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RESEARCH PAPER

Navitoclax (ABT-263) and bendamustine ± rituximab induce enhanced killing of non-Hodgkin's lymphoma tumours *in vivo*

Correspondence
Scott Ackler, Building AP9/2185, 100 Abbott Park Road, Abbott Park, IL 60064, USA. E-mail: scott.ackler@abbott.com

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S Ackler, MJ Mitten, J Chen, J Clarin, K Foster, S Jin, DC Phillips, S Schlessinger, B Wang, JD Leverson and ER Boghaert

Global Pharmaceutical Research and Development, Abbott Labs, Abbott Park, IL, USA

BACKGROUND AND PURPOSE

Bendamustine with or without rituximab provides an effective and more tolerable alternative to the polytherapy cyclophosphamide–doxorubicin–vincristine–prednisolone (CHOP) in the treatment of haematological tumours and is currently approved for the treatment of many haematological malignancies. Navitoclax (ABT-263) is a potent inhibitor of Bcl-2, Bcl-x_L and Bcl-w, which has demonstrated efficacy in haematological tumours alone and in combination with other agents. This paper describes the *in vivo* efficacy of combining either bendamustine or bendamustine plus rituximab (BR) with navitoclax in xenograft models of non-Hodgkin's lymphoma

EXPERIMENTAL APPROACH

Activity was tested in xenograft models of diffuse large B-cell lymphoma (DoHH-2, SuDHL-4), mantle cell lymphoma (Granta 519) and Burkitt's lymphoma (RAMOS). Activity was also monitored in a systemic model of Granta 519.

KEY RESULTS

Navitoclax potentiated bendamustine activity in all cell lines tested. Bendamustine activated p53 in Granta 519 tumours, concurrent with activation of caspase 3. Navitoclax also improved responses to bendamustine-rituximab (BR) in a subset of tumours.

CONCLUSIONS AND IMPLICATIONS

Navitoclax in combination with bendamustine and BR is a viable combination strategy for use in the clinic and demonstrated superior efficacy compared with previously reported data for navitoclax plus CHOP and rituximab-CHOP.

Abbreviations

BL, Burkitt's lymphoma; BR, bendamustine-rituximab; CHOP, cyclophosphamide–doxorubicin–vincristine–prednisolone; CLL, chronic lymphocytic leukaemia; CR, complete responder; DLBCL, diffuse large B-cell lymphoma; IHC, immunohistochemistry; MCL, mantle cell lymphoma; NHL, non-Hodgkin's lymphoma; ORR, overall response rate; PR, partial responder; R-CHOP, rituximab–cyclophosphamide–doxorubicin–vincristine–prednisolone; TGD, tumour growth delay; TGI, tumour growth inhibition

Introduction

Non-Hodgkin's lymphoma (NHL) is a family of lymphocyte-derived malignancies, with an estimated incidence and mortality in 2010 of 65 540 and 20 210, respectively (Howlader *et al.*, 2011). The anti-apoptotic protein Bcl-2 is frequently overexpressed in NHL, particularly in nodal follicular lymphoma where a hallmark t(14;18) translocation, placing the Bcl-2 gene under the control of the IgH enhancer region is observed in 60–90% of cases (Tsujimoto *et al.*, 1984; Sekiguchi *et al.*, 2005). Bcl-2 is also frequently up-regulated in NHL lacking this translocation and is associated with increased mortality and rate of relapse (Wei, 2004).

Navitoclax (ABT-263) is a potent small molecule inhibitor of anti-apoptotic Bcl-2 family members Bcl-2, Bcl-x_L and Bcl-w, with negligible activity against Mcl-1 or A1 (Tse *et al.*, 2008). Navitoclax competitively prevents sequestration of pro-apoptotic Bcl-2 family members by Bcl-2 or Bcl-x_L, allowing for activation of the intrinsic apoptotic programme (Tse *et al.*, 2008). Navitoclax and the closely related molecule ABT-737 have demonstrated potent single agent activity in acute and chronic lymphocytic leukaemia pre-clinically (Oltersdorf *et al.*, 2005; Lock *et al.*, 2008; Tse *et al.*, 2008; Mason *et al.*, 2009), and navitoclax replicated this result clinically (Wang *et al.*, 2008). Furthermore, broad combination activity with both cytotoxic and targeted agents in haematological tumours has been observed both *in vitro* and *in vivo* (Kang *et al.*, 2007; 2008; Kohl *et al.*, 2007; Kuroda *et al.*, 2007; 2008; Ackler *et al.*, 2008; 2010; Paoluzzi *et al.*, 2008; Stolz *et al.*, 2008; Tse *et al.*, 2008; Chen *et al.*, 2009; Jayanthan *et al.*, 2009; Mason *et al.*, 2009; High *et al.*, 2010).

Bendamustine is a bifunctional alkylating agent, possessing a nitrogen mustard moiety chemically linked to a purine analogue (Garnock-Jones, 2010). Bendamustine has limited cross-resistance to other alkylating agents such as cyclophosphamide and melphalan, and DNA cross-linking conferred by bendamustine is more durable than with other alkylators (Strumberg *et al.*, 1996). However, the molecular mechanism of action for bendamustine has not been fully characterized (Gaul *et al.*, 2008; Leoni *et al.*, 2008; Roue *et al.*, 2008). Leoni *et al.* (2008) found that bendamustine activated base excision repair genes rather than the alkyltransferase genes induced by other alkylators, and this may contribute to the efficacy of bendamustine in alkylation-resistant tumours. The efficacy of the combination of rituximab with bendamustine was synergistic *in vitro* in CD20-positive diffuse large B-cell lymphoma (DLBCL) and primary chronic lymphocytic leukaemia (CLL) lines (Chow *et al.*, 2002) and *in vivo* in a Burkitt's lymphoma (BL) model (Kanekal *et al.*, 2004). Currently, bendamustine is approved for the treatment of multiple haematological tumours, including indolent and rituximab-resistant NHL (Garnock-Jones, 2010).

R-CHOP, a polytherapy combining the anti-CD20 antibody rituximab with the cytotoxins cyclophosphamide (Cytoxan), doxorubicin (Hydroxydaunorubicin) and vincristine (Oncovin), and the immunosuppressant prednisone (P), is the current standard of care for NHL. Bendamustine in combination with rituximab (BR) was reported to have superior efficacy and tolerability in NHL patients as compared with R-CHOP (Rummel *et al.*, 2009). Previously, we have shown potentiation with the combination of CHOP and navitoclax in NHL (Ackler *et al.*, 2010). We therefore hypothesized that the combination of navitoclax with bendamustine and BR would demonstrate equivalent or superior efficacy, as compared with R-CHOP, with an improved safety profile. Therefore, this combination was tested in several models of NHL, including the DoHH-2 DLBCL model, the Granta 519 mantle cell lymphoma (MCL) model and the RAMOS BL model. Navitoclax significantly improved the durability and depth of the tumour response to bendamustine in all three models, as well as the overall response rate. The addition of rituximab also improved the responses to bendamustine, particularly in the NHL lines overexpressing myc. The addition of navitoclax further potentiated the activity of BR in DoHH-2 and Granta 519 tumours harbouring wild-type p53, but not in RAMOS with mutant p53. The potential roles of myc and p53 are discussed.

Methods

Cell culture

DoHH-2 and SuDHL-4 DLBCL, Granta 519 MCL and RAMOS BL cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 4.5 $g \cdot L^{-1}$ glucose (Sigma, St Louis, MO, USA). Cells were maintained at 37°C in 5% CO_2 and 95% relative humidity.

Reagents

Navitoclax was synthesized at Abbott Labs (Abbott Park, IL, USA), as previously reported (Park *et al.*, 2008). Bendamustine was purchased from Cephalon Inc (Frazer, MA, USA). Rituximab was purchased from Genentech Inc. (South San Francisco, CA, USA). Chemical structures for navitoclax and bendamustine are shown in Supplemental Figure S1. Phosal 50 PG was purchased from American Lecithin (Oxford, CT, USA).

Mouse xenograft trials

SCID and SCID-bg were obtained from Charles River (Wilmington, MA, USA). Ten mice were housed per cage. The body weight upon arrival was 18–20 g. Food and water were available *ad libitum*. Mice were acclimatized to the animal facilities for a period of at least one week before commencement of experiments. Animals were tested in the light phase of a 12-h light:12-h dark schedule (lights on at 06:00 h). All experiments were conducted in compliance with Abbott's Institutional Animal Care and Use Committee and the National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All the experiments were performed in SCID or SCID-bg mice. The total number of mice used in the present experiments was 400. Temperature was maintained at 25°C. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

C.B.-17 *scid* mice (DoHH-2, Granta 519) or C.B.-17 *scid*-bg mice (SuDHL-4, RAMOS) were inoculated with 1×10^6 (DoHH-2, RAMOS), 3×10^6 (SuDHL-4) or 5×10^6 (Granta 519) cells s.c. in the right flank. For flank xenografts, inoculation volume was 0.2 mL consisting of a 50:50 mixture of cells in growth medium and Matrigel (BD Biosciences, Bedford, MA, USA). Tumour volume was estimated by two to three weekly measurements of the length and width of the tumour by electronic calipers and applying the following equation: $V = L \times W^2/2$. Tumours were allowed to reach approximately 250 mm^3, and mice were size-matched (day 0) into treatment and control groups. For systemic Granta 519 tumour models, 2×10^6 cells were injected via the tail vein in 0.1 mL volume of cell medium on day 0, and treatment was initiated on day 14. All animals were ear-tagged

and monitored individually throughout the experiment. Navitoclax was administered by oral gavage once daily in a mixture of Phosal 50PG : PEG400 : ethanol as previously described (Tse *et al.*, 2008). Bendamustine and rituximab were administered i.v. at 25 and 10 mg·kg^{-1}, respectively, on day 1. Navitoclax was administered approximately 2 h before bendamustine and rituximab. All trials were comprised of 10 mice per group. Mice were humanely killed when tumours reached a size >2000 mm^3 or when any signs of distress were monitored. Signs of distress include loss of ambulation, laboured breathing or weight loss > 20% mean body weight per cage.

The % tumour growth inhibition (%TGI) was calculated as the inverse of the ratio of treated tumour size to vehicle tumour size at vehicle tumour endpoint. The % tumour growth delay (%TGD) of mono- and combination therapy was calculated as previously described (Shoemaker *et al.*, 2006). Complete responders (%CR) are defined as tumours with a volume <25 mm^3 for at least three consecutive measurements. Partial responders (%PR) are defined as tumours that dropped below 50% of their starting volume for at least three consecutive measurements, excluding complete responders. Overall response rate (%ORR) is the sum of %PR and %CR. Greater than additivity refers to combination groups which exceed the sum of each monotherapy as regards %TGI, %TGD, %CR and/or %ORR. Cures were defined by the absence of human mitochondrial staining using a mouse monoclonal antibody (ThermoFisher, Rockford, IL, USA) by immunohistochemistry (IHC) at the tumour inoculation site harvested from mice with negligible tumour burden after all measurable tumours had reached a 1000 mm^3 endpoint.

Molecular analysis

Granta 519 tumours were randomized at 500 mm^3 and treated with a single dose of bendamustine (25 mg·kg^{-1}) with or without navitoclax (100 mg·kg^{-1}), and tumours were harvested at 4, 8 and 24 h post treatment. Untreated Granta 519 tumours were used as a negative control. Tumours were divided, half-fixed in neutral-based formalin for IHC analysis, half-flash-frozen for Western blotting. Paraffin-embedded samples were routinely processed and sectioned at 5 µm, and stained for cleaved caspase 3 (Cell Signaling, Danvers, MA, USA) at a dilution of 1:100 for 1 h at room temperature. Positive binding was identified with a secondary antibody, anti-rabbit Envision Plus HRP polymer (Dako, Carpinteria, CA, USA) using 3,3′-diaminobenzidine as a chromogen.

Frozen tumours were homogenized using a Dounce homogenizer in Cell Lysis buffer (Invitrogen) containing 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA); 30 µg of protein was loaded into each well of a 10% Bis-tris NuPage gel (Invitrogen), and separated proteins were transferred to nitrocellulose membranes using the iBlot system (Invitrogen). Detection of fluorescence was performed in an Odyssey system (LI-COR, Lincoln, NE, USA) and processed using AlphaEaseFC imaging software (AlphaInnotech, San Leandro, CA, USA). Mouse anti-Noxa was purchased from Imgenex (San Diego, CA, USA). Rabbit anti-Mcl-1 and mouse anti-p53 were purchased from Santa Cruz (Santa Cruz, CA, USA). Goat anti-rabbit-AlexaFluor 680 was purchased from Invitrogen, and goat anti-mouse 800CW was purchased from LI-COR.

Statistical analysis

Significance within *in vivo* experiments for tumour growth inhibition and tumour growth delay were performed by Wilcoxon rank-sum analysis and Kaplan–Meier log-rank analysis, respectively. Significance for %CR and %ORR was performed by two-tailed Fisher's exact test. *P*-values < 0.05 were considered significant.

Results

Navitoclax potentiates both bendamustine and BR in vivo

Navitoclax administered at 100 mg·kg^{-1}·day^{-1} for 14 days demonstrated modest activity in the DoHH-2 and Granta 519 flank models, demonstrating significant tumour growth inhibition (44% and 31%, respectively) and tumour growth delay (42% and 55%, respectively), consistent with previous reports (Ackler *et al.*, 2008; 2010; Tse *et al.*, 2008). In contrast, navitoclax monotherapy was ineffective in the RAMOS model (Figures 1, 3).

A single dose of bendamustine at 25 mg·kg^{-1} demonstrated significant activity in all three tumour lines (Figure 1, Table 1). DoHH-2 was the most sensitive, with 30% ORR and a 69% inhibition in tumour growth. Growth of Granta 519 and RAMOS was also inhibited by bendamustine (%TGI of 74% and 81%, respectively), and the effect was more durable in Granta 519 (%TGD of 124%) than for DoHH-2 or RAMOS (69% and 43%, respectively).

The combination of navitoclax and bendamustine had a greater than additive response in all three models tested. Tumour growth inhibition was significantly increased to 91%, 99% and 95% for DoHH-2, Granta 519 and RAMOS, respectively. Delay in tumour growth to 1 cm^3 was also significantly enhanced by the addition of navitoclax (146%, 355% and 93%, respectively). Importantly, ORR was significantly increased in all three lines as compared with bendamustine treatment alone, and Granta 519 also demonstrated a significant increase in CR (Figure 1, Table 1).

Based on the substantial combination activity seen in Granta 519 tumours, this line was selected for further molecular analysis. Granta 519 tumours of approximately 500 mm^3 were treated with bendamustine alone or in combination with navitoclax. Tumours were harvested at 4, 8 and 24 h and analysed by IHC for caspase induction; while changes in p53, Noxa and Mcl-1 were examined by Western blotting.

Bendamustine treatment alone had little to no effect on caspase 3 cleavage in Granta 519 tumours by IHC. However, addition of navitoclax induced a significant increase in cleaved caspase 3 staining at 24 h (Figure 2A). Protein levels of the tumour suppressor protein p53 increased at 24 h post treatment with bendamustine, with or without navitoclax co-treatment (Figure 2B). Expression of noxa, a p53-inducible BH3 protein that inhibits Mcl-1, was not altered by bendamustine treatment. Mcl-1 levels also remained unchanged at

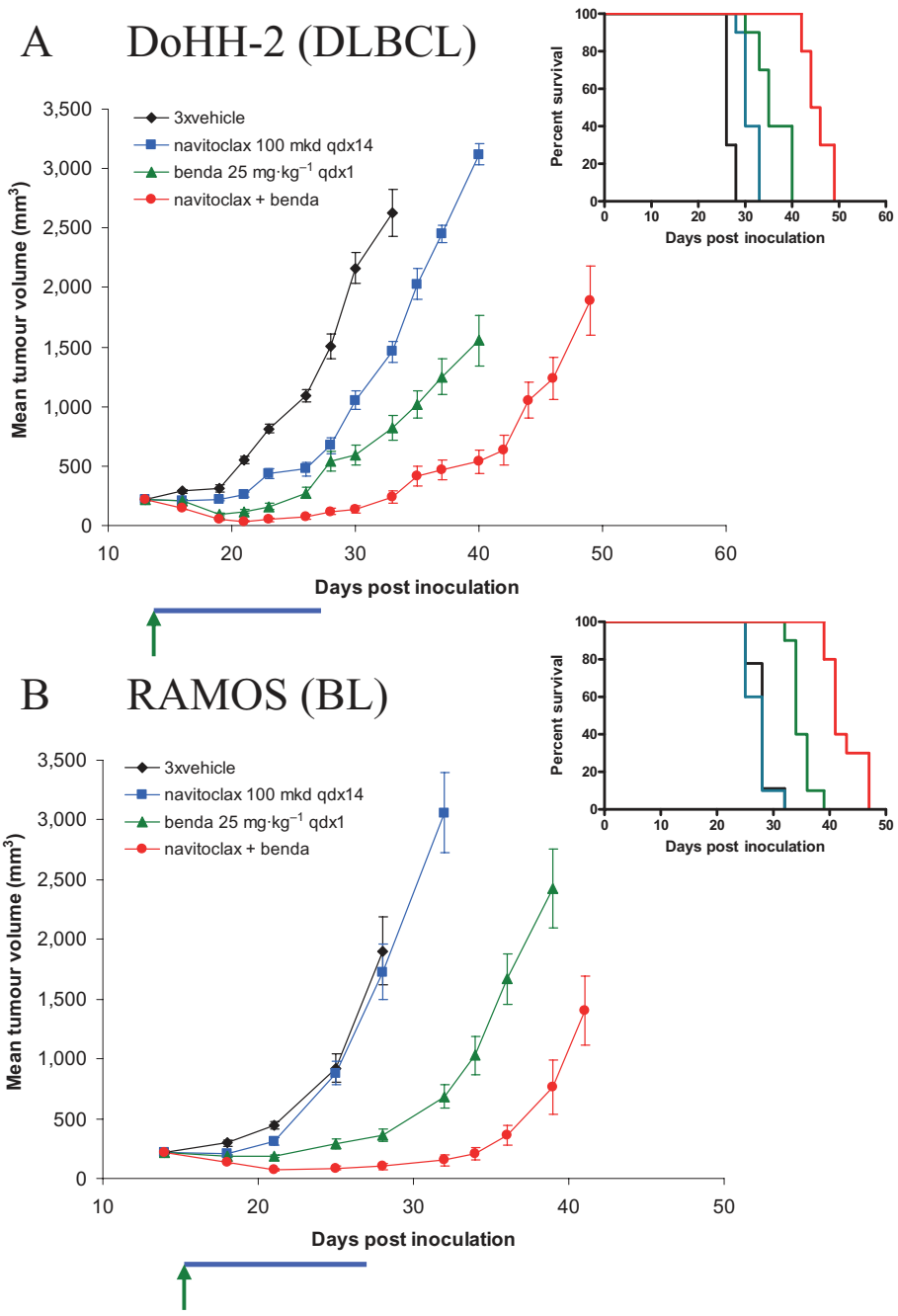


Figure 1

Xenograft sensitivity to treatment with navitoclax and bendamustine. DoHH-2 (A), RAMOS (B) or Granta 519 (C) tumours were treated with dual vehicle, navitoclax for 14 days (A,B) or 21 days (C) at 100 mg·kg^{-1}·day^{-1}, bendamustine for 1 day at 25 mg·kg^{-1} or both. Blue bar below graph represents navitoclax dosing period; green arrow represents bendamustine dosing day. Error bars represent the SEM, n = 10 mice per group. Analysis of %TGI, %TGD and response rates are shown in Table 1. All trials were performed once.

all time points. Increased p53 expression correlated temporally with the increased caspase 3 cleavage seen by IHC.

Rituximab further enhances the activity of bendamustine and bendamustine plus navitoclax in NHL tumours

The addition of 10 mg·kg^{-1} rituximab on the same day as bendamustine substantially enhanced bendamustine activity in DoHH-2 and RAMOS (Figure 3, Table 1). Strong tumour regressions were seen, with an ORR of 90% and 100%, respectively. Mice with RAMOS tumours had complete responses (CR = 80%) with BR treatment. In contrast, the addition of rituximab had little effect in the Granta 519 model, with no improvement in tumour growth inhibition, and only a minimal but significant increase in tumour growth delay (124% vs. 143%, respectively).

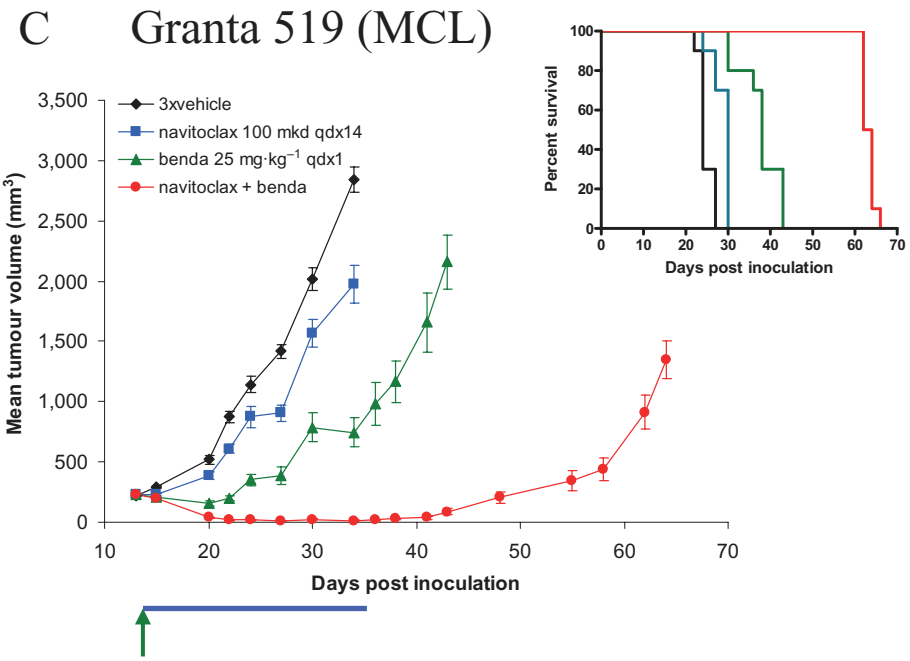


Figure 1
Continued.

The combination of navitoclax and BR produced more pronounced differences in additivity than seen with navitoclax and bendamustine. In DoHH-2, inhibition of tumour growth was increased slightly but significantly (from 97% to 99%). However, 100% CR was achieved with this combination, and the delay in tumour outgrowth was over 1100%, more than double the delay seen with BR treatment alone. Moreover, 4 of the 10 mice in this group presented with no visible tumour 169 days following initiation of therapy. Excision of the inoculation site revealed no staining for human mitochondria by IHC, suggesting that the tumours were completely eradicated in these animals. In contrast, tumour growth inhibition and delay of RAMOS tumours were not significantly increased by addition of navitoclax to BR (Figure 3, Table 1).

As with bendamustine plus navitoclax, the Granta 519 tumour response was significantly improved with the combination of navitoclax and BR. The delay in tumour regrowth was increased substantially over BR (507% vs. 355%, respectively), with equivalent tumour growth inhibition and ORR. One mouse in the triple combination group was confirmed tumour free at day 99 post-therapy initiation. Dose-response analysis of the potentiation of BR with navitoclax revealed that lowering the dose to as little as 33 $mg \cdot kg^{-1} \cdot day^{-1}$ provided similar tumour growth inhibition and delay as compared with the 100 $mg \cdot kg^{-1} \cdot day^{-1}$ dose (Figure 3C).

Effects of myc and p53 on the efficacy of a combination of BR and navitoclax

The data in Figure 3 indicate that the genetic makeup of the cell lines could influence the activity of BR with navitoclax. Two of the lines, DoHH-2 and RAMOS, harbour a myc translocation leading to myc overexpression (Taub *et al.*, 1982; Dyer *et al.*, 1996), and these lines were highly sensitive to BR therapy. Similarly, the two lines that express wild-type p53 (DoHH-2 and Granta 519) demonstrate significant improvement when navitoclax is added to BR, while the p53 mutant RAMOS tumours did not (Jones *et al.*, 2008; Dornan *et al.*, 2009). We hypothesized that myc overexpression sensitizes cells to BR therapy, while wild-type p53 was required for navitoclax to enhance responses to BR. To test this hypothesis, a second DLBCL line, SuDHL-4, was chosen, which lacks myc translocation but harbours a mutant p53 (Dornan *et al.*, 2009).

SuDHL-4 tumours responded modestly to navitoclax alone, in agreement with previous reports (Ackler *et al.*, 2008; 2010). Treatment with BR significantly inhibited tumour growth and delayed re-growth; however, this response was neither as strong nor as durable as in the myc-overexpressing line DoHH-2. The combination of navitoclax with BR demonstrated no improvement over BR alone (Figure 4), in agreement with our hypothesis.

Systemically engrafted Granta 519 tumours demonstrate an enhanced response to navitoclax plus BR

Based on the combination efficacy demonstrated by navitoclax and BR in Granta 519 flank xenografts, the efficacy of this combination was tested in the higher hurdle systemic model of Granta 519. Inoculation of Granta 519 cells i.v. induces disease in mice that, left untreated, leads to morbidity in approximately 25 days (Ackler *et al.*, 2010). Fourteen days post inoculation, mice were treated with either navitoclax, bendamustine, rituximab, BR, or navitoclax +

Table 1

Efficacy of navitoclax in combination with bendamustine or BR in NHL lines[a]

Cell line	Compound	Dose (mg·kg^{-1}·day^{-1})[b]	Avg. tumour Vol ± SE	%TGI[c]	%TGD[d]	%CR[e]	%ORR[f]	%Cure[g]
DoHH-2	navitoclax	100	1463 ± 88	44*	42*	0	0	0
(DLBCL)	bendamustine	25	819 ± 106	69*	69*	0	30	0
	bendamustine +	25+						
	rituximab	10	67 ± 14	97*	527*	0	90†	0
	navitoclax +	100+						
	bendamustine	25	237 ± 51	91*	146*	20	90†	0
		0 + 0	2628 ± 198					
	navitoclax +	100+						
	bendamustine +	25+						
	rituximab	10	18 ± 6	99*	1115*	100†	100	40
Granta 519	navitoclax	100	1975 ± 158	31*	55*	0	0	0
(MCL)	bendamustine	25	746 ± 118	74*	127*	0	0	0
	bendamustine +	25+						
	rituximab	10	635 ± 89	73*	143*	0	0	0
	navitoclax +	100+						
	bendamustine	25	14 ± 3	99*	355*	80†	100†	0
		0 + 0	2846 ± 105					
	navitoclax +	100+						
	bendamustine +	25+						
	rituximab	10	48 ± 32	98*	507*	90†	100†	10
		0 + 0 + 0	2310 ± 172					
RAMOS	navitoclax	100	1729 ± 231	9	0	0	0	0
(BL)	bendamustine	25	361 ± 51	81*	43*	0	0	0
	bendamustine +	25+						
	rituximab	10	47 ± 21	98*	393*	80†	100†	0
	navitoclax +	100+						
	bendamustine	25	99 ± 25	95*	93*	20	60†	0
	navitoclax +	100+						
	bendamustine +	25+						
	rituximab	10	10 ± 7	99	429	100	100	0
		0 + 0 + 0	1902 ± 285					

[a]Tumours were size-matched (day 0) to approximately 250 mm^3 before therapy. $n = 10$ per group.
[b]'0 + 0' is vehicle control for bendamustine + navitoclax. '0 + 0 + 0' is vehicle control for bendamustine + rituximab + navitoclax (where appropriate).
[c]% Tumour growth inhibition (measured at end of dosing period).
[d]Median % tumour growth delay for tumours to reach 1 cm^3 endpoint relative to vehicle.
[e]% Complete regression.
[f]% Overall response.
[g]As determined by the absence of human mitochondrial staining at tumour inoculation site as described in Methods.
*$P < 0.05$ versus vehicle or cytotoxic therapy (Wilcoxon rank-sum test for %TGI, Kaplan–Meier log-rank analysis for %TGD).
†$P < 0.05$ versus vehicle or cytotoxic therapy (Fisher's exact test).

bendamustine (B + N), rituximab (R + N) or BR (B + R + N); the results are presented in Figure 5. Navitoclax monotherapy was ineffective in this model, similar to previous reports (Ackler *et al.*, 2010). Monotherapy with either rituximab or bendamustine modestly but significantly increased median survival (28 and 31 days, respectively). The addition of navitoclax to either of these agents yielded no further improvement in survival. BR significantly increased survival over either bendamustine or rituximab alone, with a median survival of 45 days. The addition of navitoclax to BR induced a further increase in survival, with a median survival of 76.5 days. Additionally, two mice in the B + R + N group were still

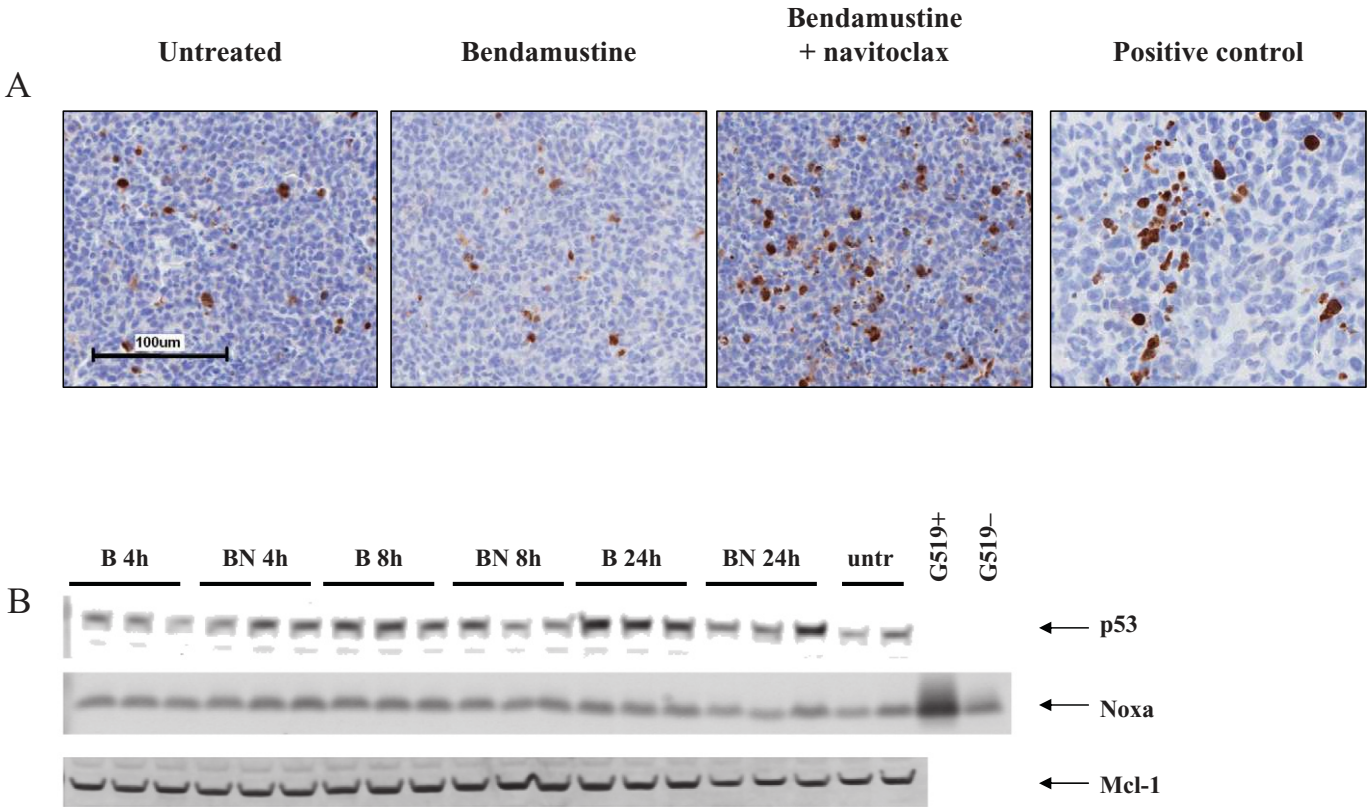


Figure 2

Molecular analysis of bendamustine in the absence and presence of navitoclax in Granta 519 flank tumours. Granta 519 tumours were treated with a single dose of bendamustine at 25 mg·kg^{-1} with or without navitoclax at 100 mg·kg^{-1}. Tumours were harvested at 4, 8 and 24 h post treatment. Naïve tumours were used as a control. (A) Immunohistochemical analysis of cleaved caspase 3. Tumours shown were harvested 24 h post therapy. CaLu-6 tumour treated with docetaxel was used as a positive control for staining. (B) Western blot analysis of expression levels of p53 and the Bcl-2 family members Noxa and Mcl-1. G519 lanes are Granta 519 cells ± bortezomib as a Noxa control. Each lane represents an individual tumour.

symptom-free 119 days following inoculation (105 days following initiation of therapy).

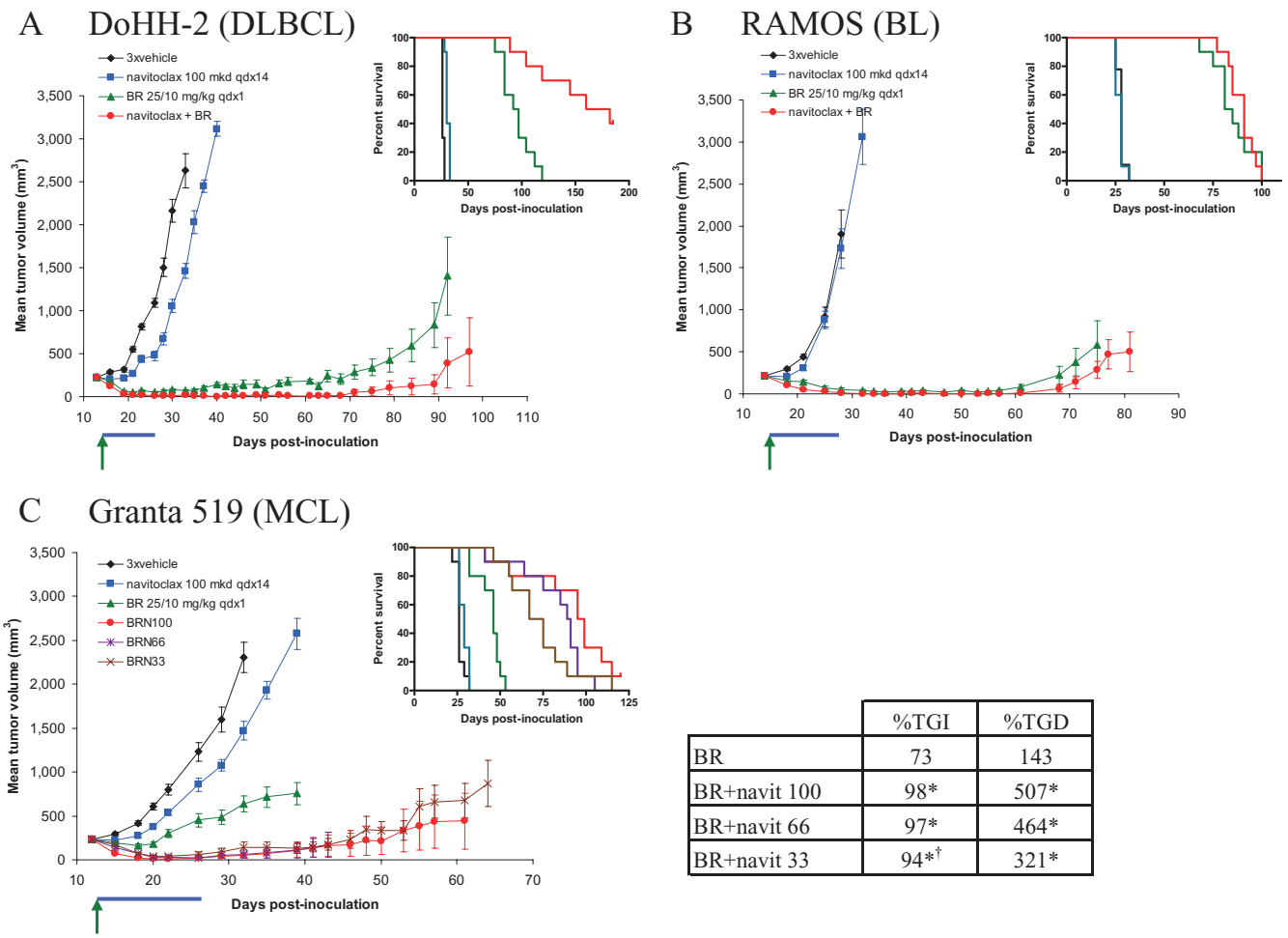
Discussion

In this paper, we describe the effects of combining bendamustine or BR with navitoclax in the treatment of NHL tumours. *In vivo* potentiation of bendamustine by navitoclax was seen in all three models tested, while potentiation of BR with navitoclax was seen only in a subset of these lines. In Granta 519 tumours, treatment with bendamustine increased p53 levels, and this was associated with enhanced cleavage of caspase 3 in a similar timeframe.

The molecular mechanism of bendamustine-induced cell killing remains unclear. Gaul *et al.* (2008) reported that myeloma cell killing was dependent on p53, while cell cycle arrest was driven by Chk2. Others have shown that in B-cell lymphoid malignancies, cell killing by bendamustine is independent of p53, and depends on the generation of reactive oxygen species (Roue *et al.*, 2008). Leoni *et al.* (2008) demonstrated that bendamustine causes cell killing by activating the DNA damage stress response and inducing mitotic catastrophe irrespective of p53 status. Our results demonstrated that bendamustine-induced killing was similar in both wild-type and mutant p53 tumours, in agreement with Leoni *et al.* (Figure 1).

Within this limited number of xenograft lines, p53 status appeared to differentiate the response of tumours treated with navitoclax and BR. DoHH-2 and Granta 519 have wild-type p53, while SuDHL-4 and RAMOS harbour mutations in p53 (Jones *et al.*, 2008; Dornan *et al.*, 2009), and significant improvement with addition of navitoclax to BR was only seen in the p53 wild-type tumours.

Furthermore, a correlation was seen between myc rearrangement and response to BR. RAMOS is a BL line, and myc rearrangement is a definitive marker within this tumour class (Taub *et al.*, 1982), and DoHH-2 harbours a complex translocation placing both c-myc and Bcl-2 under the control of the same IgH enhancer element (Dyer *et al.*, 1996). These lines responded more robustly to BR therapy than the non-rearranged myc tumour lines SuDHL-4 and Granta 519 (Figures 3,4). To our knowledge, this is the first observation of a connection between BR sensitivity and myc rearrangement. Recently, myc rearrangement was associated with a significant reduction in overall survival in DLBCL patients treated with R-CHOP (Barrans *et al.*, 2010). These data suggest that, in addition to improved tolerability of BR



	%TGI	%TGD
BR	73	143
BR+navit 100	98*	507*
BR+navit 66	97*	464*
BR+navit 33	94*†	321*

Figure 3

Xenograft sensitivity to treatment with navitoclax and BR. DoHH-2 (A), RAMOS (B) or Granta 519 (C) tumours were treated with triple vehicle, navitoclax for 14 days at 100 mg·kg^{-1}·day^{-1}, BR for one day at 25 and 10 mg·kg^{-1}, respectively, or the triple combination. In addition, the effect of different doses of navitoclax in Granta 519 for 14 days at 66 mg·kg^{-1}·day^{-1} and 33 mg·kg^{-1}·day^{-1} in combination with BR. Blue bar below graph represents navitoclax dosing period; green arrow represents BR dosing day Error bars represent the SEM, $n = 10$ mice per group. Analysis of %TGI, %TGD and response rates are shown in Table 1, analysis for lower doses of navitoclax in combination are presented in the inset. *: $P < 0.05$ versus BR alone. †: $P < 0.05$ versus BR+navit 100. All trials were performed once.

versus R-CHOP, improved outcome in resistant populations may be achieved.

Despite the small sample set provided in this report, the effects of myc rearrangement and p53 status are strikingly binary. Although practical considerations prevented us from performing sufficient xenograft trials to enable us to do a statistical comparison, and the multitude of other mutations within each tumour also probably play a role in the responses, we feel that this information provides utility as a test set for analysing data from clinical trials. As previously reported, growth rates for DoHH-2, SuDHL-4 and Granta 519 were similar (Ackler *et al.*, 2008). While DoHH-2 (rearranged myc/p53 wild-type) did demonstrate a significant enhancement of response to navitoclax plus BR, xenografts had to be followed for several weeks following treatment before separation occurred (Figure 3). Depending on the length of the clinical trial and if the increased number of treatment cycles is taken into account, this difference could be difficult to determine. Similarly, removing patients whose tumours harbour mutant p53 during meta-analysis could expose a more sensitive population within a clinical trial, and one that could be used as an exclusion criterion in future trials if warranted. Therefore, the data presented here could provide a test set to be used as a meta-analysis tool in clinical trials testing this combination. However, a larger data set is necessary to determine the durability of this trend, and clinical trials would provide sufficient numbers to test the hypothesis.

BR has been proposed to replace R-CHOP as the standard of care in NHL and other haematological tumours (Rummel *et al.*, 2009). Previously, we have reported on the efficacy of CHOP and R-CHOP in combination with navitoclax (Ackler *et al.*, 2010). The data presented here clearly demonstrate that the combination of navitoclax with BR has an advantage over CHOP in DoHH-2 both in depth of regression, durability of response and %CR. While responses in Granta 519 flank

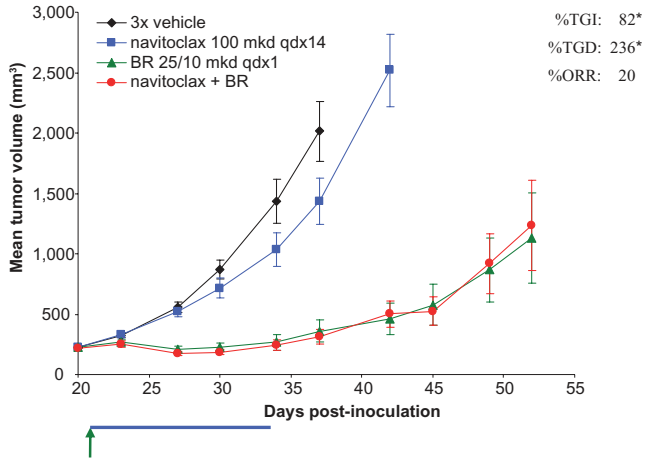


Figure 4

SuDHL-4 xenograft efficacy following treatment with BR in the absence and presence of navitoclax. SuDHL-4 tumours were size-matched and treated with vehicle, navitoclax at 100 mg·kg^{-1}·day^{-1} from days 21–34, BR at 25 and 10 mg·kg^{-1}, respectively, on day 21, or navitoclax + BR. Blue bar below graph represents navitoclax dosing period; green arrow represents BR dosing day. Error bars represent the SEM, $n = 10$ mice per group. Analysis of %TGI, %TGD and response rates (%ORR) for BR treated tumours are shown in the inset. *$P < 0.05$ versus vehicle control.

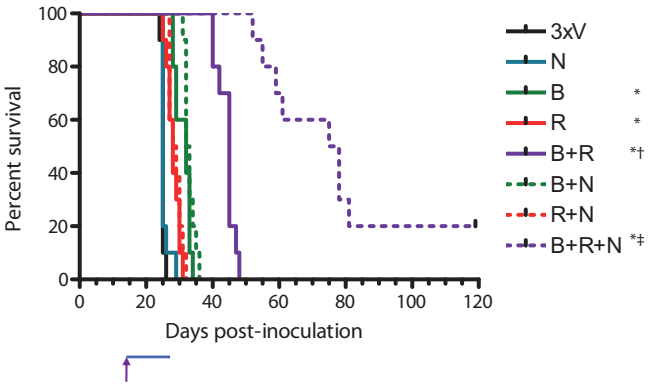


Figure 5

Sensitivity of systemically engrafted Granta 519 to treatment with navitoclax and bendamustine, rituximab or BR. Granta 519 cells were inoculated i.v. and treatment initiated 14 days later following randomization. Pharmacological agents were administered as follows (3xV: triple vehicle; B: bendamustine 25 mg·kg^{-1}, day 14; R: rituximab 10 mg·kg^{-1}, day 14; N: navitoclax 100 mg·kg^{-1}·day^{-1}, days 14–27). Bar below graph represents navitoclax dosing period; arrow represents bendamustine and/or rituximab dosing day. Animals were monitored to a morbidity endpoint. $n = 10$ mice per dose group. *$P < 0.05$ versus vehicle. †$P < 0.05$ versus bendamustine monotherapy. ‡$P < 0.05$ versus BR polytherapy.

xenografts were similar for navitoclax plus BR or R-CHOP, the activity of navitoclax plus BR in the higher hurdle i.v. systemic model was much more durable (median survival 76.5 days vs. 44.5 days for navitoclax plus R-CHOP) (Ackler *et al.*, 2010). Coupled with the improved tolerability profile of bendamustine compared with CHOP (Rummel *et al.*, 2009), BR represents a much more attractive combination therapy with navitoclax.

In conclusion, navitoclax enhances the response of NHL tumours to bendamustine in mouse xenografts. The addition of rituximab further improves the efficacy of bendamustine, with the breadth dependent on myc status, and bendamustine plus navitoclax with an apparent dependence on p53 status. The activity of this combination in Granta 519 tumours was coupled with the ability of bendamustine to induce apoptosis. These data support the testing of this combination in the clinic and warrant further studies.

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Conflict of interest

All authors are employees of Abbott Labs. Navitoclax is being developed by Abbott Labs for commercial use.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Chemical structures of navitoclax and bendamustine.

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