Synergistic inhibition of melanoma xenografts by Brequinar sodium and Doxorubicin

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ABSTRACT

Malignant melanoma continues to be a fatal disease for which novel and long-term curative breakthroughs are desired. One such innovative idea would be to assess combination therapeutic treatments – by way of combining two potentially effective and very different therapy. Previously, we have shown that DHODH inhibitors, A771726 and Brequinar sodium (BQR) induced cell growth impairment in melanoma cells. Similar results were seen with DHODH RNA interference (shRNA). In the present study, we showed that combination of BQR with doxorubicin resulted in synergistic and additive cell growth inhibition in these cells. In addition, in vivo studies with this combination of drugs demonstrated an almost 90% tumor regression in nude mice bearing melanoma tumors. Cell cycle regulatory proteins, cyclin B1 and its binding partner pcdc-2 and p21 were significantly downregulated and upregulated respectively following the combined treatment. Given that we have observed synergistic effects with BQR and doxorubicin, both in vitro and in vivo, these drugs potentially represent a new combination in the targeted therapy of melanoma.

1. Introduction

Malignant melanoma is a melanocyte neoplasm that usually takes place in the skin and occasionally harbors mutations in genes including BRAF [1,2]. Each year, the number of estimated new cases of malignant melanoma is 132,000 and approximately 48,000 patients die from malignant melanoma globally. When malignant melanoma is diagnosed at the early stage (stage 0/I), the patient’s 5-year survival rate is more than 90% after surgical excision. However, when early detection fails, it tends to invade deeply and metastasize to lymph nodes and other organs. Median overall survival rate for melanoma patients with metastasis is less than 1 year [3]. The treatment of metastatic melanoma is rapidly evolving. The potent and specific BRAF inhibitors vemurafenib and dabrafenib, as compared with cytotoxic chemotherapeutic drugs, have significantly improved response rates, along with progression-free and overall survival in patients with metastatic melanoma with BRAF V600E or V600K mutations [4,5]. However, acquired resistance to BRAF inhibitors frequently develops through reactivation of the mitogen-activated protein kinase (MAPK) pathway, resulting in a median progression-free survival of 6 to 8 months [5-8]. In addition, the use of BRAF inhibitors may result in the development of secondary skin tumors, originating from a paradoxical activation of the MAPK pathway in cells without a BRAF mutation [4,9-14]. Given that the majority of BRAF inhibitor resistance occurs through reactivation of MAPK, several potent, non-ATP competitive MEK inhibitors were developed (eg, trametinib and cobimetinib) [15] that show no off-target effects on other kinases and are currently being used in clinical trials. MEK inhibitors are able to target MAPK-dependent tumors and exhibit distinct efficacies against BRAF- and KRAS-mutant melanomas [16,17]. Studies reveal that BRAF inhibitor-resistant tumor cells are highly sensitive to MEK inhibition and demonstrate that targeted pharmacological MEK inhibition may be a highly effective therapeutic alternative in BRAF inhibitor-resistant melanoma [17]. In light of these findings, focus has turned to dual inhibition of BRAF and its downstream target, MEK [18].

The results of combination therapy of MEK inhibitors and BRAF inhibitors are promising. New clinical trials confirm improved PFS progression-free survival with concurrent inhibition of BRAF and MEK. A Phase III, randomized study, compared patients with BRAF-mutated melanomas treated with vemurafenib 960 mg twice daily versus vemurafenib plus cobimetinib 60 mg daily for 21 days followed by 7 days off. The group on combination therapy displayed prolonged median PFS (9.9 months vs 6.2 months). The latest Phase III clinical trial of dabrafenib (BRAF inhibitor) and trametinib versus monotherapy with vemurafenib showed increased median PFS (11.4 months vs 7.3 months;
HR 0.56; 95% confidence interval [CI]: 0.46–0.69; P = 0.001) and improved overall survival at 12 months with combination therapy (72% vs 65%; HR 0.69; 95% CI. 0.53–0.89; P = 0.005) [19]. Despite these clinical benefits, the emergence of resistance to BRAF inhibitors and eventually to MEK inhibitors restricts the therapeutic efficacy of these kinase inhibitors. In an attempt to overcome this resistance, more potential molecules targeting other aberrant signalling pathways are in clinical development [20,21].

We have previously shown that DHODH inhibitors such as A771726 (Leflunomide metabolite) and Brequinar sodium (BQR) expose their anti-proliferative effects on melanoma, myeloma and lymphoma cells. These inhibitors arrested cancer cells growth in S-phase, decreased c-Myc and increased p21 protein expression [22]. Furthermore, DHODH suppression by shRNA affected cell proliferation, c-Myc and its target protein, p21 in melanoma cells. In this study, we sought to investigate the effect of combining BQR and doxorubicin in vitro and in human melanoma xenograft. The combination treatment further sensitized the in vitro and in vivo melanoma tumor growth.

2. Materials and methods

2.1. Cells

Human melanoma (A375) cell line was obtained from American Type Cell Collections. A375 cells were grown in DMEM (Sigma, USA). The medium was supplemented with 10% heat-inactivated fetal bovine serum (Sigma, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, USA). Cells were maintained in a 5% CO₂ atmosphere at 37 °C.

2.2. Drugs and antibodies

A771726, BQR and PLX 4720 were pharmaceutical standard with a purity of 99.50%, 98.80% and 99.55%, respectively and these drugs were synthesized at Aurigene Discovery Technologies Limited, Bangalore, India. Doxorubicin was purchased from Celon Labs, India. Antibodies for cyclin B1, pcdc-2 and p21 and loading control, GAPDH were procured from Cell Signaling Technology, USA. Secondary antibody, anti-rabbit polyclonal antibody was secured from Sigma, USA.

2.3. Cell proliferation assay

A375 cells were seeded in 96-well plates at 1000 cells per well. The DHODH inhibitors - A771726 and BQR were dissolved in Hybrimax DMSO (Sigma, USA) whereas doxorubicin was dissolved in sterile distilled water. The additive or synergy effect between BQR and doxorubicin was determined using a non-fixed ratio method in which fixed concentrations of doxorubicin (0.3, 0.15, 0.075, 0.00375, 0.001875 and 0.0009375 μM) were added with increasing concentrations of BQR (30.0, 10.0, 3.33, 1.11, 0.37, 0.123, 0.041 and 0.014 μM) and incubated for 72 h. Each plate contained control wells for vehicle (0.5% DMSO), BQR alone and doxorubicin alone in triplicates. Cell viability was determined using Alamar blue assay. Cells were treated with 50 μl/well of 1 mg/ml resazurin (Sigma, USA) dissolved in PBS (Sigma, USA). The fluorescence reading was measured at Ex/Em of 531/595 nm. Cell viability was calculated as a percentage of fluorescence or absorbance measured in the treated wells relative to the DMSO control wells. Experiments were repeated three times and mean ± SE was calculated.

2.4. Assessment of the effect of combined drug treatments in vitro

The effect of combined drug treatment of BQR and doxorubicin were examined using the median effect analysis method described by Chou and Talalay [23]. Calculation of combination index (CI) was performed using Compusyn software (Chou-Talalay, ComboSyn) to determine whether a combination is synergistic, additive, or antagonistic by taking into account the entire shape of the growth inhibition curve [23]. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations.

2.5. In vivo toxicity assessment of BQR and doxorubicin

MTD is defined as the highest tolerable dose which does not produce major life threatening toxicity for the study duration [24]. Toxicity assessment of BQR and doxorubicin were performed using CD-1 mice, which are commonly used in toxicology and cancer research [25–27]. The MTD doses in this study were selected based on previous findings of the efficacy of BQR and doxorubicin in CD-1 mice [43–46]. Single dose of BQR (20 mg/kg) and doxorubicin (1 mg/kg) as well as in combination: BQR + doxorubicin (10 mg/kg + 1 mg/kg), BQR + doxorubicin (20 mg/kg + 1 mg/kg) were tested. Toxicity study was conducted in 5 mice per group for 14 days. Drugs were administered to CD-1 mice orally or intravenously (lateral tail vein) and toxicity was evaluated based on clinical symptoms such as lethargy, loss of body weight, diarrhoea and behavioural changes. All animal experiments were performed in accordance with the animal care and use protocol (Aurigene/IAEC/ PCD/018 E-13/09-2014) approved by the Institutional Animal Care and Use Committee, Aurigene Discovery Technologies.

2.6. Establishment of tumor xenograft

Athymic nude mice, aged 5 to 6 weeks, and weighing approximately 18–20 g from Harlan laboratories (Hyderabad, India) were housed in individually ventilated cages and were fed ad libitum with reverse osmosis treated water and gamma irradiated rodent pellet. A375 xenografts were established by subcutaneous injection of 5 × 10⁵ A375 cells suspended in 150 μl of 1:1 ratio of Hank’s balanced salt solution and extracellular matrix. The mixture was injected onto the right flank of the mice. The individual animal body weights were recorded daily before the administration of test items and the tumor volumes were measured thrice weekly. All animal experiments were performed in accordance with the animal care and use protocol (Aurigene/IAEC/PCD/018 E-13/09-2014) approved by the Institutional Animal Care and Use Committee, Aurigene Discovery Technologies.

2.7. Assessment of the effect of combined drug treatments in tumor xenograft

When the mean tumor volume reached 100–150 mm³ in size, animals were randomized based on tumor volumes into seven groups and allocated nine animals for each group. The treatment groups are as following: (i) Vehicle control (diluent control), (ii) BQR (10 mg/kg), (iii) BQR (20 mg/kg), (iv) Doxorubicin (1 mg/kg), (v) BQR + Doxorubicin (10 mg/kg + 1 mg/kg), (vi) BQR + Doxorubicin (20 mg/kg + 1 mg/kg) and (vii) PLX 4720 (Vemurafenib), a BRAF inhibitor (20 mg/kg). BQR was formulated in 0.5% CMC + 0.25% tween (20 mg/kg + 1 mg/kg) and (vii) PLX 4720 (Vemurafenib), a BRAF inhibitor (20 mg/kg). BQR was formulated in 0.5% CMC + 0.25% tween 20 and administered orally once every alternate day, Doxorubicin was formulated in 5% dextrose and administered intravenously once every alternate day. Whereas, PLX 4720 was formulated in 5% DMSO + 1% methyl cellulose and administered orally once per day. The formulations were prepared fresh and used immediately. Body weights were monitored every day and tumor volumes were measured thrice weekly. The tumor volumes were measured using the formula: [(A (length) x B (width))²]/2 [28]. The experiment was stopped at day 14, and all mice were sacrificed. The percentage of tumor growth inhibition was calculated using the following formula:

\[ \text{vehicle control TV} \text{ (day n – day 1)} - \text{[drug TV} \text{ (day n – day 1)}] / \text{[vehicle control TV} \text{ (day n – day 1)}] \times 100, \text{where TV} = \text{tumor volume and n = day of treatment} \]
2.8. Tumor lysate protein extraction and immunoblotting

Excised tumors were minced and suspended in tissue protein extraction reagent (Cell lysis buffer) (Cell Signaling Technology, USA) supplemented with protease and phosphatase inhibitor cocktails (Sigma, USA) and homogenized. The homogenized tissue was transferred to a pre-chilled microcentrifuge tube, incubated on ice for 10 min and then centrifuged at 15,000 x g for 20 min at 4 °C. The supernatant containing total cellular proteins was collected for immunoblotting analysis. Extracted protein was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Equal amount of protein was subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (GE Healthcare, USA). The membrane was blocked with Tris-buffer saline with 0.1% Tween-20 (TBST) containing 5% dry milk (Cell Signaling, USA). After blocking, the membrane was probed with appropriate primary antibodies overnight at 4 °C. On the next day, the binding of the primary antibodies was detected with anti-rabbit polyclonal secondary antibody labelled with horseradish peroxidase. The blot was developed with ECL Western blotting detection system (Pierce, USA) and subsequently this band was exposed to X-ray film. The intensity of each protein band was measured with ImageJ.

2.9. Statistical analysis

Statistical significance of the differences between control and treatment was analyzed with Student’s t-test and one-way ANOVA with Dunnett’s or Tukey’s Multiple Comparison Tests, where applicable using GraphPad Prism 6.0. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 were considered statistically significant.

3. Results

3.1. BQR in combination with doxorubicin enhanced melanoma inhibition

A375 cells were incubated with BQR or A771726 in combination with doxorubicin for 72 h. Synergistic or additive effects were achieved in most combinations except for BQR with 0.009375 μM doxorubicin. Combination of BQR with doxorubicin (Fig. 1A and B) showed greater dose-dependent proliferation inhibition than BQR alone (approximately 56.25 ± 2.6% inhibition at 30 μM) and doxorubicin alone (approximately 83.25 ± 4.33% at 0.3 μM) in A375 cells. Significant proliferation inhibition was achieved with 30 μM BQR in combination with 0.075 μM; 0.15 μM and 0.3 μM doxorubicin, with the percentage of inhibition of approximately 78.0 ± 6.03%; 91.25 ± 4.87% and 97.25 ± 0.63%, respectively in comparison to treatment with BQR alone. Whereas, the percentages of inhibition for treatment with 0.3 μM doxorubicin in combination with 30 μM and 10 μM BQR were approximately 97.25 ± 0.63 and 96.75 ± 0.85%, respectively.

3.2. BQR in combination with doxorubicin was well tolerated in mice

In vivo toxicity assessment of the single and combination of BQR and doxorubicin drugs was performed in CD-1 mice for a period of 14 days and mice weight was recorded (Fig. 2A). The tested doses of BQR and doxorubicin were well tolerated and no treatment related clinical signs or mortality was observed. Non-treated mice demonstrated a weight gain of 8% at the end of day 14. Instead of weight loss, mice with single treatment displayed a weight gain between 4-4.6% whereas mice with combination treatment saw a weight gain of no more than 1.4% (Fig. 2B).

3.3. Brequinar sodium potentiates anticancer effect of doxorubicin in A375 xenografts

The in vitro synergistic effect of BQR and doxorubicin was further evaluated for their anti-tumor activity in A375 xenograft model in athymic nude mice. PLX 4720 (Vemurafenib), a BRAF inhibitor approved for the treatment of late-stage melanoma [29] was used as the positive control. After 14 days of treatment, the tumor volume of mice administered with vehicle was 1090.0 ± 134.7 mm³ (Fig. 3A). By contrast, the tumor volumes of mice -administered with 20 mg/kg BQR; 10 mg/kg BQR + 1 mg/kg doxorubicin; 20 mg/kg BQR + 1 mg/kg doxorubicin and PLX 4720 were significantly lower than those of the vehicle control animals with tumor volumes of 404.6 ± 105 mm³; 388.6 ± 55.6 mm³; 217.2 ± 52.1 mm³ and 287.8 ± 50.7 mm³, respectively (Fig. 3A). However, there were no significant differences in the tumor volume of animals administered with single agent in comparison to the combination drugs as well as for those animals given combination drugs in comparison with PLX 4720. This is likely because of the large variances in the tumor volume between groups.

To examine further the effectiveness of the drug treatment, the percentage tumor growth inhibition (TGI) was calculated relative to the vehicle control group. At the end of 14 days treatment, mice treated with 10 mg/kg and 20 mg/kg BQR as a single agent reduced tumor growth by 52.7 ± 6.5% and 70.6 ± 10.5%, respectively whereas doxorubicin at 1 mg/kg single agent suppressed tumor growth by only 36.1 ± 4.7% relative to the vehicle control (Fig. 3B). The positive control drug, PLX 4270 resulted in tumor growth inhibition (TGI) of 82.7 ± 4.6% relative to the vehicle control. The percentage TGI with 10 mg/kg BQR + 1 mg/kg doxorubicin (72.2 ± 5.2%) was significantly higher than that of treatment with 10 mg/kg BQR alone (52.7 ± 6.5%). Similarly, the percentage TGI with 20 mg/kg BQR + 1 mg/kg doxorubicin (89.9 ± 4.5%) was significantly higher than that of the treatment with 20 mg/kg BQR alone (70.6 ± 10.5%). The combination of 10 mg/kg BQR with 1 mg/kg doxorubicin, however, yielded a TGI of 72.2 ± 5.2% only compared to the TGI of PLX 4270 (82.7 ± 4.6%). Nevertheless, the combination of 20 mg/kg BQR with 1 mg/kg doxorubicin (89.9 ± 4.5%) resulted in higher percentage TGI compared to PLX 4270 (82.7 ± 4.6%). Analysis with Compusyn showed that doxorubicin co-administered with BQR at higher dosage synergistically inhibited tumor growth (Fig. 3C). This combination not only inhibited tumor growth more effectively but also reduced vascularlarity in the tumor compared to vehicle control, BQR, doxorubicin and PLX 4720 alone, (Fig. 4A and B). The maximal body weight change of the treated mice was below 10% compared to the vehicle control treated mice, indicating a lack of cumulative toxicity during treatment (Fig. 5).

3.4. Combination of brequinar and doxorubicin treatment target cyclin B1, pdc-2 and p21 in A375 xenografts

Previously, we have shown that treatment with BQR primarily induced S-phase and G2/M cell cycle arrest and p21 protein upregulation in vitro. Here, we showed that single treatments of BQR downregulated cyclin B1 expression by approximately 1.9-fold when compared to the vehicle control group. This protein was further decreased with the combination of 10 or 20 mg/kg BQR + 1 mg/kg doxorubicin as illustrated in Fig. 6A. Notably, the combination treatments significantly suppressed the expression of cyclin B1 by 2.2-4.0-fold compared to the single treatment of 20 mg/kg BQR; by 10-12.2-fold compared to doxorubicin treatment alone and by 3.4-3.8-fold compared to vehicle control (Fig. 6A). Interestingly, treatment with doxorubicin and PLX 4720 alone did not reduce the expression of this protein.

To understand further, the binding partner of cyclin B1, pdc-2 expression was also examined. The data presented in Fig. 6B shows pdc-2 expression was downregulated in all treatment groups except doxorubicin when compared to the vehicle control group. The expression of pdc2 was markedly downregulated by 5-20-fold in the presence of 10 mg/kg BQR + 1 mg/kg doxorubicin as compared to its respective single agents and vehicle control. Similarly, this downregulated trend of pdc2 expression was observed in the combination treatment of 20 mg/
kg BQR + 1 mg/kg doxorubicin, albeit insignificant.

The expression of the cell cycle regulatory protein, p21 in all single except doxorubicin and combination treatments were decreased as compared to the vehicle control. Nevertheless, as hypothesized, the expression of this protein was significantly upregulated by 0.3-0.5-fold in both the combination treatments as compared to treatment with BQR alone (Fig. 6C). Intriguingly, this protein was not detected with PLX4720 (Fig. 6C and D).

4. Discussion

Acknowledged as one of the most aggressive skin cancers, melanoma has limited therapeutic options and its occurrence is steadily and rapidly increasing. While this is true, there has been a host of discoveries and applications of new targeted therapy agents that have brought about significant benefits. Nevertheless, resistance to chemotherapy as well as undesirable side effects remain as a monumental challenge in the clinical treatment of malignant melanoma [30]. Past studies using cell lines derived from metastatic lesions resulted in cells becoming resistant to both MEK and PI3K inhibitors when grown in 3D culture.
culture. Similar outcome was also observed in in vivo studies, where MEK inhibitor, AZD6244 led to the stabilisation of established human melanoma xenografts, but not tumor regression [31]. In other words, as the melanomas progress, there seem to be functional redundancy between the various signalling pathways in these cells [32]. Hence, targeting multiple signalling pathways simultaneously could be a better strategy to induce melanoma regression.

We have previously shown that Brequinar (BQR) produces anti-proliferative effect on melanoma cells by inducing cell cycle arrest via the DHODH-independent pathway that is associated with p21 up-regulation and c-Myc down-regulation in vitro. In addition, we have also demonstrated that exogenous uridine was able to completely reverse the anti-proliferative effect mediated by BQR at all concentrations [22]. Hence, targeting multiple signalling pathways simultaneously could be a better strategy to induce melanoma regression.

The FDA approved melanoma inhibitors such as pembrolizumab targeting the PD-1 (programmed cell death protein 1), ipilimumab targeting the CTLA-4, cytotoxic T lymphocyte-associated antigen 4, MEK inhibitor, trametinib and BRAF inhibitors, vemurafenib and dabrafenib represent an important milestone in more effective treatment of advanced melanoma [4,19,33–36]. Nevertheless, it is clear that the clinical use of these single-agent therapies have limitations. For example, ipilimumab only showed 4.5% objective response rate when used alone in a Phase II clinical trial [37]. The efficacy of vemurafenib and dabrafenib lasts only 6–8 months before the disease relapses especially in patients with metastatic melanoma [38]. With that in mind, rational combination approaches are strongly preferred in order to improve the overall patient progression-free survival (PFS), overcome or delay the development of multi-drug resistance and reduce the incidents of side effects [39–42].

In the present study, the anti-tumor effect of BQR alone and in combination with doxorubicin, an inhibitor of topo-isomerase 2 [43] was investigated. Preclinical studies of the maximum tolerated dose of BQR in several animal species have been reported previously. For instance, a dose of 193 mg/m² per day induced a 10% lethality in mice for a period of 5 days every 4 weeks. Furthermore, preclinical studies in dogs showed that they were only able to tolerate a maximum dose of 6 mg/m² per day on the same schedule [44]. Today, phase I of clinical trials with anticancer drugs recommend dosages for humans to be one third of that (13) or 2 mg/m². However, previous reports have stated that doses of 100 mg/m² (i.v.) daily for 5 days every 4 weeks had been safely administered to patients with advanced cancer and minimal prior treatment [45]. Building on from this knowledge, 2 doses of BQR were selected for this study, 10 mg/kg and 20 mg/kg.

Doxorubicin is a widely used antineoplastic agent for many cancer types. However, it is ineffective against melanoma cells due to the frequent development of resistance [46–48]. In addition to its role as a RNA-synthesis inhibitor, He et al. [49] reported that doxorubicin significantly reduced both protein and mRNA expression of DHODH in U1690 cells in a concentration-dependent manner. Further to this,
various studies have also reported that doxorubicin in combination with inhibitors such as berberine (in mouse xenograft) \[50\] and benzodiazepinedione \[51\] demonstrated a better therapeutic effect than either single drug alone.

Our findings show that BQR in combination with doxorubicin synergistically suppressed the growth of A375 cells both in vitro and in vivo within 14 days of prolonged exposure to these inhibitors without any noticeable toxic profiles. These results provide the first in vitro and in vivo evidence that BQR co-administered with doxorubicin can potentiate the RNA-synthesis inhibitor effect via the DHODH pyrimidine pathway and as such, may provide promising therapeutic benefit as a combination therapy for melanoma. The rationale of targeted therapy with multiple drugs (combined treatment) has been actively discussed and embraced in recent years. Brown et al. demonstrated that a combination of leflunomide and doxorubicin sensitized xenograft tumors derived from MDA-MB-231 cell lines. Moreover, our in vivo studies used a dosage of doxorubicin (1 mg/kg) that is approximately 0.1 times the recommended human dose based on body surface area \[52\]. The dosing schedule in this study is fairly short in comparison to various other reports. It is one that has been designed to achieve a prolonged drug exposure in an outpatient clinical setting, potentially yielding superior antitumor results.

To further elucidate the underlying mechanism of melanoma xenograft inhibition, we analysed the expression profiles of important cell cycle regulator proteins such as p21, cyclin B1 and p-cdc-2. Interestingly, a clear and significant downregulation was seen in the expression of cyclin B1 and p-cdc-2 in the combined treatment. Correspondingly, the cell-cycle regulatory protein, p21 was observed to be substantially upregulated in both the combined treatments. In the cell cycle of eukaryotes, activation of cyclin B1-cdk 1 (cyclin dependent kinase 1) also known as cdc-2 brings the onset of mitosis. Cyclin B1 is involved in the transition from G2 to M phase however it becomes unregulated in cancer cells where it is often found overexpressed. Overexpression of cyclin B1 thus leads to uncontrolled cell growth \[53–59\]. Alternatively, cdc-2 phosphorylation inhibits cell differentiation at the G2/M phase that leads to an arrest in the entry of eukaryotic cell into mitosis \[60\].

The suppression of cell proliferation by upregulation of p21 expression is a prominent mechanism activated by several chemotherapy drugs that cause genotoxic stress \[61\]. As determined in this study, the suppressed expression of cyclin B1 and pcdc-2 with concomitant increase in the expression of p21 protein especially in the combined treatment further reaffirms our earlier in vitro findings \[22\] which suggest that these inhibitors arrest cancer cell proliferation primarily at S-phase and G2/M phase. Nevertheless, our observation of a reduction in p21 expression in all treatments compared to vehicle control in general, but an increase with combination treatment, leads us to the possibility of the dual role of p21 in oncogenesis as described by Cmielova et al. \[62\]. They suggest that the expression of p21 in cancer

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**Fig. 4.** Combination of BQR and doxorubicin achieved greater therapeutic effect in melanoma xenograft in vivo. A, Representative images of the location of tumor xenografts in atymic nude mice and B, tumor regression at the end of the study. Brequinar and doxorubicin are abbreviated as BQR and Doxo, respectively.

**Fig. 5.** Percentage of body weight change in A375 xenograft mouse model during the treatment period. Mice were weighed daily and their body weight was expressed as a percentage of body weight change. Brequinar and doxorubicin are abbreviated as BQR and Doxo, respectively.
cells depends on the extent of the DNA damage. Low-level DNA damage leads to high expression of p21 and it induces cell cycle arrest whereas extensive DNA damage decreases the amount of p21 and the cancer cells undergo apoptosis. As such, the role of p21 is context dependent as it can act as pro-apoptotic or anti-apoptotic in different conditions [63]. With this in mind, these results suggest that the upregulation of p21 is a pivotal mechanism for the enhanced suppression of melanoma cells.

5. Conclusion

Findings from the present study revealed combination treatment of BQR and doxorubicin resulted in significant inhibitory effect on the growth of melanoma cells both in vitro and in vivo. The favourable synergistic effect and low toxic profiles of this treatment combination in melanoma xenograft model suggests that this combination has high potentials as targeted therapy for melanoma and warrants future pharmacokinetics and resistance studies.

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Declarations of interest

None.

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