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Identification of dual DNA-PK MDR1 inhibitors for the potentiation of cytotoxic drug activity

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ABSTRACT

Inhibition of DNA repair is an attractive therapeutic approach to enhancing the activity of DNA-damaging anticancer chemotherapeutic agents. Similarly, blockade of the multidrug-resistance protein 1 (MDR1) can overcome efflux-mediated resistance. DNA-dependent protein kinase (DNA-PK) is essential for the non-homologous end-joining DNA repair pathway. NU7441 is a potent DNA-PK inhibitor ($IC_{50} = 14$ nM) that is used widely to study the effects of DNA-PK inhibition *in vitro*. In growth inhibition studies, 1 μ M NU7441 sensitised vincristine-resistant CCRF-CEM VCR/R leukaemia cells (1200-fold resistant) to a range of MDR1 substrates, including doxorubicin (8-fold, $p=0.03$), vincristine (14-fold, $p=0.01$) and etoposide (63-fold, $p=0.02$), compared with 1.4-fold ($p=0.02$), 2.2-fold ($p=0.04$) and 3.6-fold ($p=0.01$) sensitisation, respectively, in parental CCRF-CEM cells. A doxorubicin fluorescence assay showed that in MDR1-overexpressing canine kidney MDCKII-MDR1 cells, 1 μ M NU7441 increased doxorubicin nuclear fluorescence 16-fold. NU7441 and 3 structurally-related compounds (NU7742 (an NU7441 analogue that does not inhibit DNA-PK - $IC_{50}>10$ μ M), DRN1 (DNA-PK-inhibitory atropisomeric NU7441 derivative - $IC_{50}=2$ nM) and DRN2 (DNA-PK non-inhibitory atropisomeric NU7441 derivative - $IC_{50}=7$ μ M)) all increased intracellular vincristine accumulation in the CCRF-CEM VCR/R cells to a level similar to verapamil, as measured by LC-MS. However, the non-inhibitory compound NU7742 was 5-fold less effective at sensitising CCRF-CEM VCR/R cells to vincristine than NU7441 ($p=0.008$). This paper demonstrates that NU7441 is a dual DNA-PK and MDR1 inhibitor, and this extends the therapeutic potential of the compound when used in combination with MDR substrates.

KEYWORDS: DNA-PK, MDR1, NU7441, cancer, multidrug resistance

1. INTRODUCTION

DNA is the main target of many conventional anticancer agents and therefore the inhibition of DNA repair is an attractive approach to chemosensitisation. DNA double-strand breaks are the most lethal type of DNA damage and therefore two major pathways have evolved to repair these DNA lesions; homologous recombination (HR) and non-homologous end-joining (NHEJ). DNA-PK is a serine/threonine protein kinase and is a member of the phosphatidylinositol 3-kinase like kinase (PIKK) family. DNA-PK is essential for the NHEJ repair pathway [1], and has also been implicated in HR [2], H2AX phosphorylation [3], metabolic gene regulation [4], and in the inflammatory response through interactions with NF κ B [5].

NU7441 (8-dibenzothiophen-4-yl-2-morpholin-4-yl-chromen-4-one, Fig. 1(a)) is a potent ATP-competitive DNA-PK inhibitor (IC_{50} = 14 nM) developed from the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 [6]. NU7441 has been shown to increase sensitivity to radiation and topoisomerase II poisons, and increase etoposide-induced tumour growth delay in mice bearing SW620 xenografts [7].

NU7441 is around 20-fold more selective for cellular DNA-PK over PI3K inhibition [8]. NU7742 (8-dibenzothiophen-4-yl-2-piperidin-1-yl-chromen-4-one, Fig. 2(a)) is an inactive derivative of NU7441 (IC_{50} > 10 μ M) in which the morpholine oxygen has been replaced with a methylene group, resulting in the loss of sensitisation of cells to DNA-damaging agents [9]. The introduction of a methyl substituent at the 7-position of the chromen-4-one ring generated atropisomers DRN1 (DNA-PK IC_{50} = 2 nM) and DRN2 (DNA-PK IC_{50} = 7 μ M) (Fig. 1(c)). As expected and due to restricted rotation between the chromen-4-one and dibenzothiophene rings, the DNA-PK inhibitory activity resides exclusively in the laevorotatory enantiomer DRN1 [10].

MDR1/P-glycoprotein (the product of the *ABCB1/MDR1* gene) was the first member of the (ATP)-binding cassette (ABC) superfamily of transmembrane transporters to be cloned, and is known to play a significant role in the multidrug-resistance (MDR) phenotype in cancer cells [11]. MDR1 is a 170 kDa protein located in the apical membrane of many cell types which acts as a drug efflux transporter in the liver, kidneys, gastrointestinal tract, and multiple blood barriers including the blood-brain and the blood-placental barriers [12, 13]. MDR1 is known to play a role in conferring resistance to a number of anticancer agents, including the vinca alkaloids, taxanes and anthracyclines [14].

Verapamil, a calcium channel blocker used clinically as a coronary vasodilator, was amongst the first compounds identified that could reverse MDR and potentiate the effects of MDR1 substrates such as vincristine [15, 16]. Verapamil, along with a number of other MDR1 blockers, have proved largely unsuccessful in clinical trials due to toxicity or side effects [17].

Recently, there have been a number of publications reporting that targeted anticancer therapies also reverse MDR by inhibiting the efflux functions of the ABC family of transporters, including ABCB1 (MDR1). Thus the kinase inhibitors imatinib and nilotinib (BCR-Abl inhibitors) [18], gefitinib, erlotinib and lapatinib (EGFR inhibitors) [19-21], apatinib (VEGFR2 inhibitor) [22], and sorafenib (Raf-targeting drug) [23] have all been shown to be potent inhibitors of ABCB1.

Following observations that the DNA-PK inhibitor NU7441 caused greater sensitisation to cytotoxic agents in MDR1-overexpressing versus non-overexpressing cells, this study aimed to investigate whether NU7441 was interacting with drug efflux *via* MDR1.

2. MATERIAL AND METHODS

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich (UK) unless stated otherwise and all solvents and solutions for HPLC were obtained from Fisher Scientific (UK). DNA-PK inhibitors were provided by the NICR Medicinal Chemistry group and synthesised as previously described [9, 10]. NU7441 and NU7742 were dissolved at 2 mM in DMSO. DRN1 and DRN2 were dissolved at 1.5 mM in DMSO. The structures of NU7441, NU7742, DRN1 and DRN2 are shown in Figure 1. All solutions were stored at -20 °C.

2.2. Cell lines

The CCRF-CEM acute lymphoblastic leukaemia cell line and the vincristine-resistant derivative, CCRF-CEM VCR/R, cells were used. The CCRF-CEM cells (85112105 (ECACC)) have been previously authenticated. The CCRF-CEM VCR/R cells were developed by stepwise exposure to increasing concentrations of vincristine [24] and display overexpression of P-glycoprotein and multidrug resistance. Cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10 % (v/v) foetal calf serum (Gibco, UK), penicillin (50 units/ml) and streptomycin (50 µg/ml).

The MDCKII and MDCKII-MDR1 cells were obtained from Alfred Schinkel (Netherlands Cancer Institute). These cells are a Madin-Darby canine kidney strain II cell line, the MDCKII-MDR1 subline overexpresses the human MDR1 gene due to transfection [25]. The MDCKII and MDCKII-MDR1 cells were maintained in DMEM supplemented with 10 % (v/v) foetal calf serum (Gibco, UK), 1 % (w/v) glutamine, penicillin (50 units/ml) and streptomycin (50 µg/ml).

All cells lines were maintained at 37 °C in a 5 % CO₂ atmosphere and were routinely screened for mycoplasma and used when under the 30th passage from frozen stocks.

2.3. Protein extraction and Western blotting

Whole cell extracts were treated with ice-cold Phosphosafe extraction reagent (Merck, UK) containing protease inhibitor (Roche, UK), centrifuged and supernatants were kept at -80 °C until protein quantification. Gel electrophoresis was carried out with 3–8 % (w/v) tris–acetate XT-criterion gels (Biorad, UK), and blotting was performed on nitrocellulose membranes (Hybond C Extra, Amersham Biosciences, UK). Membranes were blocked for 1 hour (TBS-T containing 5 % (w/v) BSA for phosphorylated proteins or 5 % (w/v) milk for total proteins) followed by 4 °C overnight incubation with primary antibodies against actin (CP01, Calbiochem, UK), pSer2056 DNA-PKcs (Ab18192, Abcam, UK), total DNA-PKcs (sc-9051, Santa Cruz Biotechnology, USA) or MDR1 (SC-13131, Santa Cruz Biotechnology, USA). Membranes were then washed in TBS-T and incubated with HRP-conjugated secondary antibodies (goat anti-mouse, code P0447 and goat anti-rabbit, code P0448, Dako, UK) and visualised using ECL reagent (Amersham Biosciences, UK). Images were acquired using Kodak X-ray film (Carestream Health, USA) on MediPhot 937 film developer, (Colenta, Austria) then digitally scanned. Quantification was undertaken by image acquisition of the films and densitometry analysis with a Fuji LAS-3000 Luminescent Image Analyzer (Raytek, UK). Data are displayed relative to actin loading control.

2.4. Quantitative real-time PCR

CCRF-CEM and CCRF-CEM VCR/R cell pellets were collected and the total RNA was purified using RNeasy Mini kit (Qiagen, UK). The RNA was then reverse transcribed using Quantitect RT kit (Qiagen, UK) and real time PCR was performed on 40ng cDNA using Taqman Gene Expression assays (ABCB1 assay ID: Hs00184500_m1, Beta-actin assay ID: Hs99999903_m1) and Taqman PCR master mix (Applied Biosystems, UK). The samples were run and analysed on a 7500 Fast Real Time PCR system (Applied Biosystems,UK). Results are displayed as ΔCT , which is the cycle threshold of ABCB1 normalised to β -actin, and the relative quantity (RQ) of ABCB1.

2.5. XTT cytotoxicity assay

The XTT assay (Roche, Germany) was carried out following the manufacturer's instructions. After 72-hour drug treatment, 50 μ l XTT assay reagent mix was added to each well in a 96-well plate, which was then incubated for 4 hours at 37 °C and 5 % CO₂ after which the absorbance was measured at 450 nm using a FLUOstar Omega plate reader (BMG Labtech, Germany). Growth of drug-treated cells was expressed as a percentage of control cell growth. Fold sensitisation was calculated using the GI₅₀ values of each treatment.

2.6. Doxorubicin nuclear fluorescence assay

Doxorubicin is a fluorescent P-glycoprotein substrate (λ ex: 470 nm, λ em: 595 nm). MDCKII and MDCKII-MDR1 cells were seeded in black, clear-bottomed 96-well plates (Greiner Bio-One, UK) and incubated for 3 days. Cells were then incubated for 1 hour with varying concentrations of NU7441 (0-10 μ M), verapamil (0-10 μ M) or cisplatin (10 μ M). Following incubation, 10 μ M doxorubicin was added for a further 1 hour. Cells were then washed in PBS and fixed with 10 % (v/v) formalin in PBS for

20 minutes. Cells were stained with DAPI for 20 minutes, washed again and 200 μ l PBS was added. Nuclear doxorubicin content was measured by fluorescence on a BD Pathway HT microscope (BD Biosciences, UK). Excitation was at 488 nm and a 515 nm long-pass filter was used to collect emitted fluorescence. Arbitrary fluorescence data was collected from >1000 cells in 3 separate experiments and the doxorubicin concentration was calculated as a percentage of the doxorubicin control-treated cells.

2.7. Sample Preparation for LC-MS analysis

CCRF-CEM and CCRF-CEM VCR/R cells were seeded into 6 well plates for 24 hours at a density of 1×10^6 cells/ml. The cells were then incubated for 1, 8, or 24 hours in the presence of vincristine alone at equitoxic GI_{50} concentrations (1 nM in CCRF-CEM cells and 1 μ M in the CCRF-CEM VCR/R cells) or in the presence of 1 μ M of NU7441, NU7742, DRN1, DRN2 or verapamil. Cells were centrifuged for 5 minutes and washed in PBS twice to remove all trace of media. 400 μ l 100 % methanol was added and the cells were centrifuged at $16000 \times g$ for 5 minutes. The methanol solution was transferred to glass vials and stored in a $-20^\circ C$ freezer until analysed. Standard curves for vincristine (0.01-3 μ M), NU7441 and verapamil (0.003-1 μ M) were prepared in methanol.

2.8. Liquid Chromatography-Mass Spectrometry

Analysis of samples was carried out using a liquid chromatography–mass spectrometry (LC–MS) assay validated in house based on a previously described method [26]. An API4000 LC/MS/MS (Applied Biosystems, USA) was used for analysis, with an LC-200 Micro pump, autosampler and Peltier column oven (Perkin Elmer, UK). The analytical column used was a Phenomenex Luna 3 μ C8 (2), 50 x 2

mm, (PNo. 00B-4248-B0, Phenomenex, UK) maintained at 30 °C. Elution of the analytes was performed with mobile phases of 0.02 M ammonium acetate pH 5 (pump A) and 100 % methanol (pump B). The analysis was performed using the gradient shown in Table 1, at a flow rate of 400 µl/min, and an injection volume of 10 µl. Atmospheric Pressure Chemical Ionisation was performed in positive ion mode using nitrogen gas with the following optimum settings: collision gas, 9; curtain gas, 10; ion source gas 1, 21; ion source gas 2, 45 (settings in Table 2). The temperature of the heated nebulizer was 450 °C. Analyst[®] software v1.5 (AB SCIEX, USA) was used for sample analysis, peak integration and analyte quantitation.

3. RESULTS

3.1. NU7441 sensitises MDR1-overexpressing multidrug resistant cells to a range of MDR1 substrates

Growth inhibition assays were used to investigate the effect of drug combinations on the growth of CCRF-CEM and CCRF-CEM VCR/R cells. The CCRF-CEM VCR/R cells are multidrug-resistant (MDR) through overexpression of the drug efflux transporter, MDR1, as shown in Fig. 2(a), (b) and (c). The CCRF-CEM VCR/R cells were approximately 1200-fold ($p=0.01$) resistant to vincristine compared to the sensitive CCRF-CEM cells (Fig. 2(e)) and this large difference in sensitivity is the reason that these cell lines were selected for these experiments (GI_{50} values in Table 3). The CCRF-CEM VCR/R cells were also cross-resistant to doxorubicin (52-fold resistant ($p=0.02$)) (Fig. 2(f)) and etoposide (150-fold resistant ($p=0.02$)) (Fig. 2(g)). The DNA-PK inhibitor NU7441 used at 1 μ M did not cause any growth inhibition alone in either cell line and both the CCRF-CEM and CCRF-CEM VCR/R cells showed the same growth inhibition profile in response to increasing concentrations of the DNA-PK inhibitor (Fig. 2(d)). NU7441 sensitised the CCRF-CEM VCR/R cells 14-fold to vincristine ($p=0.01$), 8-fold to doxorubicin ($p=0.03$) and 63-fold to etoposide ($p=0.02$), compared with 1.4-fold ($p=0.02$), 2.2-fold ($p=0.04$) and 3.6-fold ($p=0.01$), respectively, in the CCRF-CEM cells (Figs. 2(e) (f) (g)). These observed differences in sensitisation by NU7441 in the parental and vincristine-resistant CCRF-CEM cells to cytotoxic agents that are all known MDR1 substrates led us to the hypothesis that NU7441 interacts with MDR1.

3.2. NU7441 interacts with MDR1

The Madin-Darby canine kidney II (MDCKII) cells and their MDR-1-overexpressing counterpart, MDCKII-MDR1 cells, were used in this experiment (Fig. 3(a)) because the doxorubicin fluorescence assay must be carried out on adherent cells.

Doxorubicin is a fluorescent cytotoxic MDR1 substrate and this assay measured doxorubicin fluorescence in the nucleus. There was less doxorubicin detectable in the MDCKII-MDR1 cells compared with the MDCKII cells, reflecting the greater efflux of doxorubicin by MDR1. There was a concentration-dependent increase in doxorubicin fluorescence with the addition of the competitive MDR1 substrate, verapamil, up to 1.6-fold increase at 10 μ M verapamil (Fig. 3(c)). There was also a concentration-dependent increase in nuclear doxorubicin accumulation, i.e. inhibition of efflux, with the addition of NU7441 to a level similar to that seen with verapamil (Figs. 3(c) and (d)). There was a significant 1.2-fold increase in doxorubicin fluorescence at 1 μ M NU7441, which is the concentration previously shown to be optimal for DNA-PK inhibition with no cellular toxicity [7]. In contrast to the MDCKII-MDR1 cells there was no significant change in doxorubicin fluorescence with either verapamil or NU7441 in the MDCKII cells (Fig. 3(b)), consistent with the effect seen in the MDCKII-MDR1 cells being due to NU7441 interacting with MDR1 to block the efflux of doxorubicin from these cells.

3.3. The effect of NU7441, NU7742 and two structural atropisomeric derivatives of NU7441 on DNA-PK activity in CCRF-CEM cells

Following the observation that NU7441 was interacting with MDR1, it was necessary to determine if the effect of NU7441 was direct or mediated by DNA-PK inhibition. It is known that the morpholine group is necessary for DNA-PK inhibitory activity of NU7441 (DNA-PK IC_{50} = 14 nM) (Fig. 1(a)) [27] as replacement of the morpholine oxygen with a methylene group results in loss of DNA-PK inhibitory activity as seen

with NU7742 (DNA-PK $IC_{50} > 10 \mu M$) (Fig. 1(b)). Two atropisomeric derivatives of NU7441, DRN1 and DRN2 (Fig. 1(c)) were used in these studies [10]. Introduction of a methyl substituent at the 7-position of the chromen-4-one ring restricts rotation between the dibenzothiophene and the chromenone rings causing atropisomerism. DRN1 is the laevorotatory enantiomer, showing high potency against DNA-PK ($IC_{50} = 2 \text{ nM}$) (Fig. 1(c) (i)), whereas the dextrorotatory enantiomer, DRN2, has markedly reduced inhibitory activity ($IC_{50} = 7 \mu M$) (Fig. 1(c) (ii)).

Figure 4 shows the DNA-PK-inhibitory properties of NU7441 and the derivatives in the CCRF-CEM and CCRF-CEM VCR/R cells as measured by Western blotting (Fig. 4(a) and (b)). Both the sensitive and MDR cells have basal DNA-PK activity as demonstrated by detection of DNA-PK phosphorylated at serine 2056, a site of DNA-PK autophosphorylation. Cells were treated with GI_{50} vincristine concentrations both alone and in combination with NU7441, NU7742, DRN1, DRN2 and verapamil. NU7441 and DRN1 markedly inhibited DNA-PK activity in both cell lines (Fig. 4(c) and (d)), whereas NU7742, DRN2 and verapamil had no effect on DNA-PK phosphorylation.

3.4. Both DNA-PK inhibitory and non-inhibitory compounds increase intracellular vincristine levels in MDR cells

Following the observation that NU7441 interacts with MDR1, as shown by the doxorubicin efflux fluorescence microscopy data in the MDCKII cells treated with doxorubicin, this effect was further investigated using a more quantitative approach. The CCRF-CEM and CCRF-CEM VCR/R cells were treated with the MDR1 substrate vincristine alone and in combination with the compounds using the conditions described in Fig. 4. None of the compounds altered vincristine

concentration in the CCRF-CEM cells after 1 or 8 hours drug exposure (Fig. 5(a)). In contrast, in the CCRF-CEM VCR/R cells, all of the compounds tested increased the intracellular vincristine level when compared with vincristine alone at both 1 and 8 hours, consistent with an inhibition of vincristine efflux (Fig. 5(b)). At the concentration tested (1 μ M), NU7441 induced the highest vincristine accumulation at both 1 hour (1.3-fold; $p=0.01$) and 8 hours (2.1-fold; $p=0.04$), i.e. to a level similar to that induced by verapamil. NU7742 did not increase intracellular vincristine to the same degree as NU7441 (1.5-fold at 8 hours), showing that the morpholine group may play a role in the interaction of NU7441 and MDR1. Both DRN1 and DRN2 increased intracellular vincristine to the same degree (1.4-fold) after 8 hours, although they did not block vincristine efflux to the same degree as NU7441.

It was also possible to measure the intracellular levels of both verapamil and NU7441 in the CCRF-CEM and CCRF-CEM VCR/R cells. There was no change between 1 and 8 hours in the levels of these compounds in the CCRF-CEM cells (data not shown). In the CCRF-CEM VCR/R cells, there was no significant change in intracellular verapamil concentrations after 8 hours (Fig. 5(c)). There was however a significant increase in intracellular NU7441 levels at 8 hours, suggesting accumulation (Fig. 5(d)).

Overall, the data in Figure 5 demonstrate that inhibition of vincristine efflux by NU7441 and its derivatives is independent of their activity as DNA-PK inhibitors.

4. DISCUSSION

It is known that many conventional cytotoxic anticancer drugs are transported out of the cell by MDR1, and it has been shown that MDR1 affects both the intracellular accumulation and intra-tumour distribution of compounds [28]. The importance of the ABC transporter family has been somewhat overlooked in the development of more targeted therapies but a number of recent publications have demonstrated that targeted therapies can interact with this family of transporters [18-23, 29].

Understanding the role of drug efflux transporters is key when investigating combinations of targeted therapies with drugs that are MDR1 substrates.

Inhibition of DNA repair, particularly of double-strand break repair pathways, is an emerging therapeutic strategy in cancer treatment. In the preclinical setting, DNA-PKcs overexpression has been shown in chronic lymphocytic leukaemia patients with del(17p) (associated with p53 mutation) or del(11q) (associated with ATM mutation) chromosomal aberrations and associated with poor prognosis [9]. In addition, enhanced metastasis and poorer prognosis has been observed in patients with lower DNA-PK activity in their peripheral blood lymphocytes [30]. Furthermore, in response to DNA-damaging agents, ATM-defective cells are prevented from undergoing apoptosis and display a strong non-oncogenic addiction to DNA-PKcs [31]. Inhibition of DNA-PK means the ATM-deficient cells cannot repair double-strand breaks and leads to apoptosis and DNA-PK inhibitors have been shown to be an effective monotherapy in ATM-defective lymphomas *in vivo* [31].

NU7441 is a potent inhibitor of DNA-PK. During investigations of the effect of NU7441 on the activity of a range of chemotherapeutic agents in both parental and multidrug-resistant cells, it was noted that NU7441 sensitised the multidrug-resistant

CCRF-CEM VCR/R cells to vincristine, doxorubicin and etoposide and to a greater degree than in parental CCRF-CEM cells. The CCRF-CEM VCR/R cells are resistant to vincristine *via* overexpression of the efflux transporter MDR1 following stepwise treatment with increasing concentrations of vincristine. Therefore, a potential interaction between NU7441 and MDR1 was hypothesised.

Initially, the doxorubicin fluorescence assay demonstrated that NU7441 induces doxorubicin accumulation in a concentration-dependent manner in paired wild-type and MDR1-overexpressing MDCKII cells. The kinase inhibitor sorafenib is used to treat advanced renal cell carcinoma [32] and hepatocellular carcinoma [33], and MDR1 has been shown to play a major role in the acquired resistance phenotype in hepatocellular carcinoma [34]. Furthermore sorafenib has been shown to reverse paclitaxel resistance in MDR cells and cause a concentration-dependent increase in the uptake and retention of rhodamine 123; suggesting that sorafenib inhibits MDR1 [23]. Thus the data with NU7441 are consistent with those for sorafenib.

To investigate whether drug accumulation induced by NU7441 was linked to DNA-PK inhibition, a range of NU7441 derivatives with different DNA-PK inhibitory properties were studied. All of the compounds investigated increased intracellular vincristine concentration, as shown by LC-MS, in the MDR1-overexpressing CCRF-CEM VCR/R cells regardless of their DNA-PK-inhibitory activity.

Compounds that interact with MDR1 can do so by different mechanisms. Verapamil is known to modulate drug resistance by acting as a competitive MDR1 substrate [35]. However, the data presented here indicate that NU7441 does not act as a competitive substrate but rather as an inhibitor of MDR1. Thus NU7441 has similar

growth inhibitory activity in the sensitive and resistant cells and there was no observed reduction in intracellular levels of NU7441 over time (Figures 1(c) and 5).

Although the clinical relevance of MDR1 expression in solid tumours remains controversial, MDR1 has been shown to be relevant to acquired drug resistance in haematological cancers [36]. MDR is a biomarker for poor prognosis in patients with haematological cancers who can be cross-resistant to standard chemotherapeutic agents that are MDR1 substrates. The MDR substrates doxorubicin, etoposide and vincristine are used clinically in the treatment of haematological malignancies, such as acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) [37-39]. NU7441, in its capacity as a DNA-PK inhibitor, has been shown to sensitise cells to mitoxantrone, doxorubicin (*in vitro*) and etoposide (*in vitro* and *in vivo*) [7, 9], and this study has demonstrated that NU7441 can sensitise CCRF-CEM cells to vincristine.

In conclusion, this study highlights the role of NU7441 as a dual DNA-PK and MDR1 inhibitor, and this extends the therapeutic potential of the compound in combination with MDR substrates to sensitise both parental and MDR phenotypes.

5. ACKNOWLEDGEMENTS

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Step	Total Time (min)	A (%)	B (%)
0	0.0	80	20
1	2.0	80	20
2	7.0	10	90
3	7.5	80	20
4	11.0	80	20

Table 1: Gradient conditions of the HPLC mobile phase

Analyte	Retention Time (mins)	Q1 Mass (Da)	Q3 Mass (Da)	Declustering Potential	Entrance Potential	Collision Energy	Collision Exit Potential
Vincristine	6.58	825.39	765.4	101	10	49	26
Verapamil	6.37	455.23	164.7	91	10	41	10
NU7441	7.64	414.01	247.1	91	10	67	6

Table 2: Mass transitions and optimised MS/MS parameters for vincristine analysis

	CCRF-CEM		CCRF-CEM VCR/R	
	Drug [μ M]	Drug + NU7441 [μ M]	Drug [μ M]	Drug + NU7441 [μ M]
Vincristine GI ₅₀	0.0005 $\pm$ 0.02	0.0003 $\pm$ 0.03 ^{p=0.02}	0.61 $\pm$ 134 _{p=0.01}	0.043 $\pm$ 13.0 ^{p=0.01}
Doxorubicin GI ₅₀	0.04 $\pm$ 0.01	0.02 $\pm$ 0.002 ^{p=0.04}	2.03 $\pm$ 0.53 _{p=0.02}	0.26 $\pm$ 0.04 ^{p=0.03}
Etoposide GI ₅₀	0.33 $\pm$ 0.04	0.10 $\pm$ 0.03 ^{p=0.01}	48.4 $\pm$ 12.5 _{p=0.02}	0.93 $\pm$ 0.38 ^{p=0.02}
NU7441 GI ₅₀	4.07 $\pm$ 0.27	-	3.66 $\pm$ 0.26 _{ns}	-

Table 3: GI₅₀ values calculated from the growth inhibition curves of the CCRF-CEM and CCRF-CEM VCR/R cells treated with vincristine, doxorubicin, etoposide or NU7441 alone or vincristine, doxorubicin, etoposide in the presence of 1 μ M NU7441. GI₅₀ values were calculated from three independent experiments and are displayed as mean $\pm$ standard error. Significant differences between CCRF-CEM cells treated with drug alone and the CCRF-CEM VCR/R cells treated with drug alone were calculated using an unpaired t-test shown as subscript. Significant differences between cells treated with drug alone and cells treated with drug + NU7441 were calculated using an unpaired t-test shown as superscript. ns = non-significant.

(a) NU7441
DNA-PK; $IC_{50} = 14 \text{ nM}$

(b) NU7742
DNA-PK; $IC_{50} > 10 \text{ }\mu\text{M}$

(c) (i) DRN1
 $[\alpha_D]^{27.2} = -254$
DNA-PK; $IC_{50} = 2 \text{ nM}$

(c) (ii) DRN2
 $[\alpha_D]^{27.5} = +254$
DNA-PK; $IC_{50} = 7 \text{ }\mu\text{M}$

Figure 1: The chemical structures and DNA-PK-inhibitory activity of (a) NU7441, (b) NU7742, and (c) the atropisomers of NU7441, DRN1 and DRN2.

Figure 2: MDR1-overexpressing CCRF-CEM VCR/R cells were sensitised to MDR1 substrates to a greater degree by NU7441 when compared with parental CCRF-CEM cells. (a) CCRF-CEM and CCRF-CEM VCR/R cell lysates were prepared and MDR1 expression determined by Western blot. (b) Quantification of 3 independent Western blots of MDR1 protein level represented as fold increase relative to CCRF-CEM cells. (c) Taqman real-time PCR of CCRF-CEM and CCRF-CEM VCR/R cells examining ABCB1 mRNA expression. ΔCT is the cycle threshold of ABCB1 normalised to β -actin, as determined using the 7500 Fast RT PCR system. Relative quantity (RQ). CCRF-CEM (solid line) and CCRF-CEM VCR/R (dotted line) cells treated with (d) increasing concentrations of NU7441 or (e) vincristine, (f) doxorubicin or (g) etoposide alone (open symbols) or in the presence of 1 μM NU7441 (solid symbols) for 72 hours and growth inhibition analysed by XTT assay. The growth data are presented as a percentage of untreated control cells. Each point represents 3 independent experiments, each in triplicate $\pm$ standard error.

Figure 3: NU7441 interacts with MDR1 to block doxorubicin efflux in MDR1-overexpressing MDCKII-MDR1 cells. (a) MDCKII and MDCKII-MDR1 cell lysates were prepared and MDR1 expression determined by Western blot. (b) and (c) MDCKII and MDCKII-MDR1 cells were treated with 0.1, 0.5, 1, 5, 10 μM verapamil (striped) or NU7441 (checked) for 1 hour followed by 10 μM doxorubicin (DOX) treatment for 1 hour. Cells were fixed, DAPI stained, and DOX nuclear fluorescence detected on a BD Pathway HT microscope. Results are means $\pm$ standard error of three independent experiments. Asterisk indicates a statistically significant difference ($P < 0.05$). (d) Representative images of MDCKII-MDR1 cells showing DAPI (blue) and doxorubicin (red) fluorescence.

Figure 4: Effect of NU7441, NU7742, DRN1 and DRN2 on vincristine-induced DNA-PK activation. (a) CCRF-CEM and (b) CCRF-CEM VCR/R cells were treated for 8 hours with GI_{50} concentrations of vincristine alone (Vinc) or in combination with 1 μ M NU7441, NU7742, DRN1, DRN2 or verapamil. Western blot shows DNA-PK activation (phosphorylation at Serine 2056) in response to vincristine and the effects of the compounds on DNA-PK activation. Representative blots of three independent experiments. (c) and (d) Quantification of 3 independent Western blots for GI_{50} Vinc, NU7441 and NU7742 and 2 independent experiments for DRN1, DRN2 and verapamil displayed as a fold change in pDNA-PK level relative to the level in cells treated with GI_{50} Vinc alone.

Figure 5: Both DNA-PK inhibitory and non-inhibitory NU7441 derivatives increase intracellular vincristine levels in MDR cells. (a) CCRF-CEM and (b) CCRF-CEM VCR/R cells were treated for 1 or 8 hours with GI_{50} concentrations of vincristine (VCR) alone or in combination with 1 μ M NU7441, NU7742, DRN1, DRN2 or verapamil. Intracellular vincristine levels were measured using LC-MS. CCRF-CEM VCR/R intracellular levels of (c) verapamil and (d) NU7441 also measured using the same LC-MS method. Results are means $\pm$ standard error of three independent experiments. Asterisk indicates a statistically significant difference in fold change relative to 1 hour VCR treatment alone ($P < 0.05$).