Published OnlineFirst June 2, 2016; DOI: 10.1158/1535-7163.MCT-16-0004

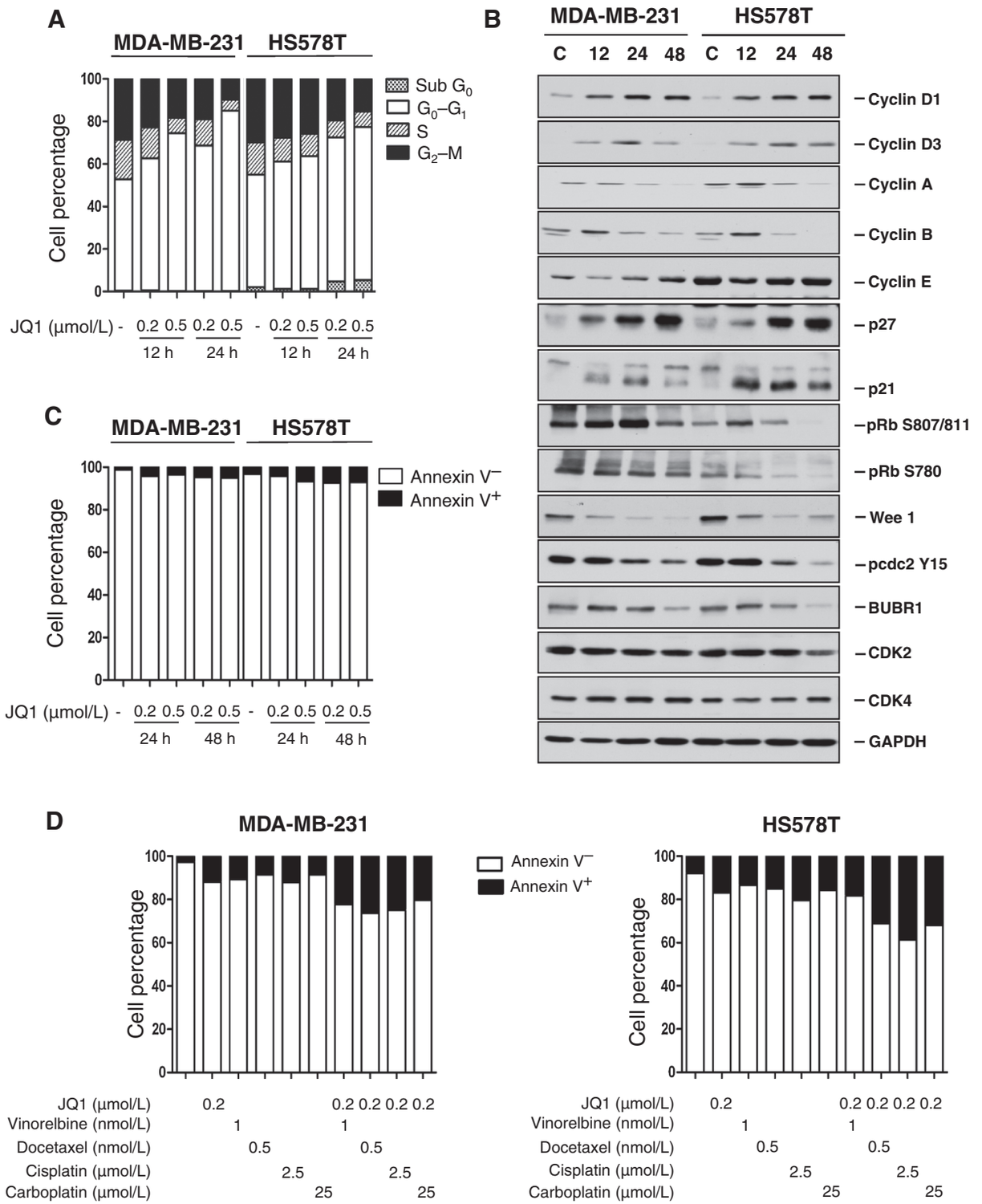

Figure 3.

Mechanism of action of JQ1: induction of cell-cycle arrest and apoptosis. **A,** flow cytometric analysis of the effect of JQ1 on cell cycle in MDA-MB-231 and HS578T. Cells were cultured in DMEM + 10% FBS + 2 mmol/L L-glutamine and treated with JQ1 at 0.2 and 0.5 μmol/L. After 12 and 24 hours of treatment, cells were trypsinized, fixed in ice-cold 70% ethanol for 30 minutes, and incubated in PI/RNAse staining solution in the dark for 1 hour at 4°C. Next, cell-cycle progression was examined on FACSCanto II flow cytometer (BD Biosciences). The percentage of stained cells in each phase was gated using the FACS Diva software and represented with a histogram. **B,** biochemical assessment of cell-cycle proteins. Expression of cell-cycle–related regulators (cyclin D1, cyclin D3, cyclin A, cyclin B, cyclin E, p27, p21, pRbS807/811, pRbS780, Wee 1, pcdc2, BUBR1, CDK2, and CDK4) was determined in MDA-MB-231 and HS578T after treatment with JQ1 at 0.5 μmol/L at 12, 24, and 48 hours by Western blot analysis. Cell lysates were prepared using a cold lysis buffer containing freshly prepared 20 mmol/L Tris-HCl (pH 7.0), 140 mmol/L NaCl, 50 mmol/L EDTA, 10% glycerol, 1% Nonidet P-40, and protease and phosphatase inhibitor cocktails. (*Continued on the following page.*)

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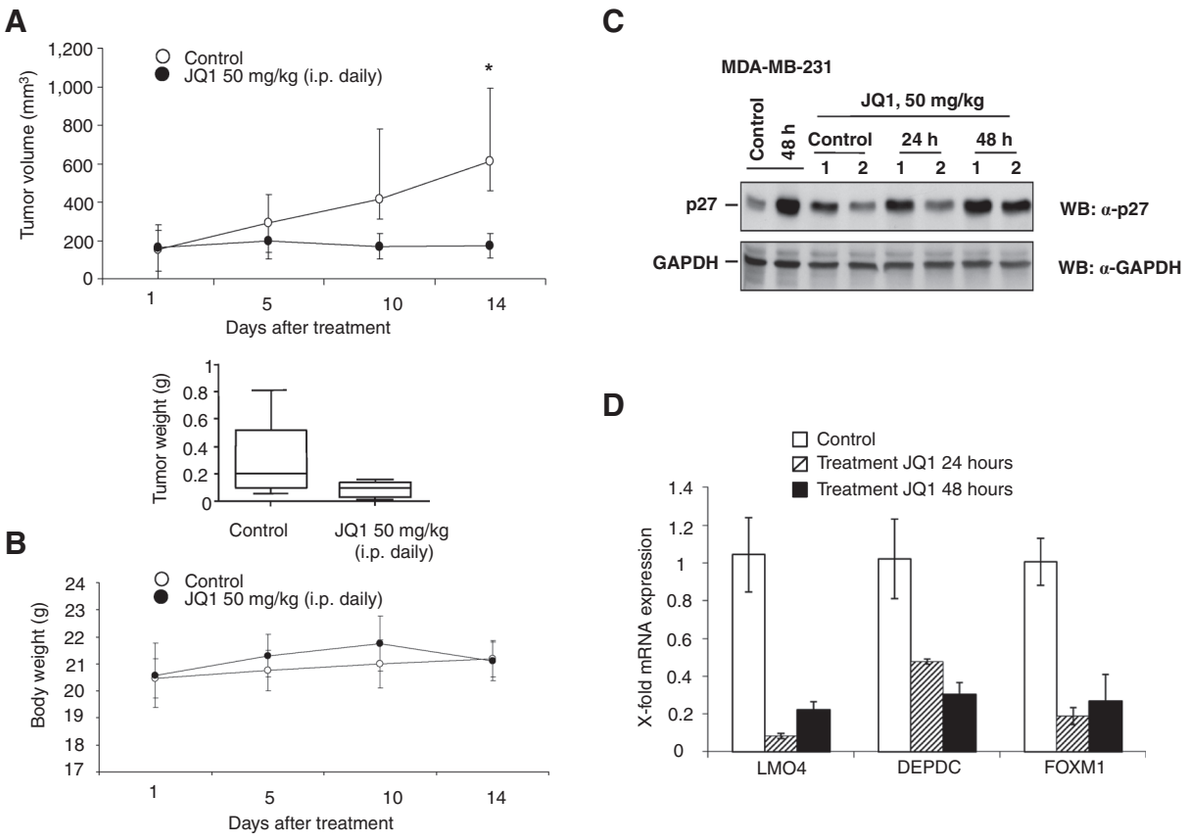

Figure 4.

In vivo antitumor activity of JQ1 and pharmacodynamic evaluation. **A,** *in vivo* effect on tumor growth and weight after JQ1 administration. For xenograft studies, MDA-MB-231 cells (2×10^6 to 5×10^6 cells in 100 μL of DMEM with 20% Matrigel) were inoculated into the mammary fat pads of mice. After approximately 2 weeks of injection, when tumors reached a volume of 150 mm^3, treatment was initiated. Mice were randomly divided into 2 groups: (i) JQ1 treatment ($n = 5$) and (ii) control group ($n = 5$). Animals were treated daily with JQ1 (50 mg/kg intraperitoneally) for 2 weeks. Tumors diameters were serially measured using the following formula: $V = (L \times W^2)/2$, where V = volume (mm^3), L = length (mm), and W = width (mm). The Student test was used to calculate statistical significance. After treatment, tumors from control and treated groups were extracted and weight. *, $P < 0.05$. **B,** body weight of nude mice in control and JQ1-treated groups among the duration of the treatment. **C,** protein expression levels of p27 in triple-negative breast tumors after JQ1 administration. Nude mice xenografted with MDA-MB-231 were treated with JQ1 50 mg/kg intraperitoneally for 24 and 48 hours. MDA-MB-231–treated *in vitro* with JQ1 at 0.5 μmol/L was used as control. Tumor samples were minced, washed with cold PBS, and homogenized in cold lysis buffer (1.5 mL/100 mg of tumor). This homogenate was centrifuged at 10,000 × *g* for 20 minutes at 4°C, and the supernatants were transferred to new tubes. The protein expression of p27 was analyzed by Western blotting as described before. GAPDH was used as a loading control. **D,** effect of JQ1 on the mRNA expression of LM04, DEPDC, and FOXM1, in treated compared with nontreated tumors. Nude mice injected with MDA-MB-231 were treated with JQ1 (50 mg/kg intraperitoneally) for 24 and 48 hours, and LM04, DEPDC, and FOXM1 transcription levels were measured by quantitative RT-PCR as previously described.

transcription factors could be involved in the modulation of other genes.

The *in vitro* evaluation of 2 novel BET inhibitors showed antitumor efficacy that was confirmed latter using *in vivo* models. Of note, as JQ1 represents a tool compound, relatively high concentrations (500 nmol/L) was required for optimal effects. Therefore, a medical useful inhibitor would need to be optimized for further development. In any case, their range of activity was still at the nanomolar range. BET inhibitors acted on the G_1 phase of the cell cycle as was observed by flow cytometry, data that are in

(*Continued*.) Cell extracts (50 μg of protein) were separated on 6% to 15% SDS-PAGE and, subsequently, immunoblotted using primary antibodies raised against the already mentioned proteins. GAPDH was used as a loading control. **C,** graphical representation of proapoptotic effect of JQ1 on MDA-MB-231 and HS578T. Cells (300,000 per 100-mm dish) treated with JQ1 at 0.2 and 0.5 μmol/L for 24 and 48 hours were incubated in trypsin-EDTA, washed twice with cold PBS, and then stained with Annexin V-DT-634 and PI solution in the dark for 1 hour. Both early (Annexin V–positive and PI-negative) and late (Annexin V–positive and PI-positive) apoptotic cells were determined using a FACSCanto II flow cytometer and were included in cell death determinations. **D,** JQ1 and chemotherapeutics (vinorelbine, docetaxel, cisplatin, and carboplatin) cooperate to induce apoptosis. MDA-MB-231 and HS578T were exposed to drugs alone and in combination with JQ1, at the indicated doses. Apoptosis was examined after 72 hours of treatment by flow cytometry using Annexin V/PI staining as previously described.

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line with previous reports in B-cell lymphomas (27, 28). The biochemical evaluation of their function showed a profound effect on proteins that regulate this phase, like an increase of p21, p27, and cyclin D, with a decrease of regulators more associated with latter phases of the cell-cycle cyclin B or BURB. The results from tumors xenografted in animals demonstrated *in vivo* an increase of p27.

An interesting finding was the synergistic interaction between BET inhibitors and chemotherapies used to treat triple-negative tumors—compounds affecting mitosis and producing DNA damage. Evaluation of cell cycle showed that both type of chemotherapies produced an arrest at G_2–M. The combination of BET inhibitors that mainly stop cells at G1 with agents that both induced an arrest at G2–M suggests that acting of different phases of the cell cycle could be an attractive therapeutic approach. Indeed, BET inhibitors alone did not induce apoptosis in a relevant manner, but their combination with the mentioned agents led cells to a programmed death.

Finally, evaluation of JQ1 in animal models showed that the drug had the same antitumor effect on xenografted tumors confirming the data observed in cell lines. Administration of JQ1 was able to induce the expression p27. Similarly, a reduction of LM04, DEPDC, and FOXM1 was observed after 24 and 48 hours of treatment.

BET inhibitors are a family of compounds with drug candidates at different stages of drug development, from drugs in preclinical stage to others like OTOX15 that has finished its phase I program with signs of activity and no unexpected toxicities (27). For triple-negative breast tumors, BET inhibitors represent a promising therapeutic option that deserves to be evaluated in clinical studies.

In conclusion, our data demonstrate the antitumor efficacy of this family of compounds paving the way of its future clinical evaluation in this unmet disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Pérez-Peña, G. Serrano-Heras, J.C. Montero, V. Corrales-Sánchez, A. Ocaña

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Pérez-Peña, G. Serrano-Heras, V. Corrales-Sánchez, A. Pandiella, A. Ocaña

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Pérez-Peña, G. Serrano-Heras, V. Corrales- Sánchez, A. Pandiella, A. Ocaña

Writing, review, and/or revision of the manuscript: J. Pérez-Peña, G. Serrano-Heras, V. Corrales-Sánchez, A. Pandiella, A. Ocaña

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Corrales-Sánchez

Study supervision: G. Serrano-Heras, V. Corrales-Sánchez, A. Ocaña

Acknowledgments

I would like to thank Pilar F. and Santiago O. for their support.

Grant Support

The study was supported by Instituto de Salud Carlos III (PI13/01444), ACEPAIN; AMAC, Diputación de Albacete and CRIS Cancer Foundation (to A. Ocaña). Ministry of Economy and Competitiveness of Spain (BFU2012-39151 and RD12/0036/0003 to A. Pandiella), and the AECC (to A. Pandiella). J.C. Montero is a recipient of a Miguel Servet fellowship program. European FEDER program.

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Received January 20, 2016; revised May 5, 2016; accepted May 17, 2016; published OnlineFirst June 2, 2016.

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Published OnlineFirst June 2, 2016; DOI: 10.1158/1535-7163.MCT-16-0004

Molecular Cancer Therapeutics

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Mol Cancer Ther 2016;15:1823-1833. Published OnlineFirst June 2, 2016.