescence was read at 590 nM (excitation 544 nm). Background fluorescence of the media without cells + Alamar Blue was taken away from each group, and the control untreated cells represented 100% cell viability. The antifungal agent miconazole (10 μM) was used as a positive control for cell death in all cell lines, resulting in 90% cytotoxicity. The cytotoxic potency of each compound was quantified by a pEC_{50} value determined by non-linear regression analysis of sigmoidal log concentration dependence curves whereby pEC_{50} is $-[-\log EC_{50}] \pm SE$ ($\log EC_{50}$ is the log [Dose] when response is equal to 50% cell viability).

[a] pEC_{50} ($-(-\log EC_{50}$ is the log [Dose] when response = 50%)) values were calculated from % cell viability versus −log concentration curves, using four concentrations in triplicate on two independent days. Data was subjected to non-linear regression analysis using a sigmoidal dose–response (Hill slope = 1) using GRAPHPAD Prism4 software (Graphpad software Inc., San Diego, CA). Miconazole (10 mM) acted as positive control for cytotoxicity resulting in 90% cytotoxicity to all cells.

[b] Compound evaluated as the free base.

[c] *c* log *P* was determined using ChemDraw Ultra version 12.

[d] Evaluated using the neutral red assay.

tin condensation, membrane blebbing and PARP cleavage (Fig. 6C and D) in the MUTU-I cell line, all conclusive with these compounds inducing Type-I programmed cell death (apoptosis). From this study it appears that the nitroethene structure may be essential for induction of apoptosis, as derivative **5d** 1,3-bis(4-chlorophenyl)-2-propanamine, although having potent antiproliferative activity in MUTU-1 cells did not induce apoptosis in these cells, however further SAR are required to confirm this observation. Further studies will also examine the ability of these derivatives to induce Type-II programmed cell death in chemoresistant BL, as a number of the nitrostyrene compounds showed potent antiproliferative activity in the DG-75 cell line, a known chemoresistant cell line previously shown to be susceptible to antidepressant induced-Type-II PCD.[24]

Investigations into the mechanism of action of these compounds eliminated a possible role for protein tyrosine phosphatases and for tubulin polymerisation effects. Examination of the morphology of MUTU-I cells treated with compounds **4a–4d** (Fig. 6D) for 2 and 4 h reveals that the mechanism of cell death is fast and potent and may involve the cell cycle and/or mitosis, implying that these derivatives most likely target a fundamental part of the BL survival pathway. Further investigations into this mechanism of action and the identification of a possible target for these nitrostyrene derivatives is of paramount importance to the potential development of these compounds as novel anticancer agents.

4. Experimental

4.1. Chemistry

Uncorrected melting points were measured on a Gallenkamp apparatus. Infra-red (IR) spectra were recorded on a Perkin–Elmer FT-IR Paragon 1000 spectrometer. ^1H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded at 27 °C on a Brucker DPX 400 spectrometer (400.13 MHz, ^1H; 100.61 MHz, ^{13}C; 376.47 MHz, ^{19}F) in either $CDCl_3$ (internal standard tetramethylsilane (TMS)) or CD_3OD. For $CDCl_3$, ^1H NMR spectra were assigned relative to the TMS peak at δ 0.00 and ^{13}C NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For CD_3OD, ^1H and ^{13}C NMR spectra were assigned relative to the centre peaks of the CD_3OD multiplets at δ 3.30 and 49.00 ppm, respectively. ^{19}F NMR spectra were not calibrated. Coupling constants are reported in Hertz. For ^1H NMR assignments, chemical shifts are reported: shift value (number of protons, description of absorption, coupling constant(s) where applicable). Electrospray ionisation mass spectrometry (ESI-MS) was performed in the positive ion mode on a liquid chromatography time-of-flight mass spectrometer (Micromass LCT, Waters Ltd, Manchester, UK). The samples were introduced into the ion source by an LC system (Waters Alliance 2795, Waters Corporation, USA) in acetonitrile/water (60:40% v/v) at 200 μl/min. The capillary voltage of the mass spectrometer was at 3 kV. The sample cone (de-clustering) voltage was set at 40 V. For exact mass determination, the instrument was externally calibrated for the mass range *m*/*z* 100–1000. A lock (reference) mass (*m*/*z* 556.2771) was used. Mass measurement accuracies of <±5 ppm were obtained. Low resolution mass spectra (LRMS) were acquired on a Hewlett–Packard 5973 MSD GC–MS system in electron impact (ESI) mode. Elemental analyses were performed on an Exetor Analytical CE4400 CHN analyser in the microanalysis laboratory, Department of Chemistry, University College Dublin. High resolution mass spectra (HRMS) were acquired on a Thermo Scientific LTQ Orbitrap Discovery system in EI mode. Flash column chromatography was carried out on Merck Kieselgel 60 (particle size 0.040–0.063 mm), Aldrich aluminium oxide, (activated, neutral, Brockmann I, 50 mesh) or Aldrich aluminium oxide, (activated, acidic, Brockmann I, 50 mesh). Preparation of compounds **2a–2f**, **3a–3c**, **3e**, **4a**, **4b**, **4c**, **5a**, **5b**, **6b**, **7b**, **12b** was carried out as described in previously reported literature.[12,19–21,46–48]

4.1.1. Preparation of 1-(4-methoxyphenyl)-2-nitroethene (2c)

Compound **2c** was synthesized according to the literature procedure[13] (eluent: 4:1 dichloromethane/hexane). Yellow crystals (70%). Mp 83–85 °C. IR ν_{max} (KBr) 1602, 1174, 1493, 1338 cm^{-1}. ^1H NMR δ ($CDCl_3$) 3.89 (3H, s, O*CH*$_3$), 6.98 (2H, d, *J* = 9.0 Hz, Ar*H*), 7.52–7.57 (3H, 2d, *J* = 9.0 Hz, Ar*H* *J* = 13.5 Hz, ArCH*CH*), 8.00 (1H, d, *J* = 13.5 Hz, Ar*CH*CH). ^{13}C NMR ppm ($CDCl_3$) 55.57, 114.95, 122.58, 131.19, 135.07, 139.08, 162.96. HRMS (ESI) calculated for $C_9H_{10}NO_3$: (M^++H) 180.0661: found 180.0664.

4.1.2. Preparation of 1-(2,4-dichlorophenyl)-2-nitroethene (2e)

Compound **2e** was prepared according to the literature procedure[12] (eluent: 7:3, hexane/diethyl ether). Yellow crystals (91%). Mp 100–102 °C. IR ν_{max} (KBr) 1337, 1587, 1634 cm^{-1}. ^1H NMR δ ($CDCl_3$) 7.34 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.42 (1H, s, Ar*H*), 7.45 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.87 (1H, d, *J* = 14.0 Hz, ArCH*CH*), 8.27 (1H, d, *J* = 14.0 Hz, Ar*CH*CH). ^{13}C NMR ppm ($CDCl_3$) 128.15, 129.32, 131.14, 131.60, 133.16, 135.60, 136.42, 142.50. HRMS (ESI) calculated for $C_8H_5NO_2Cl_2$: (M^+) 216.9697: found 216.9694.

4.1.3. General procedure A: preparation of 1-aryl-1-nitroethanes

Sodium borohydride (15.00 mmol, 5.70 g) was added to a vigorously stirred mixture of the appropriate nitroethene (3.68 mmol), silica gel (9.30 g) and propan-2-ol (100 mL) in dichloromethane (350 mL) at room temperature. The mixture was stirred at room temperature for 20 min until the solution turned colourless. Excess $NaBH_4$ was quenched with the addition of dilute HCl (50 mL). The

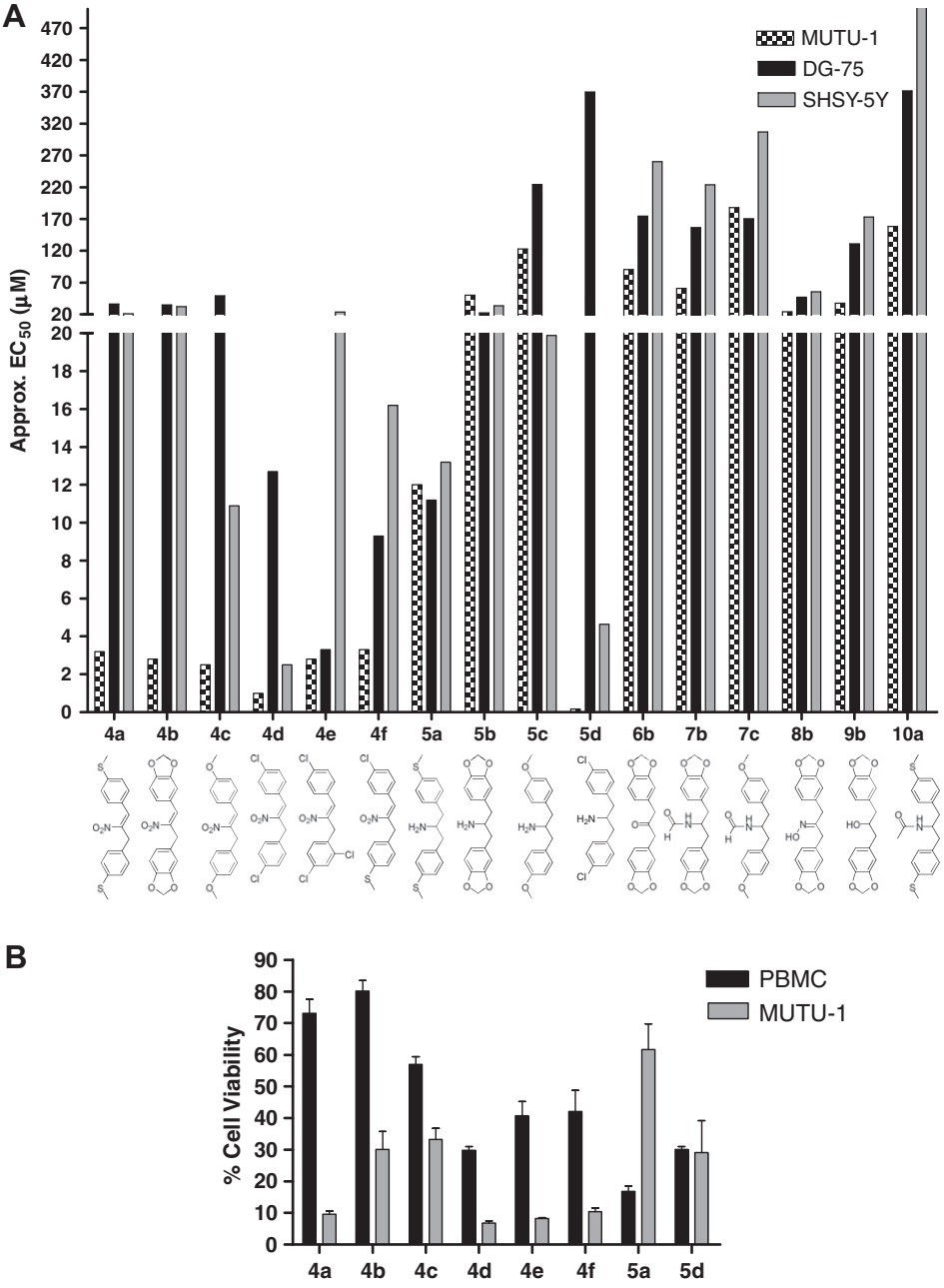


Figure 5. 1,3-Bis(aryl)-2-nitro-1-propenes and related compounds show selective toxicity toward the MUTU-1 cell line. (A and B) 1–5 × 10^4 cells/well were treated with the respective drug for 24 h. 20 μl of Alamar Blue was added and left to incubate at 37 °C in the dark for 4–6 h. Fluorescence was read using at 590 nm (excitation 544 nm). The background fluorescence of the media without cells + Alamar Blue was taken away from each group, and the control untreated cells represented 100% cell viability. The antifungal agent miconazole (10 μM) was used as a positive control for cell death in each of the other cell lines, resulting in 90% cytotoxicity. Each compound was screened over a 1 μM–1 mM concentration range in triplicate on two independent days with activity expressed as percentage cell viability compared to vehicle treated controls. The cytotoxic potency of each compound was quantified by a pEC_{50} value determined by non-linear regression analysis of sigmoidal log concentration dependence curves, whereby approx. EC_{50} values were estimated from log concentration sigmoidal dose–response curves; pEC_{50} value corresponds to $-[-\log EC_{50}] \pm SE$ ($\log EC_{50}$ is the log [Dose] when response is equal to 50% cell viability).

mixture was filtered and the silica gel washed with dichloromethane (100 mL). All organic phases were combined and washed with brine (3 × 50 mL) and water (3 × 50 mL). The organic phases were then dried over anhydrous Mg_2SO_4 and the solvent removed in vacuo. The crude product was purified by column chromatography over silica gel.

4.1.3.1. 1-(4-Chlorophenyl)-1-nitroethane (3d). Compound **3d** was prepared from 1-(4-chlorophenyl)-2-nitroethene (**2d**) (36.80 mmol, 6.76 g) according to general procedure A (Section 4.1.3). The crude product was purified by column chromatography over silica gel (eluent: 7:3 hexane/diethyl ether). Dark brown oil (82%). IR ν_{max} (film) 1552, 1376 cm^{-1}. ^1H NMR δ ($CDCl_3$) 3.31 (2H, t, J = 7.3 Hz, $ArCH_2CH_2$), 4.62 (2H, t, J = 7.3 Hz, $ArCH_2CH_2$), 7.17 (2H, d, J = 8.5 Hz, Ar*H*), 7.32 (2H, d, J = 8.5 Hz, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 32.71, 76.03, 129.15, 129.97, 133.42, 134.12. HRMS (ESI) calculated for $C_8H_8ClNO_2$: (M^+) 185.0244: found 185.0250.

4.1.3.2. 1-(2,4-Dichlorophenyl)-1-nitroethane (3e). Compound **3e** was prepared from 1-(dichlorophenyl)-2-nitroethene (**2e**) (1.38 mmol, 0.30 g) according to general procedure A (Section 4.1.3). The crude product was purified by column chromatography

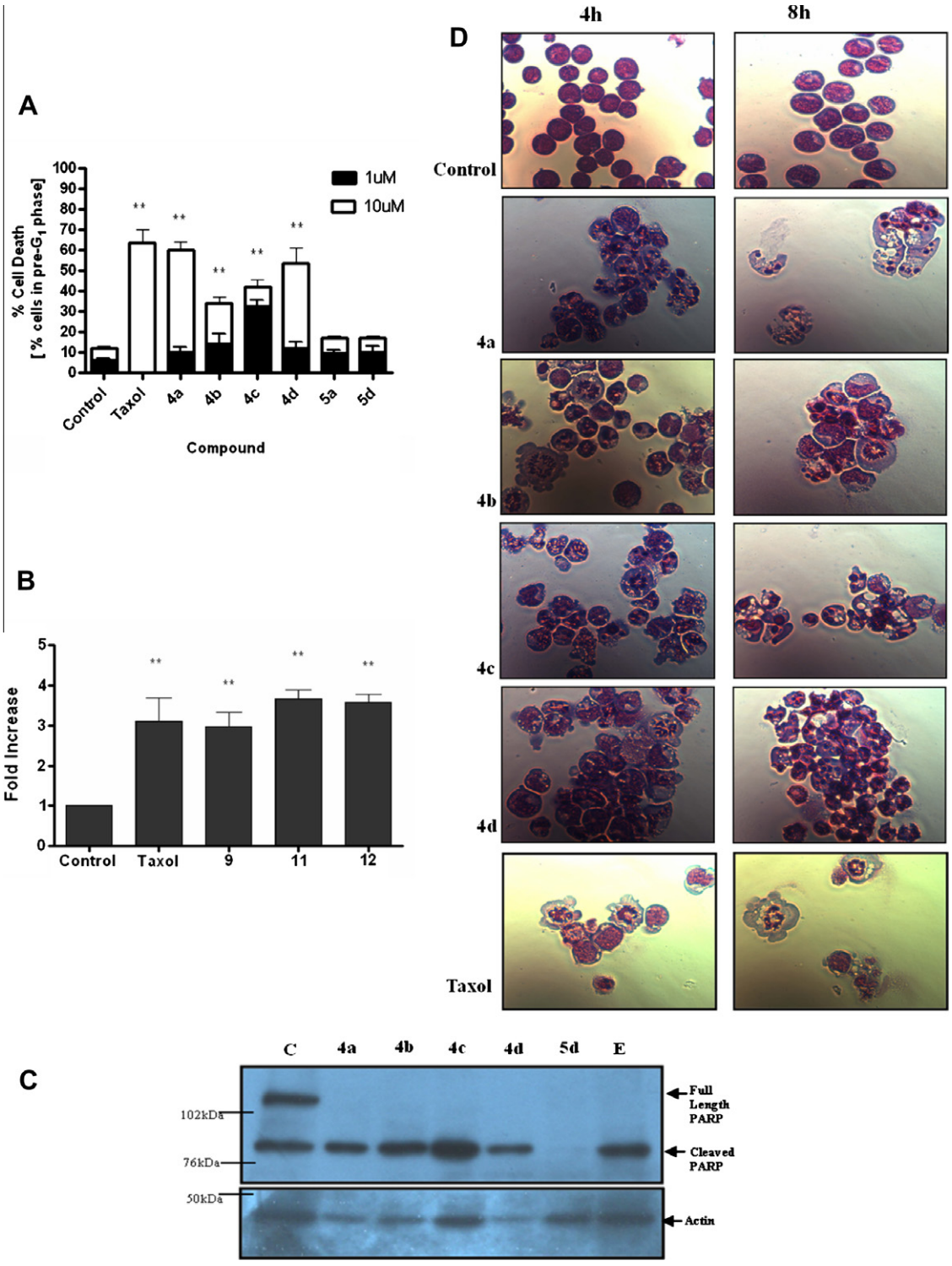


Figure 6. Compounds **4a**, **4b**, **4c** and **4d** induce apoptosis in the MUTU-I cell line. (A) 750,000 MUTU-I cells were treated with the appropriate amount of compound and incubated for 24 h. Cells were harvested, washed and fixed overnight at 4 °C. RNAse A and PI were added prior to cell cycle analysis counting 10,000 cells and analysed using CELLQUEST software package. (B) 0.5×10^5 cells were treated with compound (10 μM) for 5 h before the addition of 100 μl caspase substrate Z-DEVD-R110. Caspase 3/7 was quantified by measuring the amount of cleaved substrate at 521 nm (excitation 499 nm). (C) Samples were resolved by SDS–PAGE, transferred onto PVDF membranes and were probed with anti-PARP (recognises full length 113 kDa PARP as well as the 85 kDa cleaved form). (D) 3×10^5 cells were treated with the indicated compounds for 4 h or 8 h. Cells (150 μl) were cytocentrifuged onto microscopy slides, stained Eosin Y and methylene blue and examined under a light microscope using a 60× magnification.

over silica gel (eluent: 7:3 hexane/diethyl ether). Pale yellow oil (83%). IR ν_{max} 1549, 1369 cm^{-1}. ^1H NMR δ ($CDCl_3$) 3.24 (2H, t, J = 7.0 Hz, $ArCH_2CH_2$), 4.65 (2H, t, J = 7.0 Hz, $ArCH_2CH_2$), 7.20 (2H, d, J = 8.0 Hz, Ar*H*), 7.42 (1H, s, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 30.42, 73.53, 127.21, 129.27, 131.49, 133.82, 134.21. HRMS (ESI) calculated for $C_8H_7Cl_2NO_2$: (M^+) 218.9854: found 218.9848.

4.1.3.3. 1-(4-Fluorophenyl)-1-nitroethane (3h). Compound **3h** was prepared from 1-(4-fluorophenyl)-2-nitroethene (**2h**) (10.00 mmol, 1.67 g) according to general procedure A (Section 4.1.3). No further purification was necessary. Orange oil (85%). IR ν_{max} (film) 1378, 1552 cm^{-1}. ^1H NMR δ ($CDCl_3$) 3.21 (2H, t, J = 7.0 Hz, $ArCH_2CH_2$), 4.51 (2H, t, J = 7.0 Hz, $ArCH_2CH_2$), 7.01 (2H,

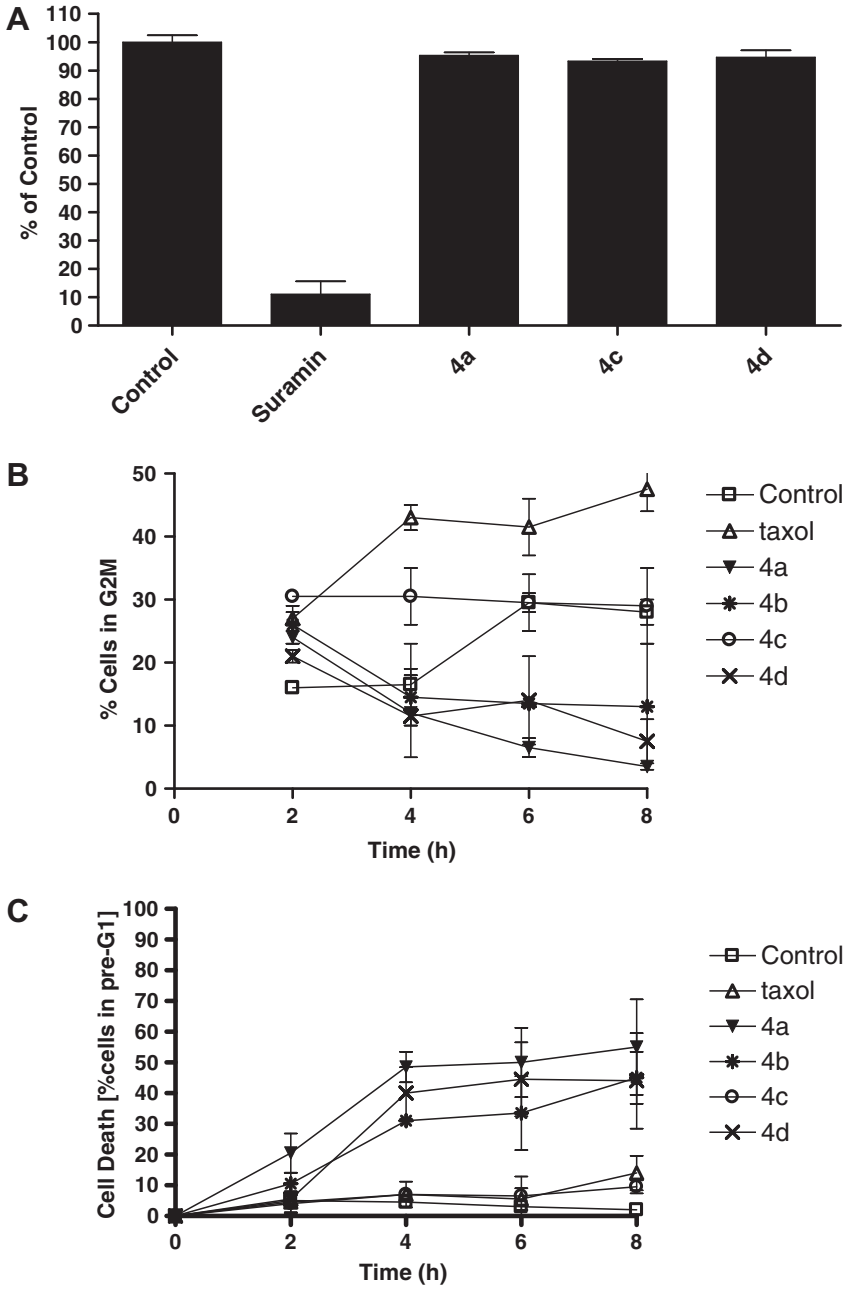


Figure 7. 1,3-Bis(aryl)-2-nitro-1-propenes (**4a**–**4d**) do not inhibit PTP1B or affect tubulin polymerisation in the Burkitt's lymphoma derived cell line MUTU-I. (A) Recombinant PTP1B was incubated with the test compound and a phosphopeptide substrate for 30 min at 30 °C. Free phosphate released was detected at 620 nm. (B) 750,000 MUTU-I cells were treated with the appropriate amount of compound and incubated for 24 h. Cells were harvested, washed and fixed overnight at 4 °C. RNAse A and PI were added prior to cell cycle analysis counting 10,000 cells and analysed using CELLQUEST software package.

d, J = 8.0 Hz, ArH), 7.38 (2H, d, J = 8.0 Hz, ArH). ^{13}C NMR ppm ($CDCl_3$) 32.53, 76.27, 115.68 (d J_{CF} = 11.6 Hz), 115.89 (d, J_{CF} = 11.6 Hz), 130.23, 131.54 (^3J = 9.0 Hz, ^4J = 3.5 Hz), 160.87, 163.31 (J_{CF} = 250 Hz). HRMS (ESI) calculated for $C_8H_8FNO_2$: (M^+) 169.0539: found 169.0544.

4.1.4. General procedure B: preparation of 1,3-bis(aryl)-2-nitro-1-propenes

A mixture of the appropriate 1-aryl-1-nitroethane (15.00 mmol), dimethylamine hydrochloride (30.00 mmol), potassium fluoride (2.25 mmol) and the appropriate aldehyde (15.00 mmol) in toluene (20 mL) was refluxed with a Dean–Stark trap for 24 h. The reaction was then diluted with toluene (100 mL) and washed with 10% aq HCl (3 × 30 mL). The organic phase was dried over anhydrous Mg_2SO_4 and solvent evaporated in vacuo. The product was purified by column chromatography over silica gel.

4.1.4.1. 1,3-Bis(4-chlorophenyl)-2-nitro-1-propene (4d). Compound **4d** was prepared from 1-(4-chlorophenyl)-1-nitroethane (**3d**) and 4-chlorobenzaldehyde (15 mmol, 2.11 g) according to general procedure B (Section 4.1.4) and purified by column chromatography over silica gel (eluent: 10:1 hexane/ethyl acetate) and recrystallized from methanol. Yellow crystals (50%). Mp 74–76 °C. IR ν_{max} (KBr) 1520, 1312 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 4.22 (2H, s, ArCH_2), 7.14 (2H, d, J = 8.5 Hz, ArH), 7.32, 7.37 (4H, 2d, J = 8.5 Hz, ArH), 7.43 (2H, d, J = 8.5 Hz, ArH), 8.28 (1H, s, ArCH).

^{13}C NMR ppm ($CDCl_3$) 32.37, 128.90, 129.20, 129.57, 130.87, 133.06, 134.56, 136.88, 149.46. HRMS (ESI) calculated for $C_{15}H_{10}Cl_2NO_2$: (M^-−H) 306.0089: found 306.0099.

4.1.4.2. 1-(4-Chlorophenyl)-3-(2,4-dichlorophenyl)-2-nitro-1-propene (4e). Compound **4e** was prepared from 1-(4-chlorophenyl)-1-nitroethane (**3d**) (2.69 mmol, 0.50 g) and 2,4-dichlorobenzaldehyde (**1e**) (2.69 mmol, 0.47 g) according to general procedure B. The product was purified by column chromatography over silica gel (eluent: 7:3 hexane/diethyl ether) and recrystallized from methanol. Yellow solid (20%). Mp 90–92 °C. IR ν_{max} (KBr) 1508, 1641 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 4.09 (3H, s, ArCH_2), 7.09 (2H, d, J = 8.5 Hz ArH), 7.23 (1H, d, J = 8.5 Hz, ArH), 7.30 (3H, d, J = 8.5 Hz, ArH), 7.55 (1H, d, J = 1.5 Hz, ArH), 8.32 (1H, s, ArCH). ^{13}C NMR ppm ($CDCl_3$) 31.83, 127.25, 128.57, 128.69, 129.73, 129.75, 131.21, 128.88, 132.66, 133.74, 135.18, 136.46, 150.68. HRMS (ESI) calculated for $C_{15}H_9NO_2Cl_3$: (M^+−H) 339.9699: found 339.9696.

4.1.4.3. 1-(4-Methylthiophenyl)-3-(4-chlorophenyl)-2-nitro-1-propene (4f). Compound **4f** was prepared from 1-(4-methylthiophenyl)-1-nitroethane (**3a**) (2.53 mmol, 0.50 g) and 4-chlorobenzaldehyde (2.53 mmol, 0.35 g) were reacted together according to general procedure B (Section 4.1.4). The product was purified by column chromatography over silica gel (eluent: 10:1 petroleum ether/ethyl acetate) and recrystallized from methanol. Yellow solid (55%). Mp 76–78 °C. IR ν_{max} (film) 1517, 1316 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.49 (3H, s, SCH_3), 4.22 (2H, s, ArCH_2), 7.13 (2H, d, J = 8.5 Hz, ArH), 7.23 (2H, d, J = 8.5 Hz, ArH), 7.40 (4H, dd, J = 15.0 Hz, J = 8.5 Hz, ArH), 8.26 (1H, s, ArCH). ^{13}C NMR ppm ($CDCl_3$) 15.44, 31.96, 126.74, 127.62, 129.04, 129.87, 130.48, 132.17, 133.80, 136.27, 136.85, 149.34. HRMS (ESI) calculated for $C_{16}H_{14}NO_2SCl$: 358.0071 (M^++K): found: 358.0321.

4.1.4.4. 1,3-Bis(4-bromophenyl)-2-nitro-1-propene (4g). Compound **4g** was prepared from 1-(4-bromophenyl)-1-nitroethane (**3g**) (1.62 mmol, 0.30 g) according to general procedure B (Section 4.1.4). The product was purified by column chromatography over silica gel (eluent: 6:1 dichloromethane/hexane) and recrystallized from methanol. Yellow solid (35%). Mp 91–93 °C. IR ν_{max} (film) 1520, 1325 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 4.19 (2H, s, ArCH_2), 7.07 (2H, d, J = 8.0 Hz, ArH), 7.28 (2H, d, J = 8.5 Hz, ArH), 7.46 (2H, d, J = 8.5 Hz, ArH), 7.57 (2H, d, J = 8.5 Hz, ArH), 8.25 (1H, s, ArCH). ^{13}C NMR ppm ($CDCl_3$) 32.00, 128.86, 129.85, 130.57, 131.67, 132.08, 134.23, 134.44, 148.93. HRMS (ESI) calculated for $C_{15}H_{10}Br_2NO_2$: (M^-−H) 393.9078: found 393.9076.

4.1.4.5. 1,3-Bis(4-fluorophenyl)-2-nitro-1-propene (4h). Compound **4h** was prepared from 1-(4-fluorophenyl)-1-nitroethane (**3h**) (2.99 mmol, 0.50 g) according to general procedure B (Section 4.1.4). The product was purified by column chromatography over silica gel (eluent: 6:1 dichloromethane/hexane) and recrystallized from methanol. Yellow solid (30%). Mp 73–75 °C. IR ν_{max} (film) 1519, 1333 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 4.24 (2H, s, ArCH_2) 7.04 (2H, dd, J_{HH} = 9.0 Hz, J_{HF} = 5.5 Hz, ArH), 7.13–7.19 (4H, m, ArH), 7.45 (2H, dd, J_{HH} = 9.0 Hz, J_{HF} = 5.5 Hz, ArH), 8.29 (1H, s, ArCH). ^{13}C NMR ppm ($CDCl_3$) 32.15, 115.80, 116.01, 116.41, 116.64 (J_{CF} = 22.0 Hz), 127.94, 128.62, 128.70 (^3J = 9.0 Hz, ^4J = 3.5 Hz), 131.31, 131.40 (^3J = 9 Hz, ^4J = 3.5 Hz), 131.84, 134.07, 160.70, 163.14 (J_{CF} = 253.00 Hz), 162.58, 165.10 (J_{CF} = 247.00 Hz, CF). HRMS (ESI) calculated for $C_{15}H_{10}F_2NO_2$: (M^-−H) 274.0680: found 274.0667.

4.1.4.6. 1-(4-Chlorophenyl)-3-(4-methylthiophenyl)-2-nitro-1-propene (4i). Compound **4i** was prepared from 1-(4-chlorophenyl)-1-nitroethane (**3d**) (2.69 mmol, 0.50 g) and 4-methylthiobenzaldehyde (2.69 mmol, 0.41 g) according to general procedure B (Section 4.1.4). The product was purified by column chromatography over silica gel (eluent: 7:3 hexane/diethyl ether) and recrystallized from methanol. Yellow needles (30%). Mp 115–117 °C. IR ν_{max} (KBr) 1525, 3091, 1654 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 2.53 (3H, s, SCH_3), 4.27 (2H, s, ArCH_2), 7.16 (2H, d, J = 8.5 Hz, ArH), 7.26 (2H, d, J = 8.5 Hz, ArH), 7.31 (2H, d, J = 8.5 Hz, ArH), 7.36 (2H, d, J = 8.5 Hz, ArH), 8.31 (1H, s, ArCH). ^{13}C NMR ppm ($CDCl_3$) 144.44, 32.11, 125.51, 127.31, 128.50, 128.65, 129.78, 132.43, 135.15, 142.84. HRMS (ESI) calculated for $C_{16}H_{14}NO_2SCl$: 319.0434 (M^++H): found: 319.0432.

4.1.5. General procedure C: preparation of 1,3-bis(aryl)-2-propanamines

To a solution of $LiAlH_4$ (15.00 mmol) in dry tetrahydrofuran (25 mL), the appropriate nitroalkene (5.00 mmol) in anhydrous benzene (20 mL) was added dropwise. After the addition, the mixture was heated at reflux for 3 h. The excess $LiAlH_4$ was quenched by addition of 1–2 mL of water, and the mixture filtered through Celite. The organic filtrate was extracted using 2 N HCl (3 × 30 mL), and the combined aqueous extracts were basified with 2 M NaOH. The free base was then extracted with ethyl acetate, dried over anhydrous Mg_2SO_4 and the solvent evaporated in vacuo. No further purification was required.

4.1.5.1. 1,3-Bis(4-methoxyphenyl)-2-propanamine (5c). Compound **5c** was prepared from 1,3-bis(4-methoxyphenyl)-2-nitro-1-propene (**4c**) (5.00 mmol, 1.49 g) according to general procedure C (Section 4.1.5). Pale yellow oil (66%). IR ν_{max} (film) 3341, 1179 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 1.40 (2H, br s, NH_2), 2.47 (2H, dd, J = 9.0 Hz, J_{gem} = 13.5 Hz, ArCH_2), 2.78 (2H, dd, J = 4.5 Hz, J_{gem} = 13.3 Hz, ArCH_2), 3.20 (1H, m, NH_2CH), 3.81 (6H, s, OCH_2), 6.87 (4H, d, J = 8.5 Hz, ArH), 7.15 (4H, d, J = 8.5 Hz, ArH). ^{13}C NMR ppm ($CDCl_3$) 42.50, 53.93, 54.81, 113.42, 129.54, 129.74, 130.99, 157.66. HRMS (ESI) calculated for $C_{17}H_{22}NO_2$: 271.1572 (M^++H): found 272.1649.

4.1.5.2. 1,3-Bis(4-chlorophenyl)-2-propanamine (5d). Compound **5d** was prepared from 1-(4-chlorophenyl)-1-nitroethane (**4d**) (5.00 mmol, 1.40 g) according to general procedure C (Section 4.1.5). Pale resin (30%). IR ν_{max} (film) 3307, 810 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 1.32 (2H, br s, NH_2), 2.55 (2H, dd, J = 8.5 Hz, J_{gem} = 13.5 Hz, ArCH_2), 2.82 (2H, dd, J = 4.5 Hz, J_{gem} = 13.5 Hz, ArCH_2), 3.24 (1H, m, NH_2CH), 7.15 (4H, d, J = 8.0 Hz, ArH), 7.30 (4H, d, J = 8.0 Hz, ArH). ^{13}C NMR ppm ($CDCl_3$) 42.97, 53.54, 128.21, 130.13, 131.77, 137.16. HRMS (ESI) calculated for $C_{15}H_{16}NCl_2$: (M^++H) 280.0660: found 280.0668.

4.1.6. 4-(3,4-Methylenedioxybenzyl)-5-(3,4-methylenedioxyphenyl)pyrimidine (14b)

Compound 14b was isolated as a byproduct from the preparation of 2-*N*-formylamino-1,3-bis(3,4-methylenedioxyphenyl)propane (**7b**) from 1,3-bis(3,4-methylenedioxyphenyl)-2-propanone (**6b**) by column chromatography over silica gel (eluent: 80:20 diethylether/hexane). Amber crystals (20%). Mp 88–90 °C. IR ν_{max} (KBr) 2779, 1609 cm^{-1}. ^{1}H NMR δ ($CDCl_3$) 4.04 (2H, s, CH_2), 5.90 (2H, s, OCH_2O), 6.05 (2H, s, OCH_2O), 6.50 (1H, dd, J = 8.3 Hz, J = 1.3 Hz, ArH), 6.61 (1H, d, J = 1.5 Hz, ArH), 6.68 (1H, d, J = 8.0 Hz, ArH), 6.71 (1H, dd, J = 7.8 Hz, J = 1.8 Hz, ArH), 6.73 (1H, d, J = 1.5 Hz, ArH), 6.90 (1H, d, J = 8.0 Hz, ArH), 8.54 (1H, s, NCHC), 9.13 (1H, s, NCHN). ^{13}C NMR ppm ($CDCl_3$) 40.17, 100.42, 100.98, 107.72, 108.13, 108.93, 109.15, 121.47, 122.53, 128.71, 131.20, 134.21, 145.78, 147.21, 147.44, 147.55, 156.70, 156.98, 165.69. LRMS: m/z 334 (M^+). Anal. Calcd for $C_{19}H_{14}N_2O_4$: C, 68.26; H, 4.22; N, 8.38. Found: C, 68.02; H, 4.31; N, 8.16.

4.1.7. 4-(4-Methylthiobenzyl)-5-(4-methylthiophenyl)pyrimidine (14a)

Compound **14a** was isolated as an impurity from the preparation of 2-*N*-formylamino-1,3-bis(3,4-methylenedioxyphenyl)propane (**7a**) from 1,3-bis(3,4-methylenedioxyphenyl)-2-propanone (**6a**) by column chromatography over silica gel (eluent: 90:10 diethyl ether/hexane). Amber solid (5%). Mp 111–112 °C (diethyl ether/hexane). IR ν_{max} (KBr) 1653, 1595 cm^{-1}. ^1H NMR δ ($CDCl_3$) 2.43 (3H, s, SC*H*$_3$), 2.54 (3H, s, SC*H*$_3$), 4.06 (2H, s, C*H*$_2$), 6.98, 7.12, 7.15, 7.32 (8H, 4d, *J* = 8.0 Hz, *J* = 8.0 Hz, *J* = 8.5 Hz, *J* = 8.6 Hz, Ar*H*), 8.54 (1H, s, NC*H*C), 9.13 (1H, s, NC*H*N). ^{13}C NMR ppm ($CDCl_3$) 15.49, 15.98, 40.49, 126.36, 126.90, 129.33, 129.54, 131.94, 134.50, 134.84, 136.53, 139.54, 157.09, 157.50, 165.83. LRMS: *m/z* 338 (M^+). Anal. Calcd for $C_{19}H_{18}NS_2$: C, 67.42; H, 5.36; N, 8.28; S, 18.95. Found: C, 67.13; H, 5.38; N, 8.08; S, 18.89.

4.1.8. 2-*N*-Formylamino-1,3-bis(3,4-methoxyphenyl)propane (7c)

1,3-(3,4-Methyoxyphenyl)-2-propanone (**6c**) (5.03 mmol, 1.51 g) and formamide (3.00 g) were heated at 190 °C for 5 h. After cooling the reaction was diluted with water (50 mL) and extracted with ethyl acetate (3 × 25 mL). The extracts were combined, washed with water (3 × 25 mL) and dried over anhydrous Na_2SO_4. Volatiles were removed in vacuo and the residue was purified by column chromatography over silica gel (eluent: diethylether). Colourless crystals (30%). Mp 134–135 °C. IR ν_{max} (KBr) 3341, 1660 cm^{-1}. ^1H NMR δ ($CDCl_3$) 2.59 (2H, m, ArC*H*$_2$), 2.75 (2H, m, ArC*H*$_2$), 3.58 (0.3H, m, NHC*H*), 3.75 (6H, s, OC*H*$_3$), 4.38 (0.7H, dd, *J* = 14.6 Hz, *J* = 7.0 Hz, NHC*H*), 5.70 (0.72H, br s, *NH*), 6.00 (0.27, br s, *NH*), 6.80 (4H, d, *J* = 8.5 Hz, Ar*H*), 7.03, 7.07 (4H, 2 d, *J* = 8.5 Hz, *J* = 8.5 Hz, Ar*H*), 7.37 (0.27H, d, *J* = 11.5 Hz, C*H*O), 7.96 (0.72H, s, C*H*O). ^{13}C NMR ppm ($CDCl_3$) 38.80, 41.12*, 50.41, 55.07, 56.27*, 113.79, 114.01*, 129.24, 129.65*, 130.12, 130.24*, 158.18, 158.35*, 160.61, 163.91. HRMS (ESI) calculated for $C_{18}H_{21}O_3Na$: (M^++Na) 322.1419: found 322.1424.

4.1.9. 1,3-Bis(3,4-methylenedioxy)-2-propanone oxime (8b)

1,3-Bis(3,4-methylenedioxyphenyl)-2-nitro-1-propene (**4b**) (1.00 mmol, 0.31 g), hydroxylamine HCl (2.36 mmol, 0.16 g), pyridine (3 mL) and ethanol (5 mL) were refluxed for 2 h. After cooling, the reaction was acidified using 10% aq HCl and extracted with dichloromethane (3 × 20 mL). The organic phases were combined, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuo. No further purification was required. Dark brown crystals (83%). Mp 106–107 °C. IR ν_{max} (KBr) 3251 cm^{-1}. ^1H NMR δ ($CDCl_3$) 3.37 (2H, s, ArC*H*$_2$), 3.58 (2H, s, ArC*H*$_2$), 5.95 (4H, s, OC*H*$_2$O), 6.63–6.77 (6H, m, Ar*H*), 8.60 (1H, br s, OH). ^{13}C NMR ppm ($CDCl_3$) 31.81, 38.93, 101.15, 108.47, 109.73, 109.94, 122.53, 130.36, 130.45, 146.64, 146.90, 148.25, 159.83. HRMS (ESI) calculated for $C_{17}H_{16}NO_5$: (M^++H) 314.1028: found 314.1032.

4.1.10. 2-(3,4-Methylenedioxybenzyl)-3-(3,4-methylenedioxyphenyl)aziridine (15b)

Compound **15b** was isolated as a byproduct from the preparation of 1,3-bis(3,4-methylenedioxyphenyl)-2-propanamine (**5b**) from 1,3-bis(3,4-methylenedioxy)-2-propanone oxime (**8b**) by column chromatography over silica gel (eluent: 90:10 diethylether/methanol). Colourless needles (62%). Mp 78–79 °C. IR ν_{max} (KBr) 3166, 2770 cm^{-1}. ^1H NMR δ ($CDCl_3$) 1.08 (1H, s, N*H*), 2.36–2.43 (3H, m, NHC*H*CH$_2$, C*H*$_2$), 3.26 (1H, d, *J* = 6.0 Hz, ArC*H*NH), 5.88 (2H, m, OC*H*$_2$O), 5.94 (2H, s, OC*H*$_2$O), 6.52 (1H, dd, *J* = 8.0 Hz, *J* = 1.5 Hz, Ar*H*), 6.60 (1H, d, *J* = 1.5 Hz, Ar*H*), 6.68 (1H, d, *J* = 8.0 Hz, Ar*H*), 6.78 (1H, d, *J* = 8.0 Hz, Ar*H*), 6.86 (1H, d, *J* = 8.0 Hz, Ar*H*), 6.90 (1H, s, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 33.93, 36.83, 38.61, 100.68, 100.85, 107.89, 108.05, 108.23, 109.18, 120.91, 121.42, 131.46, 133.56, 145.79, 146.44, 147.43, 147.46. LRMS: *m/z* 297 (M^+), 135. Anal. Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.62; H, 5.04; N, 4.56.

4.1.11. 1,3-Bis(3,4-methylenedioxy)-2-propanol (9b)

1,3-Bis(3,4-methylenedioxyphenyl)-2-propanone (**6b**) (0.67 mmol, 0.20 g) was stirred in dry MeOH (20 mL) and dichloromethane (5 mL) while $NaBH_4$ (2.74 mmol, 1.04 g) was added slowly. The reaction mixture was stirred for 2 h, checking progress by TLC. When the reaction was complete, the solvent was evaporated in vacuo. The residue was dissolved in dichloromethane and washed with water (3 × 20 mL). The solvent was evaporated in vacuo. No further purification was required. Colourless solid (90%). Mp 141–144 °C. IR ν_{max} (KBr) 3397 cm^{-1}. ^1H NMR δ ($CDCl_3$) 1.75 (1H, br s, O*H*), 2.63 (2H, dd, *J* = 4.0 Hz, *J* = 14.0 Hz, ArC*H*$_2$), 2.75 (2H, dd, *J* = 8.0 Hz, *J* = 14.0 Hz, ArC*H*$_2$), 3.95 (1H, m, C*H*OH), 5.95 (2H, s, OC*H*$_2$O), 6.68 (2H, dd, *J* = 8.0 Hz, *J* = 1.5 Hz, Ar*H*), 6.74 (2H, d, *J* = 1.5 Hz, Ar*H*), 6.76 (2H, d, *J* = 8.0 Hz, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 42.49, 73.23, 100.45, 107.85, 109.24, 121.84, 131.65, 145.74, 147.29. HRMS (ESI) calculated for $C_{17}H_{16}O_5Na$: (M+Na) 323.0895: found 323.0899

4.1.12. 2-*N*-Acetylamino-1,3-bis(3,4-methylenedioxyphenyl)-propane (10b)

To a solution of 1,3-bis(3,4-methylenedioxyphenyl)-2-propanamine (5b) (2.92 mmol, 0.87 g) in pyridine (6 mL), was added acetic anhydride (5.84 mmol, 0.59 g) and the mixture stirred at room temperature for 1 h. The reaction was acidified with 10% aq HCl (100 mL) and extracted with dichloromethane (3 × 25 mL). The extracts were combined, dried over anhydrous Na_2SO_4 and volatiles removed in vacuo, generating an amber oil which slowly solidified. Recrystallisation from methanol provided the pure product. Colourless solid (65%). Mp 133–134 °C. IR ν_{max} (KBr) 3288, 2789, 1643 cm^{-1}. ^1H NMR δ ($CDCl_3$) 1.87 (3H, s, CO*CH*$_3$), 2.66 (2H, dd, *J* = 14.1 Hz, *J* = 7.0 Hz, ArC*H*$_2$), 2.74 (2H, dd, *J* = 14.0 Hz, *J* = 6.0 Hz, ArC*H*$_2$), 4.31 (1H, m, NHC*H*), 5.17 (1H, d, *J* = 7.5 Hz, N*H*), 5.93 (4H, s, OC*H*$_2$O), 6.61 (2H, dd, *J* = 7.8 Hz, *J* = 1.5 Hz, Ar*H*), 6.67 (2H, d, H = 1.5 Hz, Ar*H*), 6.73 (2H, d, *J* = 7.5 Hz, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 22.97, 39.01, 51.06, 100.43, 107.74, 109.13, 121.75, 131.14, 145.77, 147.28, 169.00. LRMS: *m/z* 341 (M^++H) 282. Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.56; H, 5.70; N, 4.07.

4.1.13. 2-*N*-Ethylamino-1,3-bis(3,4-methylenedioxyphenyl)-propane (11b)

Compound **11b** was prepared from 2-*N*-acetylamino-1,3-di(3,4-methylenedioxyphenyl)propane (**10b**) (7.50 mmol, 2.56 g) according to general procedure C and purified by column chromatography over silica gel (eluent: methanol). Pale amber oil (33%). IR ν_{max} (film) 3283 cm^{-1}. ^1H NMR δ ($CDCl_3$) 1.02 (3H, t, *J* = 7.0 Hz, NCH$_2$C*H*$_3$), 2.57–2.68 (6H, m, NC*H*$_2$CH$_3$, ArC*H*$_2$), 2.93 (1H, m, NHC*H*), 3.73 (1H, br s, *NH*), 5.90 (4H, s, OC*H*$_2$O), 6.61 (2H, dd, *J* = 7.8 Hz, *J* = 2.0 Hz, Ar*H*), 6.65 (2H, d, H = 2.0 Hz, Ar*H*), 6.72 (2H, d, *J* = 8.0 Hz, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 15.14, 40.06, 41.46, 61.17, 100.75, 108.12, 109.37, 122.11, 130.19, 145.93, 147.64. LRMS: *m/z* 327 (M^++H) 192. HCl salt. Colourless solid (90%). Mp 164–167 °C. IR ν_{max} (KBr) 2481 cm^{-1}. Anal. Calcd for $C_{19}H_{22}ClNO_4$: C, 62.72; H, 6.09; N, 3.85. Found: C, 62.89; H, 6.11; N, 4.04.

4.1.14. 2-(*N*-Formyl-*N*-methyl)amino-1,3-bis(3,4-methylenedioxyphenyl)propane (12b)

A solution of 1,3-bis(3,4-methylenedioxyphenyl)-2-propanone (**6b**) (2.51 mmol, 0.75 g) in *N*-methylformamide (6.44 mmol, 0.38 g) and 96% formic acid (0.18 g) was stirred and heated at reflux at 150 °C for 7 h. After cooling the reaction was diluted with water (25 mL) and extracted with dichloromethane (3 × 25 mL).

The extracts were combined, washed with water (2 × 25 mL) and satd aq $NaHCO_3$ (2 × 25 mL), followed by drying over anhydrous Na_2SO_4. Volatiles were removed in vacuo and resulting residue was purified by flash chromatography over silica gel (eluent: 7:3 hexane/diethyl ether). Amber solid (58%). Mp 118–120 °C. IR ν_{max} (KBr) 2781, 1661 cm^{-1}. ^1H NMR δ ($CDCl_3$) 2.67 (0.6H, s, NCH_3), 2.79 (2.4H, s, NCH_3), 2.73–2.84 (4H, m, ArCH_2), 3.70 (0.8H, m, NC*H*), 4.63 (0.2H, m, NC*H*), 5.90 (0.6H, s, OCH_2O), 5.91 (2.4H, s, OCH_2O), 6.55 (1.6H, dd, *J* = 8.0 Hz, *J* = 1.5 Hz, Ar*H*), 6.58 (1.6H, d, *J* = 1.5 Hz, Ar*H*), 6.65 (0.4H, dd, *J* = 7.8 Hz, *J* = 1.8 Hz, Ar*H*), 6.68–6.75 (2.4H, m, Ar*H*), 7.55 (0.8H, s, *CH*O), 7.89 (0.2H, s, *CH*O). ^{13}C NMR ppm ($CDCl_3$) 24.86*, 36.77, 37.95*, 62.31*, 100.40, 100.54*, 107.74, 108.02*, 108.43*, 108.75, 121.31*, 130.77*, 131.30, 145.71, 146.00*, 147.24, 147.50*, 162.50*, 162.58 (CHO). LRMS: *m/z* 341 (M^+), 282. Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.78; H, 5.66; N, 4.02.

4.1.15. 2-*N*-Methylamino-1,3-bis(3,4-methylenedioxyphenyl)-propane (13b)

To a stirred solution of 2-(*N*-formyl-*N*-methyl)amino-1,3-bis-(3,4-methylenedioxyphenyl)propane (**12b**) (0.73 mmol, 0.25 g) in methanol (25 mL), was added concd HCl (2.5 mL) and the mixture heated at reflux for 7 h. After cooling the reaction was diluted with water (100 mL) and washed with dichloromethane (3 × 30 mL). The aqueous phase was made basic with 15% aq NaOH and extracted with dichloromethane (3 × 25 mL). The organic phases were combined, dried over anhydrous Na_2SO_4 and solvent removed in vacuo. The product was purified by column chromatography over silica gel (eluent: 60/40 diethyl ether/hexane). Pale amber oil (84%). IR ν_{max} (film) 3336, 2791 cm^{-1}. ^1H NMR δ ($CDCl_3$) 1.72 (1H, br s, N*H*), 2.39 (3H, s, *CH*$_3$), 2.57 (2H, dd, *J* = 13.8 Hz, *J* = 6.3 Hz, ArCH_2), 2.64 (2H, dd, *J* = 13.8 Hz, *J* = 6.8 Hz, ArCH_2), 2.79 (1H, m, NC*H*), 5.92 (4H, s, OCH_2O), 6.62 (2H, dd, *J* = 7.5 Hz, *J* = 1.5 Hz, Ar*H*), 6.66 (2H, d, H = 1.5 Hz, Ar*H*), 6.73 (1H, d, *J* = 8.0 Hz, Ar*H*). ^{13}C NMR ppm ($CDCl_3$) 34.09, 39.78, 62.94, 100.80, 108.16, 109.46, 122.17, 133.06, 145.95, 147.67. LRMS: *m/z* 313 (M^-−1) 178. HRMS calculated for $C_{18}H_{20}NO_4$: (M^++H) 314.1392: found: 314.1374.

4.2. Computational studies

To date no crystal structure of hSERT exists to avail of in the docking process. A recent homology model of hSERT was constructed using LeuT as a template by Jørgensen et al.[33] Previous investigation of the flexibility of the binding site in complex with the natural substrate (5-HT) determined key protein–ligand interactions.[13] The optimal 5-HT bound SERT complex was utilised as our starting point for the series of docking studies. For ligand preparation, all compounds were built and minimised using MOE v2009.10.[49] Docking was carried out using Molegro Virtual Docker (www.molegro.com), which enables both ligand and receptor flexibility to be accounted for in the docking process. All side-chains within 5Å of bound 5-HT were chosen as flexible and a search space with grid radius of 15 Å was created. No template was used in the docking process to prevent any bias, and all other parameters were retained as default. The highest ranking solutions ranked by MolDock Score with appropriate H-bonding interactions were then selected for analysis using MOE v2009.10.

4.3. Biochemistry

4.3.1. Materials

DG-75 and MUTU-I (c179) BL cell lines were gifts from Dr. Dermot Walls (School of Biotechnology, Dublin City University, Ireland) and Professor Martin Rowe (Division of Cancer Studies, The University of Birmingham, UK) respectively. The SHSY-5Y, MCF-7, MDA-MB231 and 4TI cell lines were purchased from the European Collection of Cell Cultures (ECACC). The HEK293 cell line stably overexpressing the human SERT was a gift from Dr. Patrick Schloss (Central Institute for Mental Health, Mannheim, Germany). RPMI-1640, DMEM, FBS, HEPES, sodium pyruvate, gentamycin (G418) and glutamine were from Gibco (Invitrogen. Biosciences Ltd, Ireland). Alamar Blue and LymphoPrep were from Biosciences Ltd. The neurotransmitter transporter uptake assay was purchased from Molecular Devises Inc, the Apo-ONE® homogenous caspase 3/7 assay kit was from Promega and the protein tyrosine phosphatase 1B activity kit was purchased Calbiochem. All other chemicals were purchased through Sigma–Aldrich Inc., Ireland.

4.3.2. Cell culture

The DG-75 cell line is a B-lymphocyte, Burkitt's lymphoma line derived from a metastatic pleural effusion (lung) of a sporadic case of Burkitt's lymphoma.[24] The MUTU-I (c179) cell line is an isogenic stable group I BL cell line derived from a BL biopsy.[24] The above cell lines were cultured in RPMI-1640 medium containing phenol red and supplemented with 10% (v/v) foetal bovine serum (FBS), L-glutamine (2 mM), penicillin and streptomycin (100 μg/mL). The MUTU-I c179 cell line required the additional supplements of alpha-thioglycerol (5 mM in phosphate buffered saline (PBS) with 20 μM bathocuprione disulfonic acid), sodium pyruvate (100 mM) and HEPES (1 mM). The SHSY-5Y cell line SH-SY5Y is a thrice cloned (SK-N-SH→SH-SY→SH-SY5→SH-SY5Y) subline of the human neuroblastoma cell line SK-N-SH established from a metastatic bone tumour.[12] These were cultured in DMEM: F12 (1:1) supplemented with 10% (v/v) (FBS), L-glutamine (2 mM), penicillin and streptomycin (100 μg/mL).

HEK293 cells lines stably overexpressing human SERT was cultured in DMEM supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin/streptomycin (100 mg/mL) and Geneticin (500 mg/mL). Stable expression was valid up to 30 passages.

The MDA-MB231 cell line is an estrogen receptor (ER) negative breast adenocarcinoma derived from a metastatic pleural effusion.[50] MDA-MB231 cells were cultured in DMEM, 10% (v/v) FBS, L-glutamine (2 mM) with penicillin and streptomycin (100 μg/mL). The MCF-7 cell line is an ER positive breast adenocarcinoma derived from a metastatic pleural effusion.[51] MCF-7 cells were cultured in MEM supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin and streptomycin (100 μg/mL) and 1% (v/v) non-essential amino acids. The 4TI is a cell line derived from a mouse mammary gland tumour[52] that is commonly xenografted to create disease models for breast carcinomas. 4TI's were cultured in DMEM supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin and streptomycin (100 μg/mL) and HEPES (1 mM). Cells were maintained in a 72 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 95% oxygen, and 5% carbon dioxide.

4.3.3. Generation of human peripheral blood mononuclear cells

Blood was obtained from a healthy donor, transferred into a 50 mL falcon tube and diluted 1:2 with PBS. LymphoPrep was used to separate the blood into red blood cells, white blood cell ring and serum. The blood was slowly added to 20 mL of ficoll pague plus. The tubes were centrifuged at 1700*g* for 30 min. The white blood cell ring was transferred into a new 50 mL tube. The volume was adjusted to 50 mL and the samples were centrifuged again at 1700*g* for 10 min. The supernatant was removed. This step was repeated again, the pellet was then resuspended in 10 mL of complete IMDM media (10% FBS, 0.1% Ciprofloxacin (10 mg/mL)).

4.3.4. Neurotransmitter transporter uptake assay

A novel fluorescence neurotransmitter transporter uptake assay which actively transports a biogenic amine mimic into the cell through the transporters SERT, NAT and DAT was used. The mi-

metic fluoresces once inside the cell and any external fluorescence is extinguished by a membrane impermeable masking dye. This allows the fluorescence of the cell to be measured and the SERT inhibition of the compound determined.[26]

Cells were seeded at a density of 2×10^4/well in black, clear bottomed, poly-L-lysine coated plates. After 24 h, the media was aspirated and cells were washed with 100 μl of 1× HBSS. Cells were incubated for 15–20 min with 100 μl of compound dissolved in 1× HBSS (Hanks balanced salt solution) + 0.1% BSA (bovine serum albumin), before 100 μl of the fluorescent dye was added. The fluorescence of each plate was read at 520 nm (excitation 440 nm) on a bottom up plate reader. The background fluorescence of the cells and 1× HBSS buffer was taken away from each group. Untreated cells represented 100% fluorescence/0% inhibition. Citalopram (10 μM) was used as a positive control resulting in >98% inhibition. Fluorescence for each compound was calculated as a percent of untreated cell response.

4.3.5. Neutral red assay for in vitro cytotoxicity

The Neutral red (NR) assay was carried out as previously described.[53] Briefly, 5×10^4 cells per well (200 μl) were seeded in a 96-well plate until sub-confluent (24–36 h) and treated with the appropriate compound for 48 h. Following exposure of cells to drug the supernatant was removed and the cells incubated for 3 ± 1 h with 250 μl neutral red dye solution (stock 3.3 mg/mL-prewarmed to 37 °C in media) under sterile conditions. NR solution was removed carefully and the cells washed with 200 μl of prewarmed PBS. Upon removal of all the PBS 100 μl of NR assay Solubilisation solution (50% ethanol–1% acetic acid solution in deionized water) was added to each well, the plate left to incubate in the dark for 20–30 min at room temperature with gentle shaking. The absorbance of each plate was read at 540 nm and at 690 nm (background) within 1 h. Relative cell viability was expressed as percent of vehicle treated cells. Sodium azide and Triton-X were used as positive controls for cytotoxicity, where 30 mM sodium azide and 2% Triton-X resulted in 80–90% cytotoxicity on all cell lines. Untreated cells represented 0% cytotoxicity (100% viability).

4.3.6. Alamar Blue assay for antiproliferative activity

1–5 × 10^4 cells/well were seeded in a 96-well plate and treated with the respective drug for the desired length of time. Each well was then treated with 20 μl of Alamar Blue (pre-warmed to 37 °C) and left to incubate at 37 °C in the dark for 4–6 h. Fluorescence was read using at 590 nm (excitation 544 nm). The background fluorescence of the media without cells + Alamar Blue was taken away from each group, and the control untreated cells represented 100% cell viability. The antifungal agent miconazole (10 μM) was used as a positive control for cell death in each of the cell lines, resulting in 90% cytotoxicity.

4.3.7. Statistical analysis

4.3.7.1. Non-linear regression analysis. Each compound was screened over a 1 μM–1 mM concentration range in triplicate on two independent days with activity expressed as percentage cell viability compared to vehicle treated controls. The cytotoxic potency of each compound was quantified by a pEC_{50} value determined by non-linear regression analysis of sigmoidal log concentration dependence curves whereby pEC_{50} is $-[-\log EC_{50}] \pm SE$ ($\log EC_{50}$ is the log [Dose] when response is equal to 50% cell viability) (Table 1). All data points (expressed as means ± SEM) were analysed using GRAPHPAD Prism (version 4) software (Graphpad software Inc., San Diego, CA).

4.3.7.2. Comparison of pEC_{50} values in all cell lines. To determine if the pEC_{50} value calculated for each drug differed significantly in each cell line, statistical analysis was carried out using a one way ANOVA Test comparing each pEC_{50} value. The means for different treatment groups were then compared using a two-way ANOVA test with no matching followed by a Bonferroni Post Test to compare replicate means by row to the control cell line HEK293. *P* values of <0.05 were considered to reflect a significant difference.

4.3.8. Quantification of apoptosis

4.3.8.1. Propidium iodide FACS analysis. 7.5×10^5 cells/5mL were treated with the appropriate amount of compound and incubated for a specified time. Cells were harvested by centrifugation at 300*g* for 5 min and washed with 5 mL of ice-cold PBS. The pellet was resuspended in 200 μl PBS and 2 mL of ice-cold 70% ethanol and cells were fixed overnight at 4 °C. After fixation, the cells were pelleted by centrifugation at 300*g* for 5 min and the ethanol was carefully removed. The pellet was resuspended in 400 μl of PBS with 25 μl of RNAse A (10 mg/mL stock) and 75 μl of propidium iodide (1 mg/mL). The tubes were incubated in the dark at 37 °C for 30 min. Cell cycle analysis was performed using appropriate gates counting 10,000 cells and analysed using CELLQUEST software package. Untreated cells had <5% cells in the pre-G1 phase of the cell cycle and 10 μM Taxol was used as a positive control for cell death.

4.3.8.2. Caspase 3/7 activity. Caspase 3/7 activity was assessed using the Apo-ONE® homogenous caspase 3/7 assay. Briefly, 0.5×10^5 cells were treated with compound (10 μM) for 5 h before the addition of 100 μl caspase substrate Z-DEVD-R110. After 2 h the activity of caspase 3/7 was quantified by measuring the amount of cleaved substrate at 521 nm (excitation 499 nm).

4.3.8.3. Detection of PARP cleavage by Western blot analysis. 5×10^6 cells were harvested by centrifugation at 500*g* for 5 min and the pellet washed with ice-cold PBS. Cells were resuspended in 60 μl PBS and 60 μl lysis buffer (Laemmli buffer; 62.5 mM Tris–HCl, 2% w/v SDS, 10% glycerol, 0.1% w/v bromophenol blue supplemented with protease inhibitors). Samples were prepared for SDS–PAGE resolved on a 8% loading gel and transferred onto PVDF membranes. Membranes were probed with anti-PARP (recognises full length 113 kDa PARP as well as the 85 kDa cleaved form) primary antibody followed by incubation with the corresponding IgG HRP conjugated secondary antibody. Membranes were developed using electrochemiluminescence detection.

4.3.8.4. Staining and visualisation of cells to assess programmed cell death morphology. Cells were seeded at a density of 3×10^5 cells/mL treated with the indicated compounds for the relevant time. An aliquot of cells (150 μl) was cytocentrifuged onto microscopy slides at 500*g* for 2 min using a Cytospin 3 (Shandon). The slides were removed and left to air-dry at room temperature for 2 min. Staining was carried out using the RapiDiff kit (Sigma–Aldrich) containing solution A (100% methanol), solution B (Eosin Y) and solution C (methylene blue). The cells were fixed by dipping them 10 times in solution A. The nucleus of the cells was stained pink by dipping the slide ten times in solution B and the cytoplasm stained blue by dipping eight times in solution C. Excess dye was washed off with deionized water. After allowing the slides to air dry cells were examined under a light microscope using a 60× magnification

4.3.9. Assessment of PTP1B inhibition

The ability of compounds to inhibit protein tyrosine phosphatise 1B activity was assessed using a PTP1B Assay Kit (Calbiochem). Briefly, recombinant PTP1B expressed in *Escherichia coli* was incubated with the test compound and a phosphopeptide substrate for 30 min at 30 °C. The detection of free phosphate released is de-

tected using the Malachite green assay with the absorbance read at 620 nm, quantifying the amount of phosphate released with a phosphate standard curve. The PTP1B inhibitor, Suramin was used as a positive control for PTP1B inhibition.

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