Biochemical Pharmacology 80 (2010) 31–38

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Sequential treatment with flavopiridol synergistically enhances pyrrolo-1,5-benzoxazepine-induced apoptosis in human chronic myeloid leukaemia cells including those resistant to imatinib treatment

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ARTICLE INFO

Article history:
Received 25 January 2010
Accepted 24 February 2010

Keywords:
CML
PBOX
Survivin
Flavopiridol
Apoptosis

ABSTRACT

The Bcr-Abl kinase inhibitor, imatinib mesylate, is the front line treatment for chronic myeloid leukaemia (CML), but the emergence of imatinib resistance has led to the search for alternative drug treatments and the examination of combination therapies to overcome imatinib resistance. The pro-apoptotic PBOX compounds are a recently developed novel series of microtubule targeting agents (MTAs) that depolymerise tubulin. Recent data demonstrating enhanced MTA-induced tumour cell apoptosis upon combination with the cyclin dependent kinase (CDK)-1 inhibitor flavopiridol prompted us to examine whether this compound could similarly enhance the effect of the PBOX compounds. We thus characterised the apoptotic and cell cycle events associated with combination therapy of the PBOX compounds and flavopiridol and results showed a sequence dependent, synergistic enhancement of apoptosis in CML cells including those expressing the imatinib-resistant T315I mutant. Flavopiridol reduced the number of polyploid cells formed in response to PBOX treatment but only to a small extent, suggesting that inhibition of endoreplication was unlikely to play a major role in the mechanism by which flavopiridol synergistically enhanced PBOX-induced apoptosis. The addition of flavopiridol following PBOX-6 treatment did however result in an accelerated exit from the G2/M transition accompanied by an enhanced downregulation and deactivation of the CDK1/cyclin B1 complex and an enhanced degradation of the inhibitor of apoptosis protein (IAP) survivin. In conclusion, results from this study highlight the potential of these novel series of PBOX compounds, alone or in sequential combination with flavopiridol, as an effective therapy against CML.

1. Introduction

CML represents a clonal disorder of haematological stem cells containing a constitutively active tyrosine kinase known as Bcr-Abl. This oncogenic fusion protein is characteristic of CML and is also found in 10–15% of acute lymphocytic luekaemia (ALL) patients [1,2]. Bcr-Abl confers cells with a survival advantage due to the continuous activation of many downstream signalling pathways including the signal transducer and activator transcription (STAT) and phosphatidyl inositol-3 kinase (PI3K) pathways rendering cells resistant to apoptosis [3]. The advent of the tyrosine kinase inhibitor, imatinib mesylate (STI571, Gleevec), has revolutionised CML treatment with the majority of newly diagnosed chronic phase patients achieving durable complete cytogenetic responses [4,5]. However, advanced stages of the disease, the accelerated phase and the fatal blast crisis phase, are commonly associated with the development of resistant clones that express mutants of Bcr-Abl which exhibit reduced imatinib binding compared to the unmutated protein. More than 60 such mutants have so far been identified which display various degrees of resistance to imatinib [6,7]. T315I, one of the most common resistance mutations, confers complete resistance by preventing

Abbreviations: Abl, abelson; Bcl-2, B-cell leukaemia/lymphoma 2; Bcr, breakpoint cluster region; CDK, cyclin dependent kinase; CI, combination index; CML, chronic myeloid leukaemia; DTT, dithiothreitol; FACS, fluorescent activated cell sorting analysis; FBS, foetal bovine serum; IAP, inhibitor of apoptosis protein; MTA, microtubule targeting agents; NT, no treatment; PBOX, pyrrolo-1,5-benzoxazepine; PBS, phosphate buffered saline.

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doi:10.1016/j.bcp.2010.02.019

imatinib binding as a consequence of steric hindrance and abolishment of a critical hydrogen bond. The fact that a substantial subset of CML patients fails imatinib treatment has stimulated the development of novel agents and drug combinations for the treatment of CML [8].

A novel set of MTAs, the pro-apoptotic PBOX compounds, have recently been shown by our group to induce apoptosis in a wide range of solid tumours and haematological malignancies by causing a depolymerisation of the microtubule network [9–11]. Mechanistic studies have indicated that the PBOXs induce apoptosis in CML cells by bypassing Bcr-Abl [10], and that activation of JNK signalling pathways [12] and inactivation of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL [13] are essential during PBOX-induced apoptosis. The PBOX compounds have also demonstrated efficacy in *ex vivo* chronic lymphocytic leukaemia (CLL) [14] and CML patient samples [15] and in animal models of breast cancer [16] and CML [15]. We have recently demonstrated that the PBOXs induce the formation of polyploid or large multi-nucleated cells in CML cells through endoreplication [17], a characteristic shared with other MTAs. We have also previously shown that, in a similar manner to other MTAs, a significant proportion of K562 CML cells (approximately 60%) remained resistant to our pro-apoptotic PBOXs following 48 h treatments. We therefore sought to determine whether a combination treatment with another drug could potentiate PBOX-induced apoptosis.

Flavopiridol is a potent CDK1 inhibitor which has shown activity in a wide variety of malignancies both *in vitro* and in *in vivo* animal models [18–20]. Concentrations required to inhibit CDKs were easily achieved in phase I clinical trials. However, the results of many early phase II trials using continuous infusion schedules were disappointing [21–24] and dose-limiting toxicities such as neutropenia were also evident [22,24,25]. Recently trials involving shorter infusions and higher plasma concentrations have now seen flavopiridol demonstrating strong anti-cancer activity, particularly in CLL [25,26]. The combination of this schedule of flavopiridol with other pro-apoptotic agents has also proved successful, including flavopiridol in combination with cytarabine and mitoxantrone [27] and the MTAs docetaxel [28,29] and paclitaxel together with carboplatin [30].

Survivin is a member of the IAP family that functions to prevent apoptosis by inhibiting caspases, downstream executioners of apoptosis. It is also a cell cycle-related protein, with activation occurring during mitosis via phosphorylation of Thr34 by the cell cycle complex CDK1/cyclin B1 [31]. Survivin has been associated with cytoprotection and preservation of microtubule integrity. It is expressed during development but is undetectable in most adult tissues except during mitosis. Importantly however, it is overexpressed in the majority of cancers tested including CML cells. Survivin expression in malignant cells may facilitate evasion from apoptosis and promote aberrant mitotic progression suggesting it may be a key target for anti-cancer agents [32]. The possibility of exploiting the survivin pathway for cancer therapy has been the subject of intense investigations. Inhibition of survivin expression and/or function in tumour cells by employment of survivin antisense oligodeoxynucleotides, dominant negative mutants or by siRNA has been shown to trigger apoptosis [32].

Flavopiridol has been shown to synergistically enhance the apoptotic effect of MTAs; however, the exact mechanism through which this synergism occurs remains unclear [33–35]. Some reports suggest that flavopiridol suppresses CDK1-mediated survivin phosphorylation, accelerating protein destruction through the ubiquitin-dependent proteasome and thus enhancing tumour cell apoptosis [36] while others studies imply that flavopiridol prevents MTA-induced endoreplication leading to an enhancement of MTA-induced cell death [35]. To determine if this CDK1 inhibitor can similarly enhance PBOX-induced apoptosis we characterised the apoptotic and cell cycle events associated with combination therapy of the pro-apoptotic PBOX compounds and flavopiridol in CML cell lines.

2. Materials and methods

2.1. Cell culture

K562 cells were obtained from the European Cell Culture Collection (Salisbury, UK). LAMA84 cells were kindly provided by Dr. Jane Apperley and Dr. Junia Melo, Department of Haematology, Imperial College, London, U.K. Native and mutant Bcr-Abl Baf/3 cells were described previously [37]. Cells were cultured in RPMI-1640 (Glutamax) medium supplemented with 10% foetal bovine serum (FBS) and 50 μg/ml penicillin/streptomycin. Cells were maintained in a humidified atmosphere at 95% O_2 and 5% CO_2 and passaged three times per week. Baf/3, K562 and LAMA84 cells were seeded at a previously optimised density of 2×10^5 and 3×10^5 cells/ml, respectively. Unless otherwise stated, cells were treated for the required length of time (x) with PBOX-6 or PBOX-15 and then centrifuged, resuspended in fresh medium and treated with 500 nM flavopiridol, as designated by $P6_x$,$flavo_x$ or $P15_x$,$flavo_x$. Control samples treated with either agent alone were designated $P6_x$,NT_x, $P15_x$,NT_x or NT_x,$flavo_x$.

2.2. Reagents

All antibodies were obtained from Calbiochem (Nottingham, U.K). RPMI-1640 medium was obtained from Biosciences (Dublin, Ireland) and FBS from Invitrogen (Paisley, U.K.). The enhanced chemiluminescence reagents were supplied by Amersham Biosciences (Buckinghamshire, U.K.), The BCA reagents were from Pierce (Illinois, U.S.), the polyvinylidene difluoride membranes from Millipore (Cork, Ireland), while the protease inhibitors were obtained from Roche (Clare, Ireland). Histone H1 was purchased from New England Biolabs (Herts, U.K.) and [γ^{32}P]-ATP from PerkinElmer (Dublin, Ireland). Survivin siRNA and scRNA were obtained from Cell Signaling Technology (Massachusetts, U.S.A.). All other reagents, including flavopiridol, were purchased from Sigma (Dublin, Ireland).

2.3. Flow cytometry

Following treatment with the desired compound/s, cells were harvested by centrifugation and resuspended in 100 μl ice-cold phosphate buffered saline (PBS) and fixed in ice-cold 70% ethanol (1 ml) at 4 °C. Samples were then centrifuged at 800 × g for 10 min and resuspended in 200 μl PBS containing 10 μg/ml RNase A and 100 μg/ml propidium iodide. Samples were incubated for 30 min at 37 °C in the dark. Following sample preparation, cell cycle analysis was performed at 488 nm using a Becton Dickinson fluorescent activated cell sorting analysis (FACS) Calibur flow cytometer (Becton Dickinson, CA, U.S.). The Macintosh-based application CellQuest was then used to analyse the data of 10,000 gated cells once cell debris had been excluded. The data was stored as frequency histograms.

2.4. Analysis of drug interaction

Drug interactions were determined by median dose effect analysis using the software Calcusyn (Biosoft, Cambridge, U.K.). This method is based on the drug effect equation of Chou and Talalay and can determine the degree of synergism or antagonism between two compounds by generating a combination index (CI)

value. CI values of <1, =1 and >1 indicate synergism, an additive effect and antagonism, respectively [38].

2.5. Western blot analysis

SDS-polyacrylamide gel analysis was performed as previously described [39]. Briefly, whole cell lysates were prepared in Laemmli buffer and run on 12% gels. Equal protein loading was ensured by performing a BCA protein determination assay. Samples were then transferred to polyvinylidene difluoride membranes for 1 h, blocked in 5% (w/v) marvel in Tris buffered saline, pH 7.6/0.05% Tween 20, incubated overnight with the relevant antibodies, washed, incubated for 1 h with a horseradish peroxidase-linked secondary Ab and washed again. Blots were then developed using enhanced chemiluminescense and an automated developer (Fuji X-ray film processor). Densitometry was performed using ImageJ software (NIH, U.S.).

2.6. Immunoprecipitation

K562 cells were seeded at the required density and treated as indicated. Following the treatment regimens, cells were centrifuged at 500 × *g* for 5 min and resuspended in 500 μl of lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 mM DTT, 1 mM NaF, 10 mM β-glycerophosphate and protease/phosphatase inhibitors). The cell suspension was vortexed vigorously for 10 s and incubated on ice for 30 min. The samples were then sonicated and centrifuged at 20,000 × *g* for 20 min. A BCA assay was performed on the resulting supernatants.

An aliquot of 1 μg of CDK1 antibody was used per sample. The volume of the incubation buffer was then made up to 500 μl with lysis buffer. The samples were then incubated on a roller at 4 °C for 3 h. A 50% slurry of protein A sepharose beads in PBS (50 μl) was added to the samples and incubated overnight on a roller at 4 °C. The samples were then centrifuged for 2 min at 500 × *g* to pellet the beads. The supernatant was carefully removed using gel loading tips making sure not to disturb the beads. The beads were then washed twice in 1 ml of ice-cold lysis buffer and subjected to a CDK kinase activity assay.

2.7. CDK kinase activity assay

Following the washing of immunoprecipitated beads, the beads were washed once with ice-cold kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 10 mM β-glycerophosphate and 0.1 mM sodium orthovanadate). The lysates were then resuspended in 30 μl kinase buffer supplemented with unlabelled ATP, 2 μCi [γ^{32}P]-ATP and 10 μg Histone H1. Samples were incubated at 30 °C for 30 min, following which samples were boiled at 100 °C for 3 min and added to 3× SDS sample buffer (20 μl). Samples were then run on a 12% gel, exposed to Kodak X-Omat film at −70 °C overnight and analysed for Histone H1 by autoradiography.

2.8. siRNA downregulation of survivin expression

K562 cells in the log phase of growth were transfected with 100 ng of a validated siRNA duplex to target survivin or a control scrambled (sc) RNA which does not bind to any known sequence. Cells were transfected for 24 h with a nucleofector device (Lonza, Colgne, Germany) according to the manufacturer's instructions. Cells were then examined by SDS-PAGE and Western blotting to ensure satisfactory protein knock-down. Transfected cells were then treated as required and analysed for apoptosis and G2/M arrest by flow cytometry.

2.9. Statistical analysis

The software Prism GraphPad was used to carry out statistical analysis on all samples using a Student's paired *t*-test. For illustrative purposes the *p* values are presented as $^*p < 0.05$.

3. Results

3.1. Flavopiridol synergistically enhances PBOX-induced apoptosis in CML cells

Flow cytometry demonstrated that K562 CML cells underwent a dose-dependent increase in apoptosis when treated for 18 h with PBOX-6 (1–5 μM) or PBOX-15 (100–500 nM) followed by a 24 h flavopiridol treatment (100–500 nM) when compared to either agent alone (Fig. 1A and B). Similar enhancements of PBOX-induced apoptosis were also seen in LAMA84 cells following the addition of flavopiridol (data not shown). In cells treated with PBOX-6/-15 alone, there was still a modest increase in the number of cells undergoing apoptosis 24 h after the removal of the PBOX compound from the cells, this induction of apoptosis was therefore deemed to be irreversible.

Previous combination studies involving flavopiridol and other chemotherapeutics have identified treatment order as being crucially important for apoptotic enhancement [33,34]. To determine if this was also the case with our combination treatment we decided to analyse the extent of apoptosis following treatment of cells with both compounds concurrently and in the reverse order (flavopiridol followed by PBOX-6). Results showed that cells treated with the compounds combined for 18 h ($P6 + flavo_{18}$) followed by no treatment (NT_{24}) underwent a lower percentage of apoptosis than those cells treated sequentially with 5 μM PBOX-6 followed by flavopiridol ($P6_{18}$,$flavo_{24}$), 18.7 ± 7.7% versus 54.8 ± 1.1% respectively (*p* value: 0.0435). Treatment with 5 μM

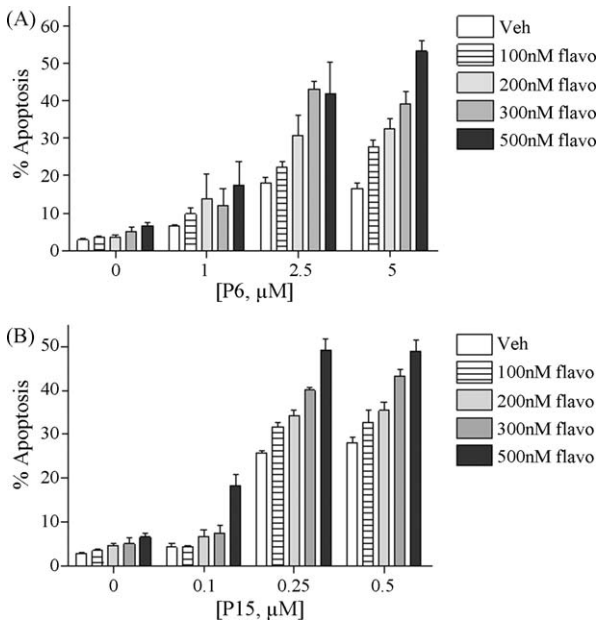

Fig. 1. Sequential treatment with PBOX-6/PBOX-15 and flavopiridol enhances apoptosis compared to either agent alone in K562 cells. K562 cells were treated for 18 h with PBOX-6 (P6) (1–5 μM) or PBOX-15 (P15) (0.1–0.5 μM), centrifuged and resuspended in fresh medium containing flavopiridol (flavo) (100–500 nM) for a further 24 h or treated with the relevant controls. After the required incubation period, cells were fixed in 1 ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the sub-G1 population. (A) PBOX-6 and (B) PBOX-15. Values represent the mean ± S.E.M. of three independent experiments.

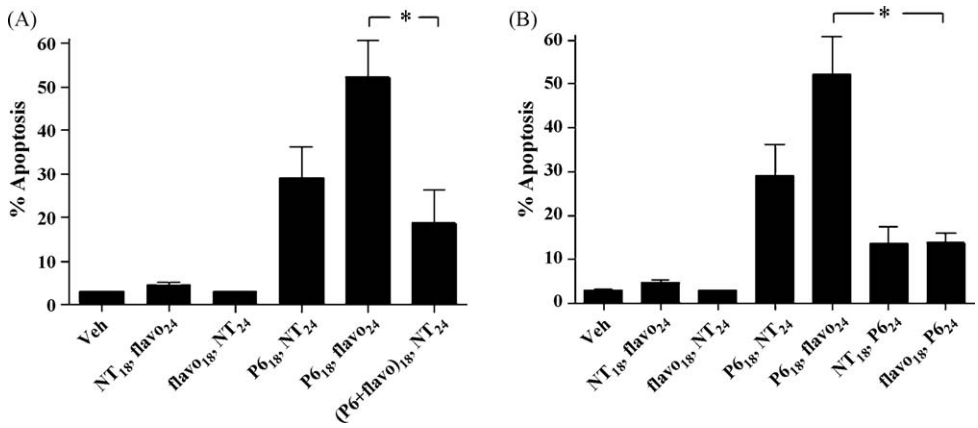

Fig. 2. Sequence-dependent administration of PBOX-6 and flavopiridol. K562 cells were treated with the indicated treatments or the relevant controls. After the required incubation period, cells were fixed in 1 ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the sub-G1 population. (A) Concurrent PBOX-6 (P6) and flavopiridol (flavo) treatment and (B) sequential treatment with flavopiridol followed by PBOX-6. Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired *t*-test, $^{*}p < 0.05$.

Table 1
CI values of PBOX-6/-15 in combination with flavopiridol in K562 CML cells.

	[PBOX, μM]	[flavo, μM]	Fa	CI value	Rating
K562	1	0.5	0.174	0.375	+++
P6	2.5	0.5	0.419	0.114	++++
	5	0.5	0.531	0.109	++++
K562	0.1	0.5	0.184	0.421	+++
P15	0.25	0.5	0.492	0.328	+++
	0.5	0.5	0.490	0.659	+++

Synergistic analysis was carried out using the software Calcusyn (Biosoft, U.K.). Data from flow cytometry experiments was used to analyse the interaction between PBOX-6 (P6)/PBOX-15 (P15) and flavopiridol (flavo) using an isobologram method described by Chou and Talalay. A CI value of >1, =1 and <1 indicates antagonism (−), an addictive effect (±) and synergism (+) respectively. Fa—fraction of cells affected by apoptosis.

PBOX-6 alone caused 29.2 ± 7.1% of cells to undergo apoptosis (Fig. 2A). Similarly, when the compounds were given in reverse order (flavopiridol first for 18 h followed by PBOX-6 for 24 h) cells underwent a lower percentage of apoptosis again compared to cells treated sequentially PBOX-6 followed by flavopiridol, 13.8 ± 2.3% versus 52.2 ± 8.6% apoptosis (*p* value: 0.0266) (Fig. 2B).

Median dose analysis showed that in all sequential PBOX-6/-15 and flavopiridol combinations tested in K562 cells, a CI value of less than 1 was obtained indicating synergism between the compounds (Table 1). Similar results were also obtained in LAMA84 cells following the sequential addition of flavopiridol to PBOX-treated cells (data not shown).

3.2. Flavopiridol enhances the apoptotic effect of the PBOX compounds in native and T315I Bcr-Abl cells

The development of the multi-inhibitor resistant T315I mutant within the Bcr-Abl gene during imatinib treatment has led to the need to develop approaches capable of overcoming this resistance phenotype. It was therefore desirable to determine if the novel PBOX/flavopiridol combination treatment may have potential in the treatment of T315I mutant cells. Murine Baf/3 cells transfected with the T315I mutant, which are completely resistant to imatinib, were used to test this hypothesis [15].

PBOX-6 (5 μM) treatment of Baf/3 cells expressing unmutated Bcr-Abl for 18 h followed by 24 h culture in drug-free medium induced 5.8 ± 1.5% apoptosis, which rose to 22.3 ± 5.4% (*p* value: 0.0378) when flavopiridol (500 nM) was added (Fig. 3A). Similar results were observed in T315I mutant cells with apoptosis increasing from 6.8 ± 0.6% to 29.0 ± 5.0% for the same treatments (*p* value: 0.0468) (Fig. 3B).

Likewise, exposure of Baf/3 cells expressing native and T315I mutant Bcr-Abl to PBOX-15 (0.5 μM) for 18 h followed by flavopiridol for 24 h resulted in an increase of apoptosis from 16.7 ± 7.3% to 35.3 ± 9.7% (*p* value: 0.0488) in native cells and from 12.5 ± 2.9 to 43.0 ± 6.1 in T315I cells (*p* value: 0.0109) (Fig. 3C and D).

3.3. An accelerated exit from G2/M transition with flavopiridol facilitates enhanced PBOX-induced apoptosis

Previously, CDK inhibitors such as flavopiridol have been shown to prevent MTA-induced endoreplication in human cancer cells [35]. We therefore examined whether flavopiridol reduces polyploidy induced by PBOX-6 in K562 cells and whether this contributes to the synergistic enhancement of PBOX-6-induced apoptosis by flavopiridol. Results showed that while flavopiridol did reduce PBOX-6-associated polyploidy in K562 cells, this reduction in the number of polyploid cells was not significant (*p* value: 0.1026) suggesting that this mechanism of action only plays a minor role in the flavopiridol enhancement of PBOX-induced apoptosis (data not shown).

Flow cytometry showed PBOX-induced apoptosis to be preceded by a G2/M arrest in K562 cells. The number of cells in the G2/M phase began to increase after 4 h reaching a peak after 18 h PBOX treatment of 66.7 ± 0.8% before dropping back to similar levels observed in vehicle samples after 42 h of 19.4 ± 2.5% (Fig. 4A). While the addition of flavopiridol to G2/M-arrested cells (18 h PBOX treatment) caused a significant increase in the number of cells undergoing apoptosis, cells treated with PBOX-6 for the longer period of 42 h (lack of G2/M arrest, Fig. 4A) and subsequently treated with flavopiridol did not undergo a significant enhancement of apoptosis (Fig. 4B).

In the absence of flavopiridol, removal of PBOX-6 caused a reduction in the number of cells in the G2/M phase of the cell cycle suggesting a requirement for PBOX-6 in maintaining G2/M arrest. Treatment with flavopiridol enhanced this exit of cells from the G2/M phase of the cell cycle (Fig. 4C). Specifically, the number of G2/M-arrested cells reduced from 56.3 ± 3.8% to 35.5 ± 4.2% following just 2 h of treatment with flavopiridol. This corresponded to a time-dependent increase in the number of cells undergoing apoptosis (Fig. 4D).

3.4. PBOX-6/flavopiridol combination treatment is accompanied by an enhanced downregulation of cyclin B1 and survivin and decreased CDK1/cyclin B1 activity

Combination studies in which cells were treated with PBOX-6 followed by flavopiridol showed an enhanced reduction in the

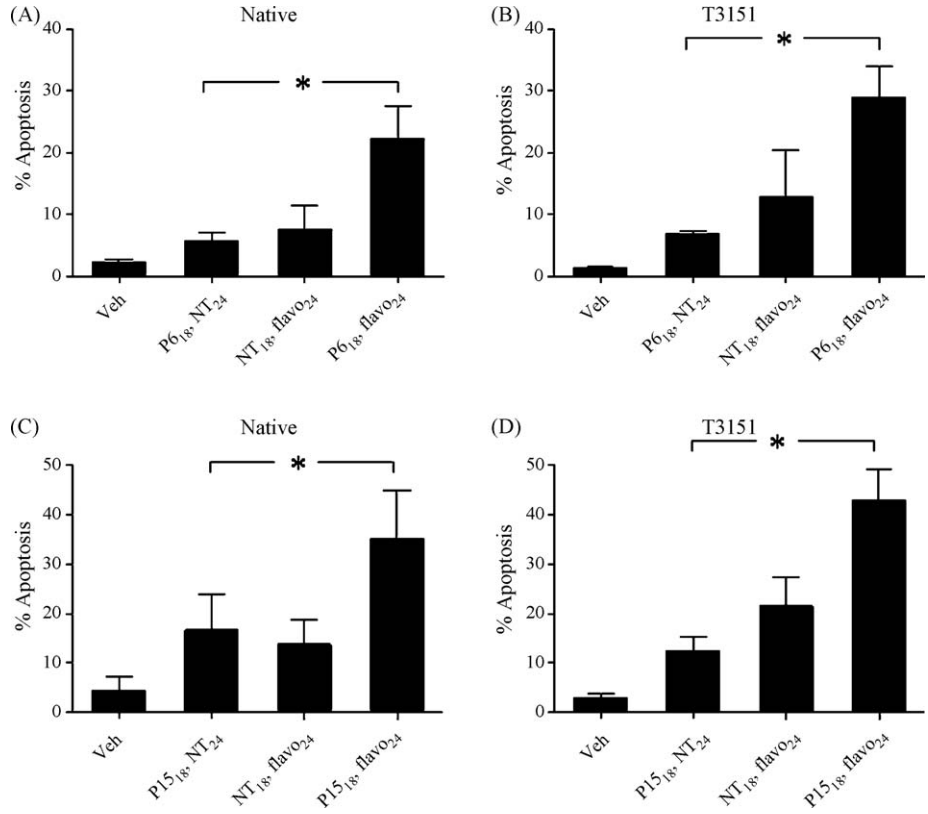

Fig. 3. Flavopiridol enhances the apoptotic effect of PBOX-6 and PBOX-15 in native and T315I Baf/3 CML cells. Native and T315I mutant Baf/3 cells were seeded during the log phase of growth and treated for 18 h with 5 μM PBOX-6 (P6) or 500 nM P15 (P15) followed by 24 h treatment with 500 nM flavopiridol (flavo) or the equivalent controls (NT). After the required incubation period, cells were fixed in 1 ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the sub-G1 population. Native cells (A and C) and T315I cells (B and D). Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired *t*-test, $^{*}p < 0.05$.

levels of cyclin B1 compared to cells treated with either compound alone (Fig. 5A). No significant reduction in the expression levels of CDK1 was observed following PBOX-6/flavopiridol treatment compared to either agent alone.

Survivin, a member of the IAP family, is known to be upregulated in the majority of cancers including CML. Activation occurs during mitosis via phosphorylation of Thr34 by the cell cycle complex CDK1/cyclin B1 and inhibition of this phosphoryla-

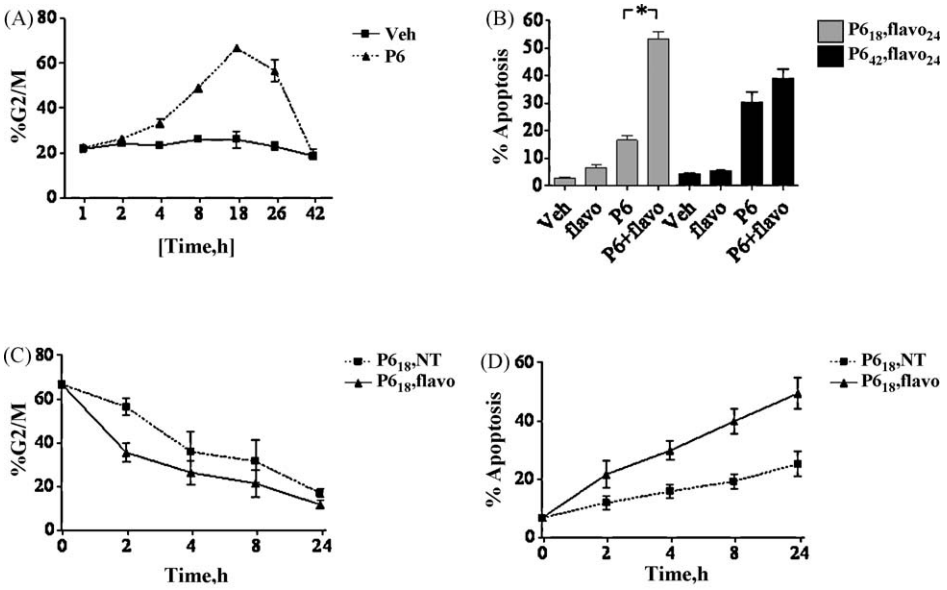

Fig. 4. An accelerated exit from G2/M transition with flavopiridol facilitates enhanced PBOX-induced apoptosis. K562 cells were treated for up to 42 h with 5 μM PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500 nM flavopiridol (flavo) for a further 24 h or treated with the required controls (NT). After the required incubation period, cells were fixed in 1 ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry for (A and C) the percentage of cells in the G2/M phase of the cell cycle and (B and D) apoptosis as determined by quantification of the sub-G1 population. Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired *t*-test, $^{*}p < 0.05$.

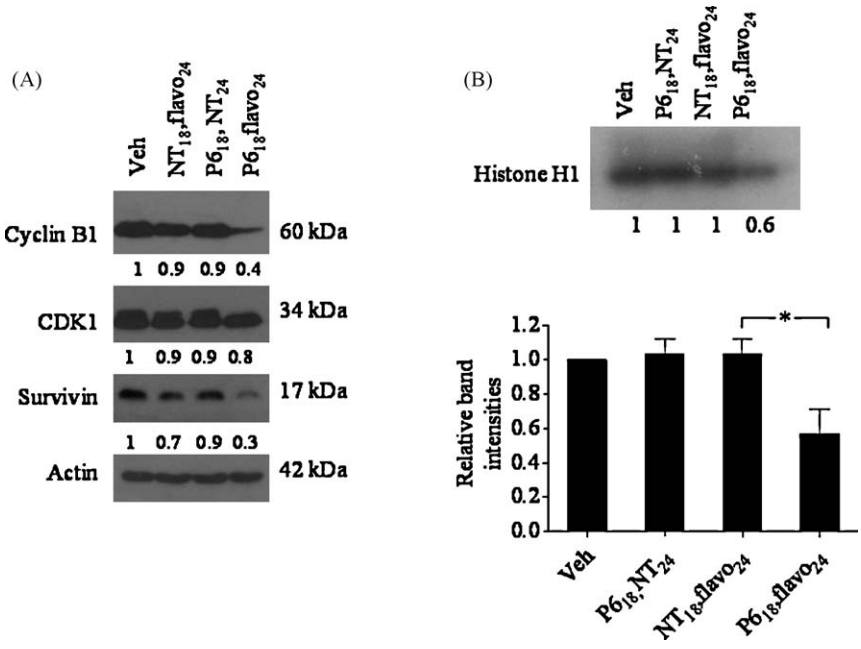

Fig. 5. PBOX-6/flavopiridol combination treatment is accompanied by an enhanced downregulation of cyclin B1 and survivin and decreased cyclin B1/CDK1 activity. K562 cells were treated for 18 h with 5 μM PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500 nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the required incubation period, samples were subjected to either (A) Western blotting and incubated with anti-cyclin B1, CDK1 or survivin mAbs overnight and enhanced with ECL, actin is shown as a loading control, band intensities were normalised by densitometry with respect to actin and expressed as fold change over vehicle control or (B) immunoprecipitation with anti-CDK1 Ab followed by a kinase activity assay using Histone H1 substrate and γ^{32}P-ATP, samples were then subjected to SDS-PAGE and detected by autoradiography, band intensities are expressed as fold change over vehicle control and depicted as a bar-graph. A representative blot/autorad of three independent experiments is depicted for each protein.

tion is associated with enhanced destruction of the protein. We hypothesised that PBOX-6, being an MTA, may have an effect on expression levels of survivin. We therefore looked at the role of survivin in PBOX-6-induced apoptosis. In the combination treatment, survivin levels were downregulated in comparison with samples treated with PBOX-6 or flavopiridol alone (Fig. 5A).

To test whether the reduced expression of survivin was correlated with a reduction of CDK1/cyclin B1 activity, we measured CDK1 activity in anti-CDK1 immunoprecipitates, using Histone H1 as a substrate. Indeed we found that CDK1 activity had significantly decreased following the combination treatment (Fig. 5B) when compared to samples treated with either PBOX-6 or flavopiridol alone (p value: 0.0198).

3.5. Inhibition of survivin by siRNA increases PBOX-6-induced apoptosis

To confirm the importance of survivin in PBOX-6-induced apoptosis, the survivin gene was knocked down for 24 h by transfection with siRNA sequences known to bind to and inhibit the targeted genes. Effective downregulation of the expression levels of the survivin protein was confirmed by Western blot analysis (Fig. 6A).

In cells treated with PBOX-6 alone, the number of apoptotic cells significantly increased from 25.0 ± 5.2% (scRNA) to 46.6 ± 1.0% (siRNA) (p value: 0.0380) in survivin knocked down cells, thus demonstrating the importance of survivin in inhibiting apoptosis. The number of apoptotic cells also increased from 51.7 ± 5.4% to 58.6 ± 7.56% in samples sequentially treated with 5 μM PBOX-6 and flavopiridol; however, this difference was not significant (p value: 0.0602) (Fig. 6B).

4. Discussion

We have recently developed a series of novel compounds known as the PBOXs that actively induce apoptosis in numerous cancer cell lines [9,40] and in primary CLL [14] and CML [15] cells. PBOX-6 has also been shown to display significant anti-tumour activity *in vivo* in an aggressive murine model of breast cancer [16] and a xenograft model of CML [15] which further highlights the

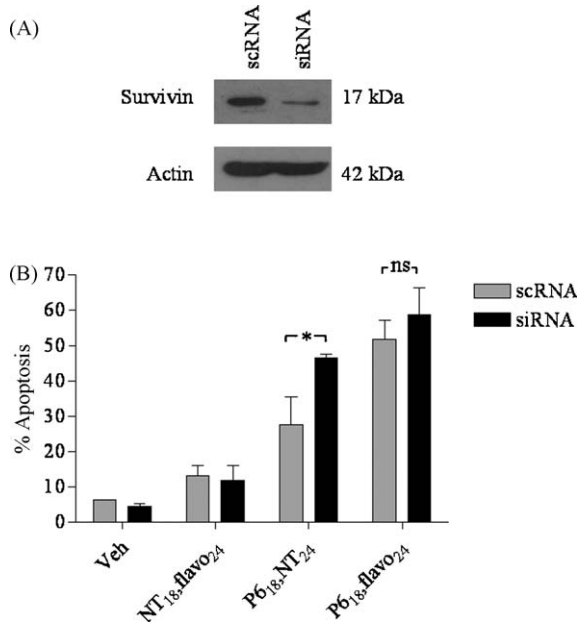

Fig. 6. Reduction in survivin levels by siRNA enhances PBOX-6-induced apoptosis. K562 cells were transfected with siRNA targeting survivin or a control scRNA for 24 h. Following this incubation, samples were analysed for (A) efficient protein knock-down by Western blotting. (B) Cells were then subsequently treated for 18 h with PBOX-6 (P6) followed by 24 h treatment with flavopiridol (flavo) or the equivalent controls (NT) and analysed by flow cytometry; apoptosis was quantified by gating cells in the sub-G1 population. Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, $^*p < 0.05$; ns, not significant.

potential of this novel class of compounds as anti-cancer agents. However, despite the potent activity displayed by the PBOXs against various cancer cell types a certain proportion of K562 CML cells remain resistant to PBOX-induced apoptosis. K562 cells are inherently resistant to a variety of chemotherapeutic agents due to the constitutive activation of Bcr-Abl and we have demonstrated that the Bcr-Abl kinase inhibitor imatinib enhances the apoptotic efficacy of the PBOXs in human CML cells [17]. However, as a significant proportion of CML patients are now gaining resistance to imatinib it was decided to examine if alternative chemotherapeutics could similarly enhance the effects of the PBOXs.

Apoptosis was synergistically enhanced in PBOX-treated CML cells following the sequential addition of flavopiridol in a dose-dependent manner as determined by flow cytometry and median dose analysis. When flavopiridol was administered prior to or concomitantly with PBOX-6/-15, no enhancement of apoptosis was observed when compared to cells treated with PBOX-6 alone. Our results therefore suggest that the order in which PBOX and flavopiridol treatments take place in CML cells is critical for mediating enhanced apoptosis. This is in agreement with previously published papers showing flavopiridol treatment to be highly sequence-dependent particularly when combined with MTAs such as paclitaxel [34]. In A549 lung carcinoma cells, sequence-dependent synergy was also observed with cytarabine, topotecan, doxorubicin, and etoposide when followed by flavopiridol treatment [33].

The T315I mutation is one of the most common Bcr-Abl mutations found in CML patients following imatinib treatment and is largely resistant to imatinib and the 'second generation' tyrosine kinase inhibitors dasatinib and nilotinib. The pro-apoptotic PBOX compounds have previously been shown to significantly induce apoptosis to a similar extent in native and T315I Bcr-Abl positive cells [15]. Our combination of PBOXs/flavopiridol also significantly increased the percentage of cells undergoing apoptosis in these cells compared to either compound alone suggesting the potential of this novel combination in the treatment of imatinib-resistant CML.

Previous research has shown that in response to MTAs, cells enter mitosis, transiently arrest and then exit without undergoing cytokinesis. A compromise in the G1 checkpoint (e.g. cells lacking p53 or pRb) results in the continuous activation of CDK2/cyclin E that leads to the endoreplication, resulting in the accumulation of polyploid cells with a >4N DNA content [35]. Flavopiridol has been shown to prevent endoreplication and development of polyploidy in cells with defective G1/S checkpoints [35]. K562 cells are p53-deficient and therefore have a compromised spindle checkpoint. As we had previously shown that PBOX-6 increases the number of polyploid cells in CML cell lines [41], it was of interest to see if flavopiridol administration could reduce this effect. Results showed that while the number of polyploid cells formed was reduced with PBOX-6/flavopiridol treatment when compared with cells treated with PBOX-6 alone, this reduction was not significant suggesting only a minor role in the mechanism through which flavopiridol synergistically enhances MTA-induced apoptosis.

Analysis of cell cycle data revealed that CML cells had undergone a G2/M arrest prior to the onset of apoptosis following treatment with PBOX-6, peaking at 18 h. The subsequent addition of flavopiridol to these PBOX-arrested samples resulted in fewer cells in the G2/M phase when compared to cells left in drug-free medium suggesting that flavopiridol accelerates the exit of cells from G2/M. Longer durations of PBOX treatment (42 h) followed by flavopiridol were not as effective, as the majority of cells treated for 42 h with PBOX-6 had already progressed through this mitotic block. This result is in agreement with a previous report demonstrating that flavopiridol facilitates exit from mitotic block induced by paclitaxel in the human gastric cancer cell line, MKN-74 [34]. It also provides an explanation for the sequential dependency of the PBOX/flavopiridol combination treatment and for the ineffectiveness of the concurrent or reverse combination.

Progression through the G2/M phase is regulated by the complex of CDK1 and cyclin B1. It was therefore necessary to examine whether PBOX-6/flavopiridol affects these cell cycle-related proteins. We found that the PBOX-6/flavopiridol combination treatment was accompanied by an enhanced down-regulation of cyclin B1 and decreased CDK1/cyclin B1 activity compared to either drug alone. Since CDK1/cyclin B1 is responsible for regulating survivin, an inhibitor of apoptosis and mitotic regulator, we hypothesised that the sequential addition of flavopiridol to PBOX-treated cells enhanced survivin degradation by inhibiting the CDK1/cyclin B1 complex thus enhancing the levels of apoptosis. In support of this, in K562 cells where survivin was knocked down using siRNA, the level of apoptosis observed was significantly enhanced after administration of PBOX-6 when compared with cells transfected with a scrambled scRNA. This is in agreement with other reports demonstrating an enhancement of apoptosis by chemotherapeutic agents in cells where survivin has been silenced [42]. It is therefore likely that enhanced degradation of survivin through inhibition of the CDK1/cyclin B1 complex plays a key role in PBOX-6/flavopiridol-induced apoptosis. Thus the accelerated inhibition of CDK1/cyclin B1 by flavopiridol prevents the phosphorylation and accumulation of survivin, removing a viability checkpoint through which it enhances apoptosis. Transcriptional repressors of survivin such as YM155 [43,44] and EM-1421 (Terameprocol) [45] have recently displayed activity in clinical trials demonstrating the importance of targeting survivin as part of an anti-cancer strategy. This is the first account of the involvement of survivin in PBOX-induced apoptotic pathways.

The molecular basis of flavopiridol-mediated anti-cancer activity has not been fully elucidated to date. It may therefore also involve mechanisms unrelated to inhibition of CDK1 activity including broad suppression of gene transcription by interfering with CDK9/cyclin T function [46]. Furthermore, flavopiridol functions as a relatively broad inhibitor of various CDKs as well as non-cell cycle regulated kinases [46], thus potentially producing a wider range of effects on cell cycle progression, gene transcription and on other signalling pathways. Despite these possible limitations, results presented herein demonstrate that one of the mechanisms by which flavopiridol enhances the pro-apoptotic effect of the PBOXs may involve loss of survivin expression. This model is consistent with other reports demonstrating the ability of flavopiridol to reduce the expression of other IAP family proteins [47].

In conclusion, we have demonstrated the ability of flavopiridol to synergistically potentiate the apoptotic efficacy of the pro-apoptotic PBOX compounds PBOX-6 and PBOX-15 in CML cell lines. Potentiation of apoptosis was highly sequence dependent. Flavopiridol reduced the number of polyploid cells formed following PBOX-6-induced apoptosis and while this was not statistically significant in the CML cells tested, it may have contributed somewhat to the enhanced levels of apoptosis. Mechanistically, the enhanced downregulation of cyclin B1, decreased activity of CDK1/cyclin B1 activity and corresponding degradation of survivin were found to be critically important events through which flavopiridol enhanced PBOX-induced apoptosis. The results obtained in this report represent the first example of successful combination of the PBOX compounds with a CDK inhibitor and highlight its potential as an effective strategy in treating multi-kinase resistant CML.

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