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| Complete List of Authors:      | Body, Simon; INSERM U1245, UNICAEN  
Estève-Arenys, Anna; Institut d’Investigacions Biomediques August Pi i Sunyer, Division of Hematology and Oncology  
Recasens-Zorro, Clara; Institut d’Investigacions Biomediques August Pi i Sunyer, Division of Hematology and Oncology  
Troussard, Xavier; INSERM U1245, UNICAEN; Laboratoire d'Hématologie Biologique  
Rouë, Gaël; Institut d’Investigacions Biomediques August Pi i Sunyer, Division of Hematology and Oncology; Hospital Vall d’Hebron, Department of Hematology  
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LETTER TO THE EDITOR

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1 Normandie Univ, INSERM UMR1245, UNICAEN, Caen, France
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4 Molecular Genetics Unit, Department of Hematology, University Hospital Vall d’Hebron, Barcelona, Spain

* Corresponding author : Brigitte Sola. Address: MICAH team, UFR Santé, CHU Côte de Nacre, 14032 Caen Cedex, France. Mail: brigitte.sola@unicaen.fr

Running head: Lack of ERβ agonist activity on MCL

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Mantle B lymphoma (MCL) is a rare, still incurable, aggressive malignant hemopathy of mature B cells [1]. In the bone marrow and secondary lymphoid organs, tumor cells are connected with their environment (stromal cells, endothelial cells, osteoblasts, osteoclasts, immune cells, etc.) by cytokines, adhesion proteins and their respective receptors [2]. These interactions are responsible for a mechanism known as CAM-DR (cell adhesion-mediated drug resistance) [2]. This is why current therapies attempt to target both tumor cells and the tumor microenvironment (TME).

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According to the dependency of MCL cells with their TME, we suspected that MCL tumor cells-stroma interactions would not be the same for MCL cells engrafted s.c. or in their physiological niche. We engineered four cell lines: JeKo1-mCherry-Luc, Z138-Luc, REC1-GFP-Luc and Granta519-GFP from their respective parental cell lines. Cells were then injected i.v. into NOD-SCID IL2Rnull (NSG) mice (n = 6). All animals recapitulated bona fide MCL disease. Tumor cells having luciferase activity were detected by bioluminescence imaging (BLI) with a
kinetics dependent of each cell line: JeKo1 being the most aggressive and REC1 the less aggressive cells (Fig S1). BLI examination indicated that tumor cells housed in hematopoietic organs (bone marrow, spleen) and, in some cases, in non-hematopoietic organs (liver, lung, kidney). The identity of tumor cells was verified at the time of mice euthanasia by cytometry analysis of CD20+ cells purified from infiltrated organs (Table 1).

We analyzed the expression of ERα and ERβ on a panel of MCL cells both at the mRNA and protein levels. ERα mRNAs were absent or very low in all cell lines (data not shown) according to previous data [5]. In contrast, ERβ was expressed in all MCL cell lines (including GFP/mCherry/Luc expressing JeKo1, Granta519, REC1 and Z138 cells) tested although at various levels (Fig 1A). We next assessed in vivo the effects of both DPN and ERB-041 (or 2-(3-Fluoro-4-hydroxyphenyl)-7)vinyl-1,3-benzoxazol-5-ol), another selective ERβ agonist [6], on MCL dissemination and growth. Z138-Luc or REC1-GFP-Luc cells were injected in NSG mice. The same day, mice were separated into three groups (n = 3) receiving: vehicle (0.01% DMSO), DPN or ERB-041 both at the concentration of 1 mg/kg. Drugs and vehicle were injected i.p. 5 days a week. BLI was recorded each week starting at day 7. Mice were euthanized at day 31 (Z138-Luc) or day 41 (REC1-GFP-Luc). As shown Fig 1B, DPN or ERB-041 treatments did not modify the engraftment properties of MCL tumor cells, since all mice were engrafted with luciferase-positive cells, or their homing, since infiltrated organs were the same among the different groups. Moreover, the number of CD45+ tumor cells in each organ was also similar between the three groups (Fig S2). In sharp contrast with published data [5], ERβ agonists have no effects on tumor cells growth in this in vivo model. In vitro, although DPN was more active than ERB-041 and, Granta519 being the cell line with the highest sensitivity, we did not observed any dose-dependent effect of ERβ agonists on cell proliferation (Fig S3).
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However, our in vitro experiments confirmed that, although ERβ are well-expressed in the MCL cell lines, ERβ agonists had no effect on proliferation, migration, invasion or VEGF production, emphasizing the in vivo results. Interestingly, we found previously that, although able to decrease multiple myeloma cell proliferation, ERβ agonists are inefficient in vivo [8].

In conclusion, our results highlight a lack of effects of ERβ agonists as single agents in mice. Nonetheless, as it has been described that estrogen play an important role in BCR pathway regulation [9], consequently further investigations would be required to assess the efficacy of ERβ agonists in association with B-cell receptor inhibitors, like ibrutinib.

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**Authors contribution**

SB, AE-A, and CR-Z performed the research and analyzed the data. XT provided essential support. GR and BS designed and supervised the study. SB and BS wrote the paper. All authors read the final version of the manuscript and agree with its content and submission.

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* in the JeKo1-mCherry-Luc series, three mice were sacrificed at day 23. ** in the Z138-Luc series, three mice were sacrificed at day 30. *** in the REC1-GFP-Luc series, three mice were sacrificed at day 33. † in the Granta-GFP series, three mice died unexpectedly at day 17, two mice were sacrificed at the same day. At the time of euthanasia, indicated organs were collected; single-cell suspensions were prepared, then stained with IOTest® CD20-PE (phycoerythrin, IM1451, FL2) or the corresponding isotype control and analyzed on an Attune acoustic focusing cytometer (Thermo Fisher Scientific). The percentage of positive cells in each organ is indicated in the table. no, no signal; nd, not done.
LETTER TO THE EDITOR

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Running head: Lack of ERβ agonist activity on MCL

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SB, AE-A, and CR-Z performed the research and analyzed the data. XT provided essential support. GR and BS designed and supervised the study. SB and BS wrote the paper. All authors read the final version of the manuscript and agree with its content and submission.

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* in the JeKo1-mCherry-Luc series, three mice were sacrificed at day 23. ** in the Z138-Luc series, three mice were sacrificed at day 30. *** in the REC1-GFP-Luc series, three mice were sacrificed at day 33. † in the Granta-GFP series, three mice died unexpectedly at day 17, two mice were sacrificed at the same day. At the time of euthanasia, indicated organs were collected; single-cell suspensions were prepared, then stained with IOTest® CD20-PE (phycoerythrin, IM1451, FL2) or the corresponding isotype control and analyzed on an Attune acoustic focusing cytometer (Thermo Fisher Scientific). The percentage of positive cells in each organ is indicated in the table. no, no signal; nd, not done.
Fig 1
Additional informations

Additional methods

**Cell culture and cell proliferation measurement**

MCL cell lines have been described previously (Moros et al, 2014). Cell lines were maintained in culture in RPMI 1640 medium (Lonza) supplemented with 10-20% FCS (PAA Laboratories), 2 mM L-glutamine and antibiotics (Lonza) in a humid atmosphere at 37°C. Cell authentication was done by short tandem repeat (STR) profiling (IdentiCell, Aarhus, Denmark).

Cell proliferation was assesses by an MTS assay (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, here the CellTiter 96® AQ™ueous One Solution Spromega) according to the supplier’s instructions.

**Transwell migration and invasion assays**

For migration assay, 5 x 10^5 cells in serum-free medium plus 0.5% BSA were added to the top chamber of 24-well inserts (Costar Transwell® Permeable Support, pore size 5 μm). For invasion assay, the same number of cells were seeded in transwell inserts coated with extracellular matrix ECM obtained from Engelbrecht-Holm-Swarm mouse sarcoma (Sigma-Aldrich). Medium supplemented with SDF1 (200 ng/ml) or not (for control), was added in the bottom chambers. Plates were incubated for 4 h (migration) or 24 h (invasion) at 37°C. Migrating and invaded cells present in the lower chambers were counted by flow cytometry. The accuracy of cell count was checked by the use of fluorescent particles added in the chambers (Accucount Fluorescent particles, SpheroTech).

**Tube formation assay**
HUVECs were plated onto a layer of Matrigel (BD Bioscience) at a density of $4 \times 10^4$ cells/well in 24-well plates. Immediately after, cells were treated with supernatants of DPN/ERB-041-treated (100 nM or 1 μM for 48 h) or vehicle-treated MCL cells. Cultures were observed 24 h later under an inverted microscope (Olympus IX2-SL, X 40 magnification) and the number of formed capillary tubes counted on 11 representative fields. A junction was recorded when a tube was bound to, at least, one another tube.

**Western blotting**

Proteins were purified from cultured MCL cell lines including GFP/mCherry/Luc-expressing cells with the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific), quantified, separated by SDS-PAGE, blotted onto nitrocellulose paper and incubated with antibodies against ERβ (sc-8974, Santa Cruz Biotech.) or β-actin (sc-47778) as a control for gel loading and transfer. Our procedure has been described previously (Moros et al, 2014).

**MCL cells engraftment in immunocompromised mice**

*In vivo* experiments were conducted in accordance with the recommendations of EEC (86/609/CEE). They were approved by the Animal Experimental Ethics Committee of our institution (UB-CEEA agreement 251/15 in Spain). Z138-GFP-Luc, REC1-GFP-Luc, JeKo1-mCherry-Luc, Granta519-GFP cell lines were engineered using lentiviruses or retroviruses. Male NSG (n = 6 per group) mice (age 6-8 weeks) were injected i.v. with $10^7$ cells in a final volume of 100 μl. Tumor engraftment was determined weekly by bioluminescence imaging (BLI). Mice were injected i.p. with 75 mg/kg of D-luciferine and then analyzed with a Aequoria Luxiflux bioluminescent device quipped with an ORCA-ER camera (Hamamatsu). Photon emissions were captured through the whole mouse body. At the end of the
experiment mice were euthanized. Then, infiltrated organs were collected; single-cell suspensions were prepared, then stained with IOTest® CD45-PE (phycoerythrin, IM1451, FL2) or the corresponding isotype controls as recommended by the supplier.

For ERβ agonists treatment, mice were either engrafted with Z138-Luc or REC1-GFP-Luc. The same day, mice were separated into three groups (n=3 in each one), one receiving DPN (1 mg/kg), another ERB-041 (1mg/kg), and the last one vehicle (0.1% DMSO). Mice were treated five days a week. Mice of the Z138 group were euthanized at day 29, of the REC1 group, at day 39. BLI was recorded as mentioned before.

Additional figure legends

Supplemental Fig S1

Six-week old NOD-scid IL2rγ null (NSG) male mice were injected in the caudal vein with 10⁷ JeKo1-mCherry-Luc, Z138Luc, or REC1-GFP-Luc cells. Mice were weekly analyzed for BLI starting one week after cells injection.

Supplemental Fig S2. Effect of DPN and ERB-041 treatment on MCL tumor cell infiltration in vivo.

NSG mice were injected intravenously with Z138-Luc cells and received a daily dose of DPN (D, 1 mg/kg), ERB-041 (E, 1 mg/kg) or the equivalent volume of vehicle (V), for up to 14 days. Mice were then sacrificed and human CD45+ malignant B cells were recounted on a cytometer from spleen, brain, bone marrow extracts and in peripheral blood samples, as described in the methods section. Shown are the relative mean fluorescence intensity (MFI-R) values between CD45-derived fluorescence and isotypic control, calculated in the different compartments and for each treatment group (statistical significance: * p < 0.05).

Supplemental Fig S3
Granta519-GFP, JeKo1-mCherry-Luc, REC1-GFP-Luc and Z138-Luc cells were either treated with the vehicle (0.1% DMSO) or various concentrations (0.1-10^4 nM) DPN or ERB-041 for 48 h. Cell proliferation was assessed using an MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). The experiment was performed twice with triplicate samples with similar results. The means ± SD from one representative experiment are reported on the histograms. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant with the Student’s t-test.

**Supplemental Fig S4**

**A. Left panel.** JeKo1 and Z138 cultured cells were were seeded into the top chamber of transwell inserts. Inserts were transferred into wells containing medium with SDF1 or without SDF1 (Ctrl) and cells were allowed to migrate for 4 h. Cells present in the bottom well were then counted. Three independent inserts were seeded per experiment; the experiment was performed three times. Mean numbers of migrating cells ± SD were plotted on the graph. **Middle and right panels.** Granta519 and JeKo1 cells were treated in vitro with vehicle or ERB-041 (0.1 or 1 μM) for 48 h or 72 h. Then they were seeded and assayed for migration as described before. Three independent inserts were seeded per experiment; the experiment was performed three times. The percentage of migrating cells ± SD was plotted on the graph. **B. Left panel.** JeKo1 cells were seeded in transwell inserts coated with extracellular matrix (ECM) proteins. Inserts were transferred into wells containing medium with SDF1 or without SDF1 (Ctrl). Cells were allowed to invade ECM for 24 h. The number of invaded cells is plotted on the graph. The experiment has been carried out twice with triplicate samples. **Middle and right panels.** JeKo1 and Z138 cells were treated with vehicle, DPN (0.1-1 μM) or ERB-041 (0.1-1 μM) for 48 h and seeded in ECM-coated transwell inserts. Inserts were incubated for 24 h and the number of cells present in the bottom wells...
counted. The experiment was repeated three times with triplicate samples. The mean ratios (treated vs. ctrl) ± SD are plotted on the graph. ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001 in t-tests.

Supplemental Fig S5

A. HUVECs were plated onto a layer of Matrigel (BD Bioscience) at a density of 4 x 10⁴ cells/well in the presence of supernatants from JeKo1 and Granta519 cells previously treated with ERB-041 (0.1 or 1 μM, 4 h) or with vehicle as a control. After a 24 h-incubation period, cells were photographed under a microscope (Olympus IX2-SL). The number of branch points (circled in red) with at least two branches, corresponding to newly formed capillary tubes was counted on representative fields. The experiment has been done twice with triplicate samples.

B. The quantification of produced VEGF was done using the Quantikine ELISA kit (R&D systems) according to the manufacturer’ instructions on a Victor X4 plate-reader (Perkin Elmer). The standard curve was drawn from the OD at 450 nm of calibrated VEGF concentrations ranging from 10³ to 15.6 pg/ml.

Additional reference

Fig S1

JeKo1-mCherry-Luc  
Z138-Luc  
REC1-GFP-Luc

no signal  
no signal  
no signal
Fig S2
Fig S3

Granta519-GFP
JeKo1-mCherry-Luc
REC1-GFP-Luc
Z138-Luc

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**Fig S4**

**Leukemia and Lymphoma**

48 h

% of migrating cells (treated vs. ctrl)

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<th>JeKo1</th>
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72 h

% of migrating cells (treated vs. ctrl)

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% of migrating cells (treated vs. ctrl)

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Number of cells

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**Z138**

Number of cells

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**JeKo1**

Number of cells

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**Fig S4**
**Fig S5**

URL: http://mc.manuscriptcentral.com/glal