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Immunoregulatory protein B7-H3 promotes growth and decreases sensitivity to therapy in metastatic melanoma cells

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Summary
B7-H3 (CD276) belongs to the B7 family of immunoregulatory proteins and has been implicated in cancer progression and metastasis. In this study, we found that metastatic melanoma cells with knockdown expression of B7-H3 showed modest decrease in proliferation and glycolytic capacity and were more sensitive to dacarbazine (DTIC) chemotherapy and small-molecule inhibitors targeting MAP kinase (MAPK) and AKT/mTOR pathways: vemurafenib (PLX4032; BRAF inhibitor), binimetinib (MEK-162; MEK inhibitor), everolimus (RAD001; mTOR inhibitor), and triciribidine (API-2; AKT inhibitor). Similar effects were observed in melanoma cells in the presence of an inhibitory B7-H3 monoclonal antibody, while the opposite was seen in B7-H3-overexpressing cells. Further, combining B7-H3 inhibition with small-molecule inhibitors resulted in significantly increased antiproliferative effect in melanoma cells, as well as in BRAFV600E mutated cell lines derived from patient biopsies. Our findings indicate that targeting B7-H3 may be a novel alternative to improve current therapy of metastatic melanoma.

KEYWORDS
AKT/mTOR inhibitors, B7-H3/CD276, MAPK, melanoma, targeted therapy, vemurafenib

INTRODUCTION

The only chemotherapeutic Food and Drug Administration (FDA)-approved drug for treatment of malignant melanoma is the alkylating agent dacarbazine (DTIC), although its efficacy is modest (Bhatia, Tykodi, & Thompson, 2009). In BRAF-mutated melanomas, targeted therapy has improved survival of patients with advanced metastatic melanoma (Eggermont & Robert, 2011; Vennpureddy, Thumallapally, Motiil Nehru, Atallah, & Terjanian, 2016). BRAFV600E is mutated in approximately 45% of cutaneous and 10% to 20% of mucosal or acral melanomas (Goldinger, Murer, Stieger, & Dummer, 2013; Karachaliou et al., 2015), with other commonly mutated or amplified genes being NRAS and c-KIT, and less frequently HRAS (Albino, Le Strange, Ollif, Furth, & Old, 1984). Thus, new alternative therapeutic alternatives are needed for melanoma patients lacking BRAF mutation. Resistance to targeted therapy is a major issue in melanoma, usually correlated with reactivation of MAPK and increase in AKT activation. Hence, inhibitors of MEK and the PI3K/AKT/mTOR pathway are being tested clinically in combination with other inhibitors (Ascierto et al., 2013; Fedorenko, Gibney, Sondak, & Smalley, 2015; Greger et al., 2012; Thumar, Shahbazian, Aziz, Jilaveanu, & Kluger, 2014). Further, the newly approved immunotherapies using PD-1 and CTL-4 checkpoints inhibitors (Ott, Hodi, & Robert, 2013; Pardoll, 2012) have shown great promise in the clinic, as has blocking PD-1 in combination with its ligand B7-H1/PD-L1 (Dossett, Kudchadkar, & Zager, 2015; Mahoney, Freeman, & Mcdermott, 2015).

B7-H1/PD-L1 is a member of the B7 family of proteins and is frequently overexpressed in melanoma (Kakavand et al., 2015). Proteins in the B7 family are important immune response regulators (Flies & Chen, 2007) and can mediate metastasis-related signals and support tumor development (Leung & Suh, 2014). Another family member, B7-H3, is also highly expressed in melanoma cells...
and implicated in tumor immunity (Chapoval et al., 2001; Loo et al., 2012; Tekle et al., 2012; Wang, Chong et al., 2013; Xu, Cheung, Guo, & Cheung, 2009; Yi & Chen, 2009). Subcellular localization of B7-H3 is important for exerting its function, and it is predominantly found in the cell membrane and cytoplasm, but also in the nucleus, of tumor cells (Chen, Tekle, & Fodstad, 2008; Ingebrigtsen et al., 2014; Liu et al., 2013; Wang, Zhang et al., 2013). Additionally, B7-H3 has been detected in exosomes, and as soluble isoforms in serum (Chen et al., 2013; Kshirsagar et al., 2012; Marimpietri et al., 2013; Zhang et al., 2008).

B7-H3 affects sensitivity to various drugs and targeted therapies in several cancer types (Jiang, Liu, Liu, Zhang, & Hua, 2016; Liu et al., 2011; Nunes-Xavier et al., 2016; Zhang et al., 2015a,b; Zhao et al., 2013), but has not been addressed in malignant melanoma. In this study, we have assessed the role of B7-H3 in the sensitivity of melanoma cells to the chemotherapeutic agent DTIC, and to clinically relevant MAPK and AKT/mTOR inhibitors: vemurafenib, binimetinib, everolimus, and triciribidine. We found that low expression or inhibition of B7-H3 renders the cells more sensitive to these drugs in addition to decreasing their glycolytic capacity. Our results suggest that targeting B7-H3 may be a novel supplement to improve current anticancer therapies in metastatic melanoma.

2 | RESULTS

2.1 | B7-H3 promotes growth and glycolysis of melanoma cells

To study the role of B7-H3 in melanoma cell growth, we used FEMX-1 and SKMEL-28 cells with knockdown or overexpressed protein levels.
Warburg effect in breast cancer cells (Lim et al., 2016; Nunes-Xavier et al., 2016). Hence, we analyzed glycolysis by measuring extracellular acidification rate (ECAR) upon B7-H3 knockdown in two different melanoma cell lines (Figures 1e and 2a, right panel). shSCR and shB7-H3 cell variants had similar ECAR levels in basal and in glucose-induced glycolysis. However, shB7-H3 cells, in contrast to shSCR cells, did not respond to the addition of the oxidative phosphorylation inhibitor oligomycin, thus failing to induce the full glycolytic capacity of the cells. This suggests that the cell glycolytic reserve is lower in B7-H3 knockdown cells. Similar ECAR profile to that of B7-H3 knockdown was found in the presence of an inhibitory anti-B7-H3 monoclonal antibody (α-B7-H3, BRCA84D) (Figure S2). This shows that inhibiting B7-H3 expression in melanoma cells leads to decreased Warburg effect.

Expression of B7-H3, phospho-ERK1/2 (pERK1/2), ERK1/2, phospho-AKT (pAKT), and AKT was compared in four different melanoma cell lines: FEMX-1 (BRAF wt, HRASG12V mutated), SKMEL-28 (BRAFV600E mutated, RAS wt), HHMS (BRAF wt, RAS wt), and MeWo (BRAF wt, RAS wt) by immunoblot analysis. High expression of B7-H3, as well as expression of activated ERK1/2 and AKT, was detected in all parental cells (Figure 2b). The presence of α-B7-H3 (BRCA84D) caused a significant and antiproliferative effect in all four cell lines, as measured by cell viability and confluence (Figure 2c and Figure S3). We also observed some antiproliferative effect in WM1366 (BRAF wt, NRASQ61L) and WM902b (BRAFV600E mutated, RAS wt) melanoma cells in the presence of an α-B7-H3 (22F2) (Figure 2d). This is consistent with the effect of shB7-H3 described above.

Figure 2 Immunoblot and proliferation analysis of melanoma cell lines. (a) Left panel: Immunoblot of B7-H3 and tubulin expression from total lysates from SKMEL-28 shSCR and shB7-H3 cells. Plots show quantified immunoblot bands from B7-H3/tubulin, in arbitrary units (A.U.) from three independent experiments ± S.E.M. Middle panels: Relative proliferation and cell confluence of SKMEL-28 shSCR and shB7-H3 cells. Average relative proliferation and cell confluence of three independent experiments were measured by the MTS assay after 3 days in culture or by growing the cells in IncuCyte FLR or IncuCyte ZOOM Kinetic Imaging System (Essen BioScience) ± S.D. Results are shown from one representative experiment. Right panel: ECAR was measured by the Seahorse Extracellular Flux Analyzer XF96e in SKMEL-28 shSCR and shB7-H3 cells ± S.D. Note significant reduction in glycolytic capacity in SKMEL-28 shB7-H3 cells compared with shSCR cells. (b) Immunoblot of B7-H3, tubulin, pERK1/2, ERK1/2, pAKT, and AKT expression from total lysates from FEMX-1, SKMEL-28, HHMS, and MeWo melanoma cells. Plots show quantified immunoblot bands from B7-H3/tubulin, pERK/ERK, and pAKT/AKT A.U. from two independent experiments ± S.E.M. (c, d) Relative proliferation was measured in FEMX-1, SKMEL-28, HHMS, and MeWo melanoma cells in the presence of 100 ng/ml B7-H3 inhibitory antibody (α-B7-H3, BRCA84D) (c), or in WM1366 and WM902b melanoma cells in the presence of 10 μg/ml B7-H3 inhibitory antibody (α-B7-H3, 22F2) (d). Results are shown from one representative experiment. Statistically significant results (p < .05) are marked with *
2.2 | B7-H3 knockdown and inhibition increase the sensitivity of FEMX-1 melanoma cells to DTIC chemotherapy and MEK and AKT/mTOR inhibitors

The role of B7-H3 in FEMX-1 (HRASG12V mutated) cells on drug sensitivity was studied by measuring cell confluence and proliferation of FEMX-1 shSCR and shB7-H3 cells to DTIC chemotherapy and to targeted therapy with the MEK inhibitor binimetinib. FEMX-1 shB7-H3 cells were more sensitive than shSCR cells to DTIC (2.01 ± 0.29-fold) and more sensitive to binimetinib (1.34 ± 0.08-fold) (Figure 3a, and Figures S4A and S4B). Additionally, the antiproliferative effect of binimetinib treatment was further increased by the simultaneous presence of α-B7-H3 (1.57 ± 0.30-fold) (Figure 3b). In accordance with this, in B7-H3-overexpressing FEMX-1 cells, the increased B7-H3 expression led to reduced sensitivity to binimetinib (0.84 ± 0.04-fold) (Figure 4b). Immunoblot analysis of pERK1/2 and ERK1/2 in FEMX-1 shSCR and shB7-H3 cells treated with single agents indicated a differential and independent growth inhibitory effect of DTIC with no significant change in pERK1/2 activation, whereas in binimetinib-treated cells, an expected inhibitory effect on pERK1/2 activation was seen (Figure 3c). FEMX-1 shSCR and shB7-H3 cell variants treated with the combination of DTIC and binimetinib showed an increased growth inhibition in the FEMX-1 shSCR cells (Figure 3a), but not in shB7-H3 cells. This suggests that B7-H3 affects drug sensitivity through common effectors of both agents.

We also tested FEMX-1 cell sensitivity to the mTOR and AKT inhibitors, everolimus and triciribidine. FEMX-1 shB7-H3 cells, as well as FEMX-1 parental cells in the presence of α-B7-H3, were more sensitive to everolimus (1.17 ± 0.11-fold and 1.62 ± 0.41-fold, respectively) (Figure 4a, c) and to triciribidine (Figures S5A, S5B, and S5C) than control cells. In contrast, B7-H3-overexpressing cells were less sensitive to everolimus (0.89 ± 0.04-fold) (Figure 4b). Moreover, the parental HHMS and MeWo (BRAF wt and RAS wt) cells also showed increased sensitivity to both binimetinib and everolimus in the presence of B7-H3 antibody (Figure 4d, e). That the combination of these two inhibitors did not increase growth inhibition in the FEMX-1 shSCR and vector control cells suggests a similar mechanistic action of the inhibitors on the growth inhibition (Figure 4a, b).

2.3 | B7-H3 knockdown and inhibition affect sensitivity of SKMEL-28 melanoma cells to vemurafenib, binimetinib, and everolimus

The role of B7-H3 in SKMEL-28 (BRAFV600E mutated) cell sensitivity to the BRAF inhibitors vemurafenib, binimetinib, and everolimus was studied using cell confluence and proliferation assays. shB7-H3 cells were more sensitive to all three inhibitors as compared to control
Taken together, these results suggest that targeting of B7-H3 could be a suitable alternative for treatment of B7-H3-positive malignant melanomas.

shSCR cells (Figure 5a, b, and Figures S6A and S6B). A significant increased sensitivity of SKMEL-28 cells to binimetinib (1.19 ± 0.02-fold), vemurafenib (1.11 ± 0.02-fold), and everolimus (1.13 ± 0.07-fold) inhibitors was also seen in the presence of α-B7-H3 antibody (Figure 5c). As opposed to the results for FEMX-1 cells, an additional inhibitory effect of combination treatment of binimetinib together with everolimus, as well as for the combination of vemurafenib and everolimus, was observed for SKMEL-28 cells (Figure 5a, b).

2.4 | Targeting B7-H3 in patient-derived BRAFV600E mutated metastatic melanoma cell lines

In Patient 1-, Patient 3-, and Patient 4-derived cell lines (BRAFV600E mutated, RAS wt), we observed activation of ERK1/2 and AKT as well as high expression of B7-H3 protein (Figure 6a). All cell lines responded well to the α-B7-H3 treatment (Figure 6b, c), but showed a clear heterogeneity in response to treatment with the targeted therapies. Interestingly, Patient 1-derived cell line, which had the highest expression of B7-H3, was less sensitive to all targeted therapies, but responded to α-B7-H3, although less than the other two cell lines (Figure 6c). These differences are likely due to other cell type-specific factors. In all three patient-derived cell lines, we observed a weak yet significant additive antiproliferative effect of α-B7-H3 treatment in combination with vemurafenib and binimetinib. In cell lines from patients 1 and 4, we also observed a significant additive antiproliferative effect of α-B7-H3 in combination with everolimus (Figure 6c). Taken together, these results suggest that targeting of B7-H3 alone, or in combination with current anticancer therapies, could be a suitable alternative for treatment of B7-H3-positive malignant melanomas.

3 | DISCUSSION

B7-H3 exerts a pro-oncogenic role in cancer cells, is implicated in tumor immunology (Nygren, Tekle, Ingebrigtsen, & Fodstad, 2011; Wang, Kang, & Shan, 2014), and has been proposed as target in many types of solid cancer (Picarda, Ohaegbulam, & Zang, 2016). In accordance with this, we found an antiproliferative effect on various melanoma cell lines by B7-H3 knockdown by short hairpin RNAs as well as upon treatment with a monoclonal antibody targeting B7-H3. Currently, targeting B7-H3 by the use of an antibody (enoblituzumab, MGA271, MacroGenics; (Loo et al., 2012)) is being tested in phase I clinical trials in patients with B7-H3-positive cancers, including melanoma, as single agent (ClinicalTrials.gov: NCT01391143, NCT01918930), or in combination with checkpoint inhibitors for CTLA-4 (ipilimumab; NCT02381314) or PD-1 (pembrolizumab; NCT02475213).

We have previously reported that breast cancer cells with knockdown of B7-H3 showed increased sensitivity to paclitaxel chemotherapy and to AKT/mTOR inhibitors (Liu et al., 2011; Nunes-Xavier et al., 2016). Here, we show that low expression or inhibition of B7-H3 in melanoma cells renders them more sensitive to DTIC and to the inhibitors vemurafenib, binimetinib, everolimus, and triciribidine, targeting either MAPK or AKT/mTOR pathway. To our knowledge, this is the first time B7-H3 expression is linked to regulation of the sensitivity to both vemurafenib and binimetinib. Additionally, vemurafenib-resistant patient biopsy melanoma cell lines responded well to inhibition of B7-H3, as well as to binimetinib and everolimus. However, the Patient 1-derived cell line showed weaker response to α-B7-H3 therapy compared to the other patient-derived cell lines despite having high B7-H3 expression levels. This is in agreement with our observation that this
weak but significant enhanced growth inhibition upon treatment with combinations of vemurafenib and binimetinib with everolimus was observed (Figure 5). Interestingly, RAS mutations in melanomas have been found to activate both MAPK and PI3K/AKT/mTOR pathways, as opposed to BRAF that seems to only activate the MAPK pathway (Downward, 2003; Lasithiotakis et al., 2008). The cross talk between MAPK and PI3K/AKT/mTOR pathways in FEMX-1 cells would then depend on both MEK and AKT activation as inhibition of both causes a similar antiproliferative effect. However, treatment with B7-H3 antibody or knockdown of B7-H3 increased the growth inhibition to each of the monotreatments, but not in combination treatment, indicating that B7-H3 exerts a similar effect on both pathways.

We did not observe significant differences in cell cycle distribution upon B7-H3 knockdown (data not shown). Of note, the reduced proliferation rate in B7-H3-inhibited and knockdown cells may be associated with their loss of glycolytic capacity. Glycolytic capacity has also been proposed to be a predictor of drug sensitivity in tumor models (Mookerjee, Nicholls, & Brand, 2016). It has been shown that B7-H3 suppresses Nrf2 activity, eventually leading to promotion of aerobic glycolysis (Lim et al., 2016). Thus, loss of B7-H3 may reduce cell proliferation and increase drug sensitivity through the inability to generate enough energy of growth.

The subcellular localization of B7-H3 could be important for its functional role in tumorigenesis, but its intracellular localization has not previously been addressed in detail in melanoma cells. Here, B7-H3 was found mainly in the cell membrane, but also in the cytoplasm of melanoma cells. Importantly, we present evidence that the cytoplasmic localization of B7-H3 is within intracellular vesicles, that is, lysosomes and late endosomes, and that B7-H3 is present in extra-cellular vesicles. Exosomal localization has previously been reported only by mass spectrometry analyses of exosomes from melanoma cells (Lazar et al., 2015; Rappa, Mercapide, Anzanello, Pope, & Lorico, 2013). As the exosomal sorting of proteins is a tightly regulated process (Villarroya-Beltri, Baixauli, Gutierrez-Vazquez, Sanchez-Madrid, & Mittelbrunn, 2014), our findings that exosomes from B7-H3-overexpressing melanoma cells have an increased expression of B7-H3 propose an active sorting of B7-H3 to these vesicles and that B7-H3 might act at a distance via exosomes. Also, this opens the possibility of B7-H3 as a marker for detection of metastatic melanoma by non-invasive techniques.

Together, these results unveil a novel role for B7-H3 in melanoma sensitivity to chemotherapy and targeted therapy and support the hypothesis that targeting B7-H3 could be beneficial in metastatic melanoma treatment.

4 | METHODS

4.1 | Cell culture, plasmids, immunoblot, antibodies, and reagents

FEMX-1 and HHMS cell lines were previously established from metastatic lesions of malignant melanoma patients treated at the Oslo University Hospital Radiumhospitalet (Fodstad et al., 1988; Lillehammer et al., 2005). MeWo and SKMEL-28 cells were purchased

FIGURE 5 Proliferation of SKMEL-28 shB7-H3 cells treated with BRAF, MEK, and mTOR inhibitors. Relative proliferation of the cells was measured by MTS assay after 3 days of treatment of SKMEL-28 shSCR and shB7-H3 cells with 0.5 μM vemurafenib, 200 nm everolimus, and combination of 0.5 μM vemurafenib and 200 nm everolimus (a), or in SKMEL-28 cell variants shSCR and shB7-H3 with 0.5 μM binimetinib, 200 nm everolimus, and combination of 0.5 μM binimetinib and 200 nm everolimus (b), or in parental SKMEL-28 with or without 0.5 μM binimetinib, 1 μM vemurafenib, and 200 nm everolimus with or without the presence of 100 ng/ml B7-H3 monoclonal inhibitory antibody (α-B7-H3, BRCA84D) (c). In all experiments, DMSO was used as a vehicle control. All MTS data are average of three independent experiments ± S.E.M., normalized, and relative to untreated cells. Statistically significant results (p < .05) are marked with *
from ATCC. WM1366 and WM902b melanoma cells were kindly provided by Prof. M. Herlyn (Wistar Institute, Philadelphia, PA, USA). All parental and variants of the melanoma cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. BRAFV600E mutant patient-derived cell lines (patients 1, 3, and 4) were established from biopsies before vemurafenib treatment as previously described (Lai, Jiang, Farrelly, Zhang, & Hersey, 2012) and were a kind gift from Prof. P. Hersey (Kolling Medical Research Institute, Royal North Shore Hospital, University of Sydney, Australia). These were grown in DMEM (Invitrogen) supplemented with 2 mM L-glutamine and 5% FBS (for patients 1 and 3) and 10% FBS (for Patient 4). Generation of shSCR and shB7-H3 cell variants was previously validated for specific knockdown as previously explained (Tekle et al., 2012). The mammalian expression plasmid to generate stable overexpressing cell lines was previously explained (Nunes-Xavier et al., 2016). Whole-cell protein extracts were prepared for total cell lysis, and immunoblot were performed as described previously (Nunes-Xavier et al., 2010; Nygren et al., 2014). Antibodies used for Western blotting were as follows: B7-H3 (AF1027, R&D), pERK1/2 (9101, Cell Signaling), ERK1/2 (sc-93 and sc-154, Santa Cruz), pAKT (4060, Cell signaling), AKT (9272, Cell Signaling), CD63 (ab59479, Abcam), tubulin (CP06, Millipore). B7-H3 inhibitory monoclonal antibody, BRCA84D, was kindly provided by MacroGenics, and 22F2 was from Dr. Reinhard Zeidler. Protein concentrations from total cell lysates were measured using The Pierce® BCA Protein Assay Kit (Thermo Scientific, USA). Binimetinib (MEK-162, MedChem Express), vemurafenib (PLX4032, Selleckchem), everolimus (RAD001, InvivoGen), and triciribidine (API-2, Sigma-Aldrich) were used at indicated concentration, during the indicated times. Dimethyl sulfoxide (DMSO) was used as a control, at the same final concentration as resuspended and diluted drugs. Drug responses were plotted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA), and IC50 (half maximal inhibitory concentration) values were obtained for DTIC, vemurafenib, binimetinib, everolimus, and triciribidine in the cell variants and are presented in Table S1.

4.2 Exosome purification and analysis

FEMX-1 cells was plated in T160 flasks and grown in 20 ml RPMI-1640 cell culture media with exosome-depleted FBS (GIBCO) for 3 days. Exosomes were purified by sequential centrifugation of cell culture supernatant, first by centrifugation at 1000 g for 5 min, then 2500 g for 10 min. To remove larger cell debris and possible apoptotic bodies, supernatant was centrifuged at 20,000 g for 20 min (Beckman Coulter, JA-25.50 rotor). Finally, exosomes were collected by centrifugation at 100,000 g for 70 min (Beckman Coulter, 70Ti rotor) and washed in 10 ml PBS. Exosome fraction was verified by electron microscopy, lysed, and analyzed by immunoblot.

4.3 In vitro proliferation, cell confluence, and colony formation assays

Cells (5 × 10^5 cells) were plated in 96-well culture plates in media and treated with DMSO, DTIC, vemurafenib, binimetinib, everolimus, or...
triciribidine after 21 hr and processed after 72 hr post-treatment. Cell proliferation was determined by CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (MTS, Promega Corp, Madison, WI, USA). Absorbance was measured at 490 nm using Wallac Victor2 1420 Multilabel Counter (PerkinElmer, USA). The data are presented as the average absorbance ± S.E.M corrected for background from at least three independent experiments. Cell confluence was measured growing the cells in IncuCyte FLR or IncuCyte ZOOM Kinetic Imaging System (Essen BioScience) that estimate cell growth and number. Cells were scanned every 3 hr during the times indicated. For colony formation assays, 500 cells were plated in six-well culture plates in media with DMSO or DTIC and processed after 14 days post-treatment. Colonies were stained as described previously (Nunes-Xavier et al., 2010). The data are presented as cell confluence ± S.D. from one representative experiment. Assays were performed in at least triplicate wells three times for each cell line at separate days. Fold change in sensitivity ± S.E.M. for all compounds in the cell variants were quantified and are presented in Table S2.

4.4 Extracellular acidification rate

XF96 glycolysis stress test was performed using Seahorse Extracellular Flux Analyzer XF96e to measure the extracellular acidification rate (ECAR) according to the manufacturer’s instructions. Cells were seeded in Seahorse plate 48 hr after splitting and cultured overnight to 80% confluence. Before measurement, the culture medium was replaced with cellular assay medium (Seahorse Bioscience) supplemented with 2 mM glutamine and incubated for 1 hr in a CO₂-free incubator. Assays were performed according to Seahorse protocols with the final concentrations of 10 mM glucose, 1 μM of oligomycin, and 100 mM of 2-deoxy-D-glucose (2-DG) and were performed in at least triplicate wells in three independent experiments for each cell line and condition at separate days ± S.D.

4.5 Statistical analysis

Error bars in results represent data average ± standard deviation (S.D.) or standard error of the mean (S.E.M.) for results showing an average of indicated number of independent experiments. Two-tailed student t test was used to evaluate statistical significance. p values of p < .05 were considered significant and marked in the results with an asterisk.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


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