The BET-bromodomain inhibitor JQ1 mitigates vemurafenib drug resistance in melanoma
Beizhaoa, Xiankui Chengkap and Xiyuan Zhoua

Inhibition of BRAF improves therapeutic efficacy of BRAF-mutant melanoma. However, drug resistance to BRAF inhibitor is inevitable, and the drug resistance mechanisms still remain to be elucidated. Here, BRAFV600E mutant cells A375 and SK-MEL-28 were chosen and treated with BRAF inhibitor vemurafenib, and the results showed that the ERK signaling pathway was blocked in these cells. Then, vemurafenib-resistant cells were constructed, and we found that drug resistance-related gene P-gp was overexpressed in the two cell lines. In addition, the histone acetylation was significantly increased on the P-gp promoter region, which suggested that the epigenetic modification participated in the P-gp overexpression. Furthermore, JQ1, a bromodomain inhibitor, was added to the vemurafenib-resistant cells and sensitizes the vemurafenib-induced melanoma cell apoptosis. In C57BL/6 mice intravenously injected with vemurafenib-resistant melanoma cells, cotreatment of vemurafenib and JQ1 also severely suppressed melanoma lung metastasis. Taken together, our findings may have important implications for the combined use of vemurafenib and JQ1 in the therapy for melanoma treatment. Melanoma Res 2018;28:521–526 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: BET inhibitor, BRAF inhibitor, cancer drug resistance, melanoma

*Institute of Dermatology and Venereology, Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital, Chengdu and ‡Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China

Correspondence to Xiyuan Zhou, MD, Institute of Dermatology and Venereology, Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital, No. 32 Western 2nd Section, 1st Circle Road, Chengdu 610072, Sichuan, China
Tel/ fax: + 86 288 739 4473; e-mail: 496811361@qq.com

Received 13 March 2018 Accepted 23 July 2018

Introduction
Despite the high cure rates associated with the early diagnosis and removal of melanoma, patients with stage IV metastatic disease have a 5-year survival expectancy of ∼18% [1]. Therefore, it is very crucial to develop an effective treatment for inhibiting melanoma metastasis. Recent studies have reported that ∼37–59% of melanomas contain a mutation in the gene that encodes BRAF [2], which was associated with younger age at diagnosis and poorer survival [3]. In recent years, targeting BRAF or MEK alone and cotargeting BRAF and MEK using specific inhibitors have become the standard of care for patients with late-stage mutant BRAF melanomas [4]. However, the benefits are often of limited duration owing to rapid development of resistance [5].

Vemurafenib, an oncogenic V600-mutant BRAF inhibitor, could cause an improvement in the response and survival rates in patients with V600-mutant BRAF melanoma, but vemurafenib resistance formation remains inevitable [6]. Resistance mechanisms involve activation of alternative kinases and nonrelated compensatory pathways [7]. For instance, P-gp could transport multiple types of chemotherapeutic drugs out of cells, and this process is coupled with the energy of ATP hydrolysis on the ATPase domain [8]. Therefore, inhibition of these transporters will restore the sensitivity of drug-resistant cancer cells to chemotherapeutic agents and may permit a successful chemotherapy regimen for patients with vemurafenib resistance.

Epigenetic dysregulation in melanoma is an emerging field of research. Paoluzzi et al. [9] have elucidated a role for epigenetic regulators and histone variants in the pathogenesis of melanoma and demonstrated a critical role for the bromodomain (BrD)-containing protein in melanoma maintenance [10]. BrD and extraterminal domain (BET) family play roles as epigenetic ‘readers’, which could bind to acetylated lysine residues of histones, to which they recruit chromatin-modifying enzymes to effect transcriptional changes [11]. Recently, small molecule inhibitors have been developed that displace BRD-containing proteins from chromatin. In particular, JQ1 is a small molecule that binds competitively to bromodomains with high potency for BRD4, and selectivity for BET proteins [12]. However, in the process of acquired vemurafenib-resistant melanoma, whether BRD-containing proteins exert their function was still unknown, and whether JQ1 could be used as the antitumor agent needs to be explored.

In the current study, we will not only provide the molecular mechanism of P-gp-related drug resistant in melanoma with vemurafenib-resistant but also demonstrate that combined targeting of BRAF and BET proteins impairs the drug
resistance in vivo, which might have a therapeutic potential for the treatment of vemurafenib-resistant melanoma.

Materials and methods

Cell culture

Human melanoma cell lines A375 and SK-MEL-28 were obtained from ATCC and cultured following ATCC recommendations. Cells were subcultured in DMEM (Gibco, Life Technology, Carlsbad, California, USA) by adding 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) and incubated at 37°C in a CO2 incubator (5%).

Vemurafenib was purchased from Selleckchem (Houston, Texas, USA), and A375 and SK-MEL-28 were cultured in complete DMEM medium with 10-μmol/l vemurafenib to induce drug-resistant cells as previously reported [13,14]. After 10 passages, the cells resistant to vemurafenib were screened and then cultured with DMEM medium with 10 μmol/l vemurafenib continually to keep the characteristics.

Cytotoxicity test

The effect of vemurafenib or JQ1 (Selleckchem) on cell viability was assessed by MTT assay. In brief, A375 or SK-MEL-28 cells were suspended in DEMM to 5 × 10^4 cells/ml, and aliquots (5 × 10^3 cells/100 μl/well) were put into each well of a 96-well plate. One day later, the media were changed with different concentrations of vemurafenib and incubated for another 24 h. After exposure, the supernatant was removed, and 100 μl of MTT solution (5 mg/ml in medium) was added per well and incubated for 4 h. After incubation, the MTT solution was discarded, and the formed crystal was dissolved in DMSO (100 μl). The optical density of each well was determined at 540 nm.

Apoptosis assay

FITC-annexin apoptosis detection kit (BD Biosciences, San Jose, California, USA) was used to evaluate apoptosis. The experiment was performed according to the manufacturer’s instructions. In summary, cells were seeded at a density of 2.5 × 10^5 in a six-well plate and were treated with vemurafenib with or without JQ1 for 24 h. Cells were harvested and washed twice with ice-cold PBS and then resuspended in 1 × binding buffer followed by incubation with annexin V/PI solution for 15 min at room temperature. The samples were immediately analyzed by flow cytometry using a C6 Flow Cytometer system (BD Biosciences). At least 2 × 10^5 cells were analyzed for each sample.

Western blot analyses

Whole cell extracts (lysate) were prepared from 1 × 10^6 cells in lysis buffer to determine the expression of protein. A total of 30 μg of proteins from each sample was subjected to 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Target proteins were probed with specific antibodies-P-gp (sc-71557), caspase 3 (sc-22139) and GAPDH (sc-367714) (Santa Cruz Biotechnology, Santa Cruz, California, USA). Gels were stripped and reprobed with antibodies against GAPDH to ensure equal loading. Western blotting bands were scanned, and gray density was quantified using Image J software (NIH, Bethesda, Maryland, USA). Relative expression of the target proteins was standardized with the GAPDH, and the ratio of control groups was set as 1. For the p-ERK with both total ERK and GAPDH as controls, the total ERK was used to standardize. The relative expression levels were shown as numbers below each target protein blots.

Histone acetylation detection

The histone H3 total Acetylation Detection Fast Kit (ab115124) was purchased from Abcam (Pudong, China). Specific buffers ESC1 and ESC2 were added into each well, together with the 5 μg of the histone extract into the sample wells. The diluted standards were added, and strips were incubated at room temperature for 1 h. After washing and aspiration, diluted buffer was added to each well and incubated at room temperature for 60 min. The wells were aspirated, and color reagent and stop solution were added. The obtained yellow color was read on a microplate reader at 450 nm within 2–15 min.

Mouse melanoma lung metastasis model

C57BL/6 mice, 5–6 weeks old, were purchased from the Chengdu Shuoda Laboratory Animal Company, and all animal experiments were approved by the Animal Ethics Committee of Sichuan Academy of Medical Sciences and complied with its regulations. A mouse model of melanoma lung metastasis was constructed by injection of vemurafenib-resistant A375 or SK-MEL-28 cells into mice (5 × 10^5 cells/mouse) through tail vein. Compound treatment was started from the next day after melanoma cells injection. The mice were intravenously injected with normal saline, vemurafenib (10 mg/kg), JQ1 (5 mg/kg), or their combination every 3 days, correspondingly. Mice were killed after 4-week treatment, and the lungs were separated to examine the number of lung metastasis nodules. Then the lungs were homogenized and incubated in 1 μmol/l NaOH containing 10% DMSO at 80°C for 2 h to measure the melanin content [15]. Then the homogenate was centrifuged, and the absorbance of supernatant was read at 490 nm. The lung of each mouse was fixed by formalin and examined by immunohistochemistry staining assay.

Statistical analysis

All statistical analyses were performed with SPSS 17.0 program (SPSS Inc., Chicago, Illinois, USA). All measurement data were represented as the mean ± SD. The differences between groups were analyzed using Student’s t-test (only two groups) or one-way analysis of variance (more than two groups). P value less than 0.05 was considered statistically significant.
Results

Vemurafenib inhibited melanoma cell growth by ERK signaling pathway

To test the efficiency of vemurafenib on melanoma cells, the BRAFV600E mutant cells A375 and SK-MEL-28 were chosen for the study. These cells were treated with BRAF inhibitor vemurafenib (10 μmol/l) for up to 24 h, and MTT assay indicated that the cell growth rate was significantly downregulated in a time-dependent manner (Fig. 1a). As shown in the previous study, the ERK signaling pathway was involved in the process of BRAF inhibitor [16]. Our western blot result also confirmed that ERK signaling pathway was blocked by vemurafenib in both cell lines, as the phosphorylation status of ERK1/2 was suppressed to 20 and 10% in the A375 and SK-MEL-28 cells compared with the mock control, respectively (Fig. 1b).

P-gp played roles in vemurafenib resistance of melanoma cells

To explore the mechanism of vemurafenib resistance in melanoma cells, the vemurafenib-resistant cell lines were constructed as previously reported, and MTT assays showed that the vemurafenib-resistant cell lines displayed significantly higher survival rate than the normal cells when exposed to vemurafenib (Fig. 2a), and the half maximal inhibitory concentration was much higher in the vemurafenib-resistant cell lines compared with normal ones (A375 group: from 7.55 to 57.29 μmol/l; SK-MEL-28 group: from 6.40 to 35.38 μmol/l) (Fig. 2b). P-gp played important roles in many kinds of cancer drug resistance; the immunoblotting assay showed that the expression of P-gp was significantly upregulated in the vemurafenib-resistant cell lines than normal ones in both the A375 and SK-MEL-28 cells (Fig. 2c). Furthermore, we found that the histone H3 acetylation status was greatly upregulated in the vemurafenib-resistant cell lines; however, the H3 acetylation status maintained lower status in the normal parental cells (Fig. 2d), which indicated that epigenetic modification might occur in the process of acquired vemurafenib resistance in melanoma cells.

JQ1 alleviated vemurafenib resistance in melanoma cells

The small molecule JQ1 was added to the vemurafenib-resistant melanoma cells A375 and SK-MEL-28. The MTT assays showed that the JQ1 could significantly sensitize the antitumor effect of vemurafenib in the resistant cell lines (Fig. 3a), and the half maximal inhibitory concentration was greatly decreased in the JQ1 and vemurafenib combination group compared with the Vemurafenib solo treatment group (A375 group: from 7.55 to 2.85 μmol/l; SK-MEL-28 group: from 6.40 to 3.29 μmol/l) (Fig. 3b). In the molecular biology level, the P-gp protein level was much lower in the JQ1-treated cell lines compared with the mock group (Fig. 3c), and the acetylation status on H3 histone displayed lower enrichment in JQ1-treated cells than the control group (Fig. 3d).

JQ1 sensitized vemurafenib-induced apoptosis in melanoma cells

Functionally, whether JQ1 could influence the biological behavior was detected by flow cytometry assay, and the results showed that addition of JQ1 could significantly increase the apoptosis rate in both A375 and SK-MEL-28 cells, and a synergetic antimelanoma effect of JQ1 and vemurafenib could be elicited (Fig. 4a and b). In addition, the cleaved caspase 3 was greatly upregulated in response to the JQ1 stimulation by western blot assay, which confirmed the data from flow cytometry assay (Fig. 4c).

Vemurafenib and JQ1 blocked melanoma lung metastasis in vivo

To test the efficacy of JQ1 combined with vemurafenib in preventing melanoma lung metastasis, vemurafenib-resistant A375 or SK-MEL-28 cells were intravenously injected into C57BL/6 mice, respectively, which were then treated with vemurafenib and combination of JQ1...
P-gp plays roles in vemurafenib resistance of melanoma cells. A375 and SK-MEL-28 cell lines with vemurafenib resistance were constructed, and then treated with vemurafenib for 24 h. (a) Cell growth inhibition curves of vemurafenib as noted by MTT assay, and (b) the half maximal inhibitory concentration values of vemurafenib were evaluated in A375 and SK-MEL-28 cells by MTT assay. Data from growth-inhibition assays were modeled using a nonlinear regression curve fit with a sigmoid dose–response. (c) P-gp protein levels in A375 and SK-MEL-28 parental normal cells (N) or vemurafenib-resistant cells (R) were analyzed by western blot, and GAPDH was used as the endogenous control. (d) The histone H3 acetylation levels in A375 and SK-MEL-28 parental normal cells (N) or vemurafenib-resistant cells (R) were measured by histone acetylation detection assay. *P < 0.05 versus the control.

JQ1 could eliminate vemurafenib resistance in melanoma cells. Vemurafenib-resistant A375 and SK-MEL-28 cell lines were treated with 100 μmol/l vemurafenib (Vem) with or without increasing dosage of JQ1 (Vem + JQ1) for 24 h. (a) Relative cell growth inhibition rates of combination of vemurafenib and JQ1 as noted by MTT assay. (b) The calculated half maximal inhibitory concentration values of combination of vemurafenib and JQ1 in A375 and SK-MEL-28 cells. Data from growth-inhibition assays were modeled using a nonlinear regression curve fit with a sigmoid dose–response curve. (c) Cell lysates were subjected to western blot for detecting P-gp protein level, and GAPDH was used as endogenous control, and (d) the histone H3 acetylation was measured by histone acetylation detection assay. *P < 0.05 versus the control; #P < 0.01 versus the control.
and vemurafenib for 4 weeks, respectively. Compared with control, cotreatment with JQ1 exhibited more efficiency in reducing pulmonary metastatic nodules and almost blocked melanoma lung metastasis (Fig. 4d and e). The result was further confirmed by the melanin content determination (Fig. 4f). All the aforementioned results signified that JQ1 could improve the vemurafenib resistance in vivo.

Discussion

Melanoma is the least common type of skin cancers, which accounts for ~1% of cases; however, it is responsible for most deaths owing to skin cancer [17]. The advanced and metastasized melanoma always indicates poor survival and lacks effective drugs in clinic [18]. Thus, drug combination is reasonably raised as a promising strategy for melanoma metastasis [19]. For example, Zhou et al. [20] demonstrated that the combination therapy of PKCζ and COX-2 inhibitors could significantly inhibit melanoma metastasis in vitro and in vivo, which promised an efficient strategy for treatment of melanoma metastasis in clinic.

BRAF is an intracellular kinase in the mitogen-activated protein kinases pathway. BRAF is involved in regulating important cell functions such as cell growth, division, differentiation, and apoptosis [21]. Variations in the kinase domain of BRAF have been associated with various cancers. The most common BRAF variant, V600E, which was detected in ~50% of melanomas, could constitutively activate the kinase and cause cell proliferation in the absence of growth factors that would normally be required [22].

Vemurafenib is a BRAF kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with the BRAFV600E variant, as detected by an FDA-approved test. Both targeted therapy with vemurafenib and immunotherapy regimens (e.g. nivolumab plus ipilimumab) have been shown to improve overall survival in patients with metastatic melanoma compared with chemotherapy [23,24]. However, there are no randomized trials that compare targeted therapy with immunotherapy so far, and only very few data have been reported regarding the appropriate combinations and sequencing of these therapies for patients with a BRAFV600E variant. In the current study, we investigated the effect of combining the BET inhibitor JQ1 with the BRAF inhibitor vemurafenib in-vitro models of BRAF-mutant melanoma, which was consistent with the findings of Paoluzzi et al. [9]. Furthermore, the result showed that the vemurafenib resistance was resolved with combination of JQ1 and vemurafenib in BRAF-mutant cell lines.

The protein product of ABCB1, P-gp, is a member of a class of glycoproteins that export xenobiotic agents across the cytoplasmic membrane. P-gp is normally expressed in the liver, pancreas, kidney, and gut, but is also aberrantly
upregulated in certain solid tumors and hematologic malignancies by a variety of mechanisms [25]. Its presence in certain cell types acts to reduce intracellular accumulation of anticancer drugs, resulting in relative resistance to those agents. Tsai et al. [26] demonstrate that BRAFV600E induces the expression of the multidrug resistance gene ABCB1 and its product, P-gp. In the current study, we found that P-gp expression was significantly upregulated in vemurafenib-resistant melanoma, whether in vitro or in vivo, suggesting that targeting of P-gp might improve the therapeutic efficiency. Through the in-vitro test, we found that silencing P-gp could greatly sensitize the vemurafenib-induced apoptosis in A375 and SK-MEL-28 melanoma cell lines.

To sum up, our results show that silencing P-gp with JQ1 significantly antagonizes vemurafenib-mediated cancer drug resistant in-vitro by epigenetic modification on the P-gp promoter histones. In the current study, we have only tested the biological function of JQ1 and vemurafenib combinations in cell lines and mice model; therefore, future in-vivo studies will be important for identifying the optimal BET-BRD inhibitor for combination therapy in patients with melanoma.

Conclusion
Our findings may have important implications for use of combination of JQ1 and vemurafenib in the treatment of melanoma.

Acknowledgements
This study was supported by the General Program of the Health and Family Planning Commission of Sichuan Province (no. 17PJ153).

B.Z. and X.C. performed the research and analyzed the data; X.C. and X.Z. designed the research study; and B.Z. and X.Z. prepared the manuscript.

Conflicts of interest
There are no conflicts of interest.

References
1. Balch CM, Gershenwald JE, Soong SJ, Thompson JF. Update on the melanoma staging system: the importance of sentinel node staging and primary tumor mitotic rate. J Surg Oncol 2011; 104:379–385.
2. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002; 417:949–954.
3. Keller HR, Zhang X, Li L, Schaeider H, Wells JW. Overcoming resistance to targeted therapy with immunotherapy and combination therapy for metastatic melanoma. Oncotarget 2017; 8:75675–75686.
4. Liu F, Jiang CC, Yan XG, Tseng HY, Wang CY, Zhang YY, et al. BRAF/MEK inhibitors promote CD47 expression that is reversible by ERK inhibition in melanoma. Oncotarget 2017; 8:69477–69492.
5. Carlino MS, Long GV, Kefford RF, Rizos H. Targeting oncogenic BRAF and aberrant MAPK activation in the treatment of cutaneous melanoma. Crit Rev Oncol Hematol 2015; 96:385–398.
6. Qiu JG, Zhang YJ, Li Y, Zhao JM, Zhang WJ, Jiang QW, et al. Trametinib modulates cancer multidrug resistance by targeting ABCB1 transporter. Oncotarget 2016; 5:15494–15509.
7. Ozben T. Mechanisms and strategies to overcome multiple drug resistance in cancer. FEBS Lett 2006; 580:2903–2909.
8. Heise R, Amann PM, Ensslen S, Marquardt Y, Czaja K, Joussen S, et al. Interferon alpha signalling and its relevance for the upregulatory effect of transporter proteins associated with antigen processing (TAP) in patients with malignant melanoma. PLoS One 2016; 11:e0148325.
9. Paoluzzo L, Hanniford D, Sokolova E, Osmian I, Darvishian F, Wang J, et al. BET and BRAF inhibitors act synergistically against BRAF-mutant melanoma. Cancer Med 2016; 5:1183–1193.
10. Segura MF, Fontanals-Greva B, Gazeli-Sovran A, Guajardo MV, Hanniford D, Zhang G, et al. BRD4 sustains melanoma proliferation and represents a new target for epigenetic therapy. Cancer Res 2013; 73:6264–6276.
11. Floyd SR, Pacold ME, Huang Q, Clarke SM, Lam FC, Cannell IG, et al. The bromodomain protein Brd4 insulates chromatin from DNA damage signalling. Nature 2013; 498:246–250.
12. Ambrosini G, Sawle AD, Musi E, Schwartz GK. BRD4-targeted therapy induces Myc-independent cytotoxicity in Gna11-mutantveal melanoma cells. Oncotarget 2015; 6:33397–34309.
13. Sinnberg T, Makino E, Krueger MA, Velic A, Macek B, Rothbauer U, et al. A nexus consisting of beta-catenin and Stat3 attenuates BRAF inhibitor efficacy and mediates acquired resistance to vemurafenib. EBioMedicine 2016; 8:132–149.
14. Molinoli L, Costanza M, Sharma GG, Viltadi M, Massimino L, Citterio S, et al. Concomitant BCORL1 and BRAF mutations in vemurafenib-resistant melanoma cells. Neoplasia 2018; 20:467–477.
15. Chang MS, Choi MJ, Park SY, Park SK. Inhibitor effects of Hoelen extract on melanogenesis in B16/F1 melanoma cells. Phytother Res 2010; 24:1359–1364.
16. Burgeiro A, Gajate C, Dakir el H, Villa-Pulgarin JA, Oliveira PJ, Molinillo F. Involvement of mitochondrial and B-RAF/ERK signaling pathways in berberine-induced apoptosis in human melanoma cells. Anticancer drugs 2011; 22:507–518.
17. Belley-Cote EP, Hanfl H, D’Aragon F, Eikelboom JW, Anderson JL, Borgman M, et al. Genotype-guided versus standard vitamin K antagonist dosing algorithms in patients initiating anticoagulation. A systematic review and meta-analysis. Thromb Haemost 2015; 114:768–777.
18. Loquai C, Schmidtmann I, Garzaroli M, Kaatz M, Kahler KC, Kurschat P, et al. Interactions from complementary and alternative medicine in patients with melanoma. Melanoma Res 2017; 27:238–242.
19. Połkowski M, Czapiewska E, Kozłowska-Wojsiecówka M. Drug combinations as the new standard for melanoma treatment. Curr Treat Options Oncol 2016; 17:61.
20. Zhou P, Qin J, Li Y, Li G, Wang Y, Zhang N, et al. Combination therapy of PKCζeta and COX-2 inhibitors synergistically suppress melanoma metastasis. [Internet]. J Exp Clin Cancer Res 2017; 36:115.
21. Dean L. Vemurafenib therapy and BRAF and NRAS genotype. In: Pratt V, McLeod H, Dean L, Malheiro A, Rubinstein W, editors. Medical genetics summaries. Bethesda, MD: National Center for Biotechnology Information (US); 2012-2017.
22. Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. J Clin Oncol 2011; 29:1239–1246.
23. Fisher R, Larkin J. Vemurafenib: a new treatment for BRAF-V600 mutated advanced melanoma. Cancer Manag Res 2017; 9:243–252.
24. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med 2015; 373:23–34.
25. Wang S, Wang A, Shao M, Lin L, Li P, Wang Y. Schisandrin B reverses doxorubicin resistance through inhibiting P-glycoprotein and promoting proapoptotic-mediated degradation of survivin. Sci Rep 2017; 7:8419.
26. Tsai YT, Lozanski G, Lehman A, Saks EJ, Herfein E, Salunke SB, et al. BRAFV600E induces ABCB1/P-glycoprotein expression and drug resistance in B-cells via AP-1 activation. Leuk Res 2015; [Epub ahead of print].