Endogenous and combination retinoids are active in myelomonocytic leukemias

Orsola di Martino,[1] Haixia Niu,[2] Gayla Hadwiger,[1] Heikki Kuusanmaki,[3] Margaret A. Ferris,[4] Anh Vu,[1] Jeremy Beales,[1] Carl Wagner,[5] María P. Menéndez-Gutiérrez,[6] Mercedes Ricote,[6] Caroline Heckman[3] and John S. Welch[1]

[1]Department of Internal Medicine, Washington University, St Louis, MO, USA; [2]Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; [3]Institute for Molecular Medicine Finland, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland; [4]Department of Pediatrics, Washington University, St Louis, MO, USA; [5]School of Mathematical and Natural Sciences, Arizona State University, Phoenix, AZ, USA and [6]Myocardial Pathophysiology Area, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

 doi:10.3324/haematol.2020.264432

Received: June 26, 2020.

Accepted: November 16, 2020.

Pre-published: February 4, 2021.

Correspondence: *JOHN S. WELCH* - jwelch@wustl.edu

Supplemental Methods

Reagents

Bexarotene was from LC Laboratories (Woburn, MA, USA). Corn oil, alpha-Tocopherol, ATRA, and pIpC were from Sigma-Aldrich (St. Louis, MO, USA). Tamibarotene, BMS753, BMS961, GW7647, Pioglitazone, GW3965, BMS493, HX531, LG100754, 9cisRA, Am80, LG268, flurobexarotene, SR11237, Hx630 were from Tocris (Bristol, UK). NRX194204 was from Axon Medchem (Reston, VA, USA). CW103-4 was synthesized by Carl Wagner. ATRA 21-day release pellets were from Innovative Research of America (Sarasota, FL, USA). Targretin capsules were a gift from Gary Landreth, Indiana University. Anti-RXRA antibody (H-10) and GAPDH Antibody (FL-335) were from Santa Cruz Biotechnology (Dallas, TX, USA). Cytokines and Methylcellulose complete media were purchased from R&D Systems (Minneapolis, MN, USA).

Plasmid and cell lines

The pBABE-RXRA plasmid was a gift from Vivek Arora, Washington University. The Gal4-SMRT, Gal4-NCOR, SMRT-VP16, NCOR-VP16, VP16-RARA, and VP16-RXRA constructs were gifts from Mitchell Lazar, University of Pennsylvania. MSCV-TLS-ERG plasmid was a gift from Catherine Carmichael, Monash University. MSCV-Notch1 plasmid was a gift from Grant Challen, Washington University. THP1 and 293T cells were obtained from ATCC. Monomac6, OCI-AML-3, and MOLM13 cells were obtained from DSMZ.

Genomic DNA extraction and analysis

Genomic DNA was isolated from mice BM and spleen using the Qiagen EZ1 DNA Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA was amplified using similar strategies as (23).

Immunoblot

Total protein extracts were lysed in RIPA buffer (Cell Signaling, Danvers, MA, USA) including 1× cocktails of protease and phosphatase inhibitors (Sigma Aldrich) and gel electrophoresis was performed as (12). Images were acquired by myECL Imager (Thermo Fisher).

Retrovirus production and transplantation

Retrovirus production and transduction were performed exactly as previously described (14). Kit+ BM cells were spinfected as before (14) and injected by tail vein into sublethally irradiated recipient mice. Upon transplant, mice were treated with Bactrim in the drinking water for 14 days to limit infection complications. Mice treated with ATRA received a 21-day-release pellet (Innovative Research of America, Sarasota, FL) subcutaneously implanted.

Mammalian two-hybrid assay

293T cells were co-transfected with UAS-GFP reporter, “bait”, and “prey” plasmids using Lipofectamine 3000 (Thermo Fisher). Six hours after transfection, cells were washed and fresh media added. Cells were collected and analyzed by flow cytometry 72 hours after transfection.

Differentiation, apoptosis and cell cycle analysis

MLL-AF9 leukemia cells were treated with ATRA/bexarotene and cytospins stained using Wright-Giemsa (Sigma Aldrich). Images were obtained with a Nikon Microphot-SA microscope. Colony forming was evaluated by plating 2,000 cell/plate in 1.1 ml of complete mouse methylcellulose medium (R&D Systems). Colonies were counted on day 7. To evaluate the cell cycle progression *MLL-AF9* cells were treated with ATRA/bexarotene at indicated concentration cells and stained with FxCycle-Violet

(Biolabs, Ipswich, MA, USA). The proliferation rate was analyzed on a ZE5 Cell Analyzer (Biorad) at the indicated time points. For apoptosis analysis, *MLL-AF9* cells were treated with ATRA/bexarotene at indicated concentration and cell viability was analyzed using the Annexin-V assay Kit (BD Pharmingen, Franklin Lakes, NJ, USA) on a ZE5 Cell Analyzer (Biorad). To quantify caspase activity cell titers were determined after 48hrs of ATRA/bexarotene treatment using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) and caspase activity was determined using the Caspase-Glo 3/7,8 and 9 Assay Kits (Promega). Caspase activity was normalized to CellTiter-Glo viability activity to account for differences in cell number.

Data analysis

Flow cytometry data were analyzed with FlowJo software version 10. Synergy was calculated with results from SynergyFinder.fimm.fi (24). Crystal structure data from PDB: 4K4J were visualized in Pymol (Schrodinger, Inc, New York, NY, USA). Gene expression data from AML cases in TCGA were retrieved, and the FPKM values for each gene were converted to Z-scores. Two Pearson correlation coefficients were calculated for each gene, one comparing against the expression of RARA, and one comparing against the expression of RXRA. Statistical analysis was performed using Prism (GraphPad, San Diego, CA, USA). T-test and ANOVA tests were performed, as appropriate. TGCA AML dataset available on https://leylab.shinyapps.io/TCGA_AML_Web_App/. All studies were performed in triplicate unless otherwise indicated. Error bars represent standard deviation. Data points without error bars have standard deviations below GraphPad's limit to display.

Supplemental Figure 1. Retinoid receptor expression in human samples. Hierarchically clustered heat map showing scaled expression of *RARA, RXRA,* and cell surface markers with positive or negative correlation in TCGA AML RNA Seq samples (n = 197, (7)). Pearson correlation coefficient (PCC) for are indicated in the table. Note that RARA and RXRA are variably, but coordinately expressed (PCC 0.73) in AML, and they strongly correlate with expression of myelomonocytic markers (CD14, ITGAM, ITGB2,CD86, etc), but negatively correlate with expression of immature markers (KIT, CD34,CRLF1, etc). B-G. Expression profile of retinoid receptors in total bone marrow cells in the same 197 human AML cases and sorted normal bone marrow cells. FAB is indicated on X-axis. NC: bispecific leukemia. CD34: CD34+ cells. Pro: promyelocytes. PMNs: neutrophils. One-way ANOVA with Tukey post-test was positive (** $p < 0.01$ or *** $p < 0.001$) for all pairwise comparisons with neutrophils in panels B, E, and F. Similar results are observed in additional publicly available datasets with greater granularity of subset flow-based analysis (http://servers.binf.ku.dk/bloodspot/).

Supplemental Figure 2. Schema of reporter assay and ex vivo validation. The Gal4-DNA binding domain (DBD) is fused to the RXRA ligand-binding domain (LBD). The Gal4 DBD recognizes the yeast UAS promoter sequences. (A) Ligand binding (black circle) to RXRA LBD repositions the AF2 domain (helix 12), displaces corepressors, recruits coactivators (CoA), and permits transactivation of UAS-GFP reporter. (B) When no ligands are present, the AF2 domain is extended, corepressors are bound, and the UAS-GFP reporter is not active. (C) The AF2 deletion removes helix 12. Without ligand-dependent repositing of helix 12, corepressors remain bound, coactivators are not recruited, and the UAS-GFP reporter is not transactivated. Corepressors NCOR and SMRT each have two nuclear receptor binding domains and are illustrated interacting with a homodimer element. The multimerized UAS promoter contains five UAS sequences and alternative multimer Gal4-RXRA binding configurations are possible. (D-

E). *MLL-AF9* leukemia cells derived from *UAS-GFP* bone marrow and transduced with *MSCV-Flag-Gal4-RXRA-IRES-mCherry* retrovirus *(MLL-AF9 Gal4-RXRA* cells*)* or *MSCV-Flag-Gal4-RARA-IRES-mCherry* retrovirus *(MLL-AF9 Gal4-RARA* cells*)* were treated as indicated and the percentage of GFP+ cells was assessed 48hrs after by flow cytometry. The calculated IC_{50} is indicated. (F) Table of RXR agonists used in D-E.

Supplemental Figure 3. Serial replating of MLL-AF9 Gal4-RXRA leukemia cells treated with ATRA and bexarotene. (A-B) *MLL-AF9 Gal4-RXRA* cells were treated as indicated in duplicate, and the percentage of GFP and mCherry and the GFP median fluorescence intensity was measured by flow cytometry at indicated time points.

Supplemental Figure 4. Pharmacological targeting RXRA and RARA *in vitro*. (A-B) *MLL-AF9* leukemia cells were treated as indicated for 48 hours, replated and re-treated with fresh compounds, and total viable cells in 50 µl assessed in duplicate after 96 hours of total treatment. Compounds used include (A) RXR agonists HX630, fluorobexarotene, SR11237, LG268, CD3254, NRX194204; (B) RXR agonists in combination with the pan-RAR agonist ATRA; (C) pan-RAR-inverse agonist BMS493, RARA-specific agonist tamibarotene, pan-RXR competitive antagonist HX531, pan-RXR antagonist LG100754, pan-RXR agonist 9cis retinoic acid (9cisRA); (D) pan-RAR-inverse agonist BMS493 in combination with ATRA/bexarotene; (E) PPARA-specific agonist GW7647 in combination with bexarotene; (F) PPARG-specific agonist pioglitazone in combination with bexarotene.

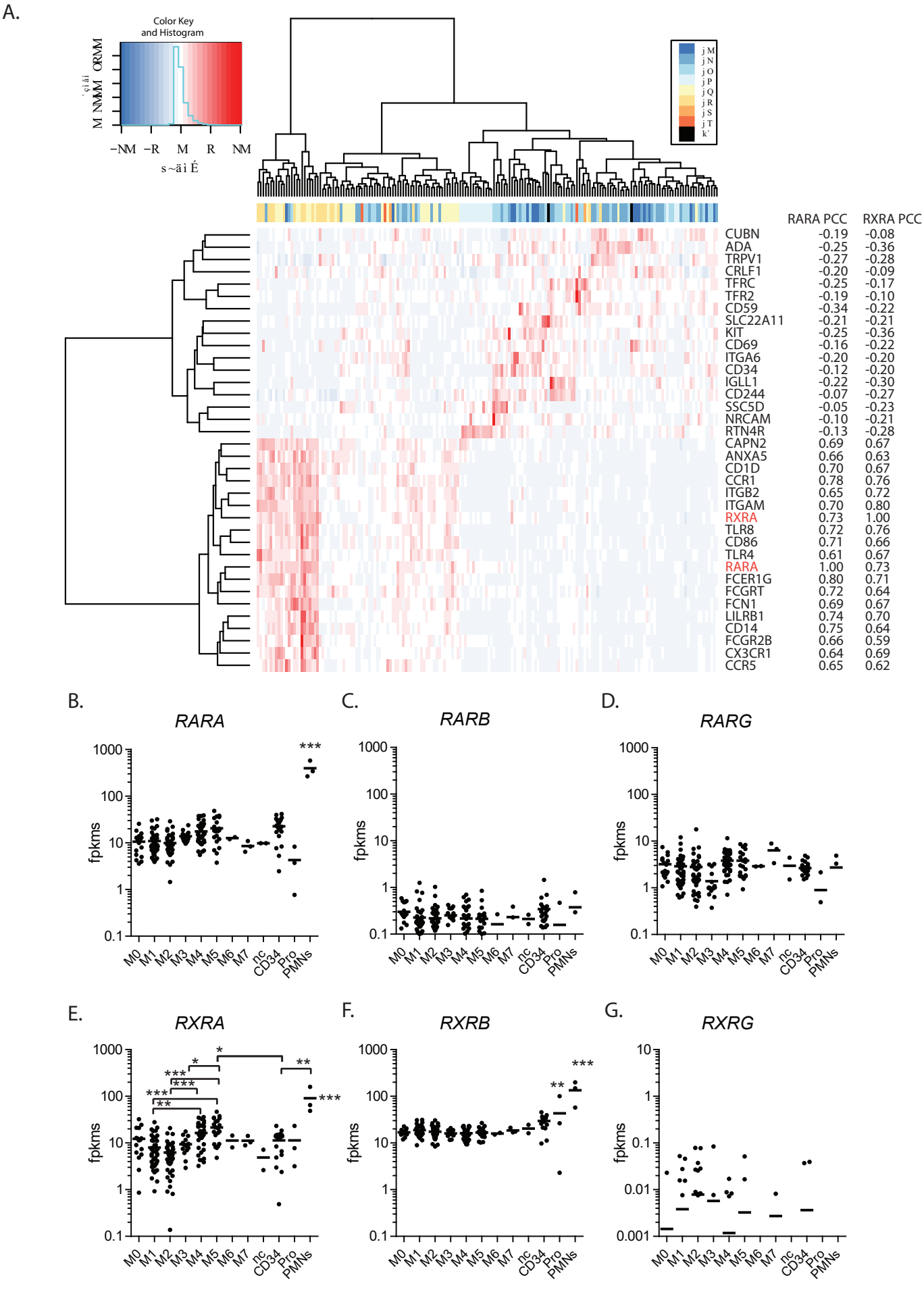
Supplemental Figure 5. RXRA mutations structural position and expression level. (A-B) Position of ligand-binding domain mutations that were analyzed in Figure 3g overlayed on structural results of the human RXRA structure (grey) with Nuclear receptor coactivator 2 peptide (orange) and an RXRA ligand (cyan, PDB: 4K4J). Amino acid color schema reflects schema in Figure 3g and are shown in ball and stick format. Green: L276/V280. Blue: K440. Brown: 453. Light blue: V298/L301. Red: Y402. Yellow: R426.

A. 4K4J in standard nuclear receptor position. B. 4K4J rotated 120 degrees about the vertical axis. Note that L276/V280 (green), V298/L301 (light blue), and E453 (brown) all localize within a co-activator binding plane, and mutations at these positions have been implicated in reduced co-activator binding. K440 (blue) occurs at the end of helix 11, and has been implicated in co-repressor binding. R426 (yellow) and Y403 (red) have been implicated in heterodimerization. R316/L326 (light green) are amino acids with direct

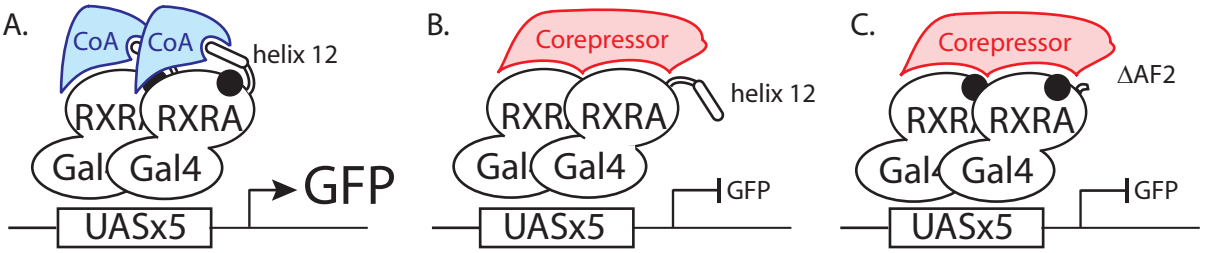
contact with the ligand. (C) Western blot analysis using whole-cell lysates from RXR-KO *x MLL-AF9* leukemia cells transfected with retroviruses encoding *MSCV-RXRA-IRES-mCherry* vectors and indicated mutations. Un-transduced RXR-KO x *MLL-AF9* leukemia cells were used as negative control*.* 48 hours after transduction, protein extract was collected and a Western blot was performed using anti-RXRA antibody (H-10 SantaCruz Biotechnology). Western blotting with anti-GAPDH antibody serves as a loading control (lower panel).

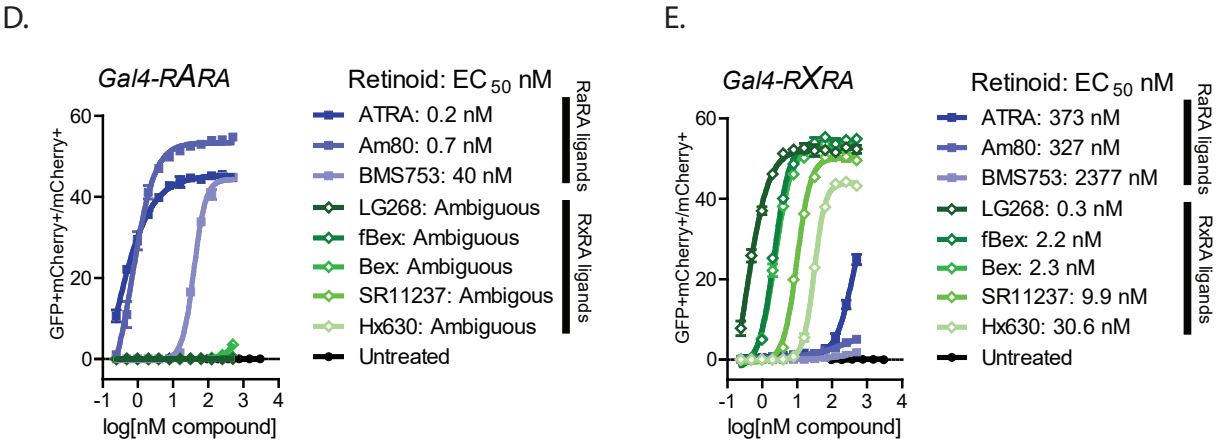
Supplemental Figure 6. CW103-4 biological activity *in vitro*. (A) Colony Forming Units (CFUs) in methylcellulose per 2000 *MLL-AF9* leukemia cells treated as indicated and assessed in triplicate. (B) Annexin V staining of *MLL-AF9* leukemia cells after 48 hours of CW103-4 treatment in triplicate. (C) Relative activity of caspases 3/7, in *MLL-AF9* leukemia cells after 48 hours of CW103-4 treatment in triplicate. (D) Photographs of MLL-AF9 colonies treated as indicated for 7 days. (F-H). *MLL-AF9* leukemia cells derived from *UAS-GFP* bone marrow and transduced with *MSCV-Flag-Gal4-PPARD-IRES-mCherry*, *MSCV-Flag-Gal4-LXRA-IRES-mCherry or MSCV-Flag-Gal4-LXRB-IRES-mCherry* retrovirus were treated as indicated in triplicate. The percentage of GFP+ cells was assessed 48hrs after transfection by flow cytometry. ** $p < 0.01$, ***$p < 0.001$, T-test with Welch's correction relative to control.

Supplemental Figure 7. **Effect of *in vivo* ATRA and bexarotene treatment.** Cohorts of 5 wild type FVB mice were orally gavaged with ATRA (0.831mg) plus bexarotene (1 mg) or vehicle control 5 days per week for 3 weeks and complete blood counts assessed: (A). The percent of blood cells. (B) The white blood cell count in K/µl (WBC) (C). The hemoglobin level in g/dL (Hb) (D). The mean corpuscular volume in fL (MCV). (E) Tthe number of platelets (PLT) in K/ul. (F) Representative photographs of mice from the Kaplan–Meier survival curve analysis in Figure 7C. (G) Representative photographs of mice from the Kaplan–Meier survival curve analysis in Figure 7E.



Supplemental Figure 1.



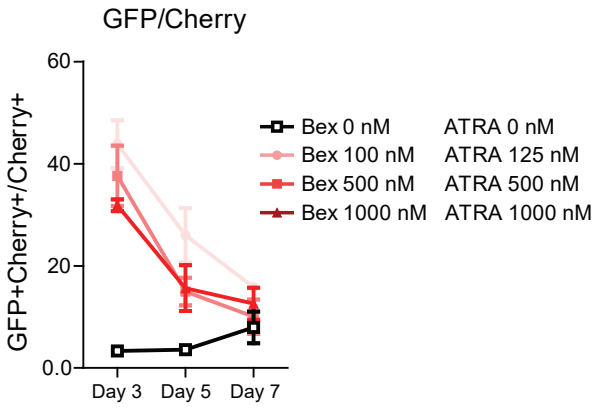


F.

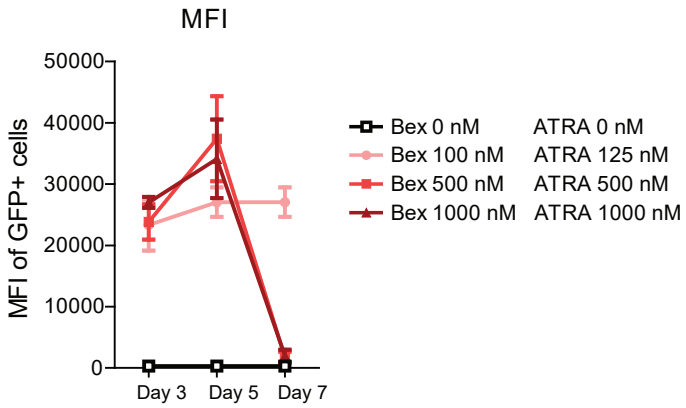
Ligand	***Nuclear Receptors***	***Primary use***
ATRA (All-*trans*-retinoic acid)	Pan RAR agonist (RARα/β/γ)	Acute promyelocytic leukemia
AM80 (Tambirotene)	RARα/β	Acute promyelocytic leukemia
BMS753	RARα selective agonist	N/A
LG268	Pan RXR agonist (RXRα/β/γ)	N/A
f-Bex (fluorobexarotene)	Pan RXR agonist (RXRα/β/γ)	N/A
Bexarotene (Targretin)	Pan RXR agonist (RXRα/β/γ)	Cutaneous T-cell Lymphoma
SR11237	Pan RXR agonist (RXRα/β/γ)	N/A
Hx630	Pan RXR agonist (RXRα/β/γ)	N/A

Supplemental Figure 2

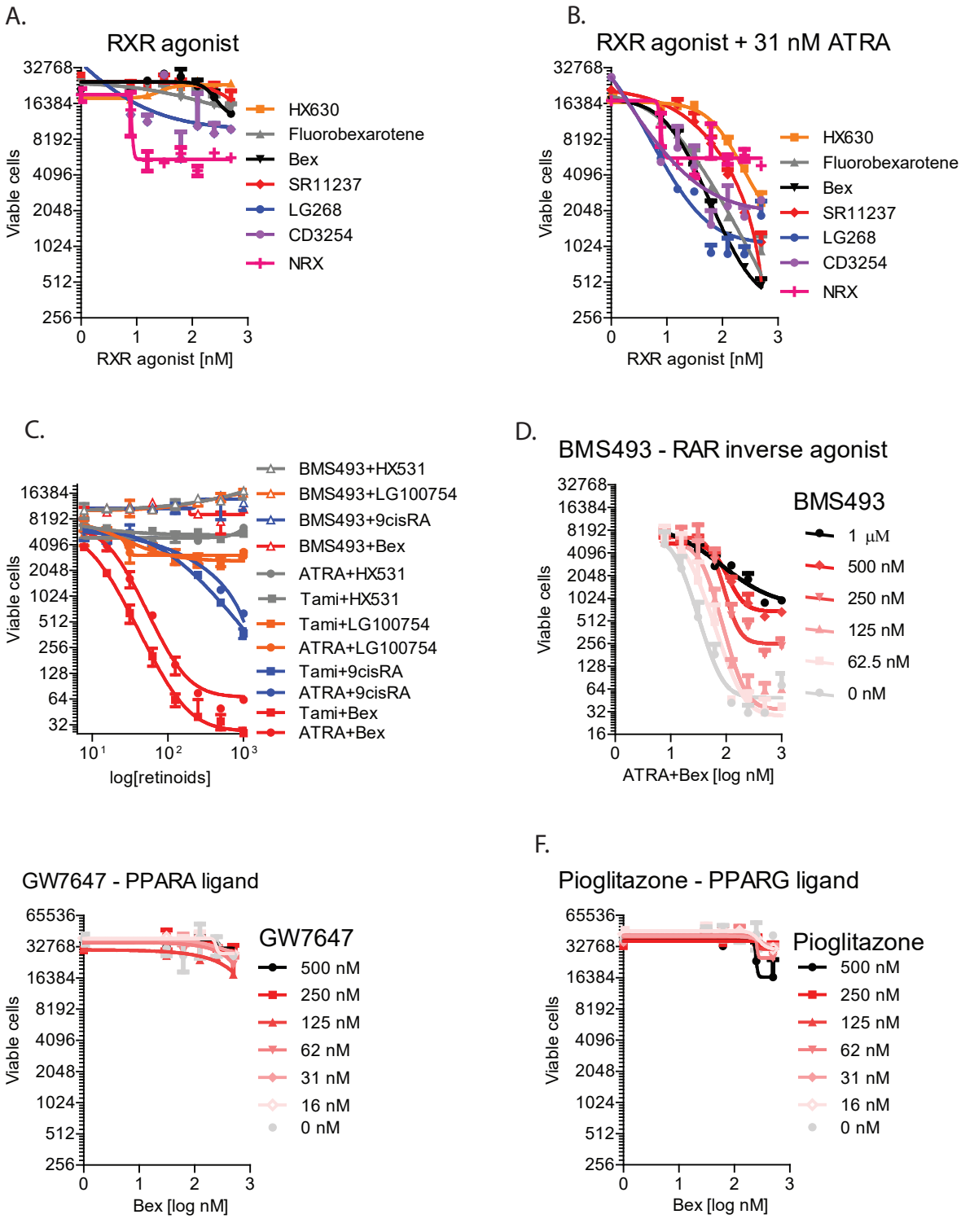
A.



B.

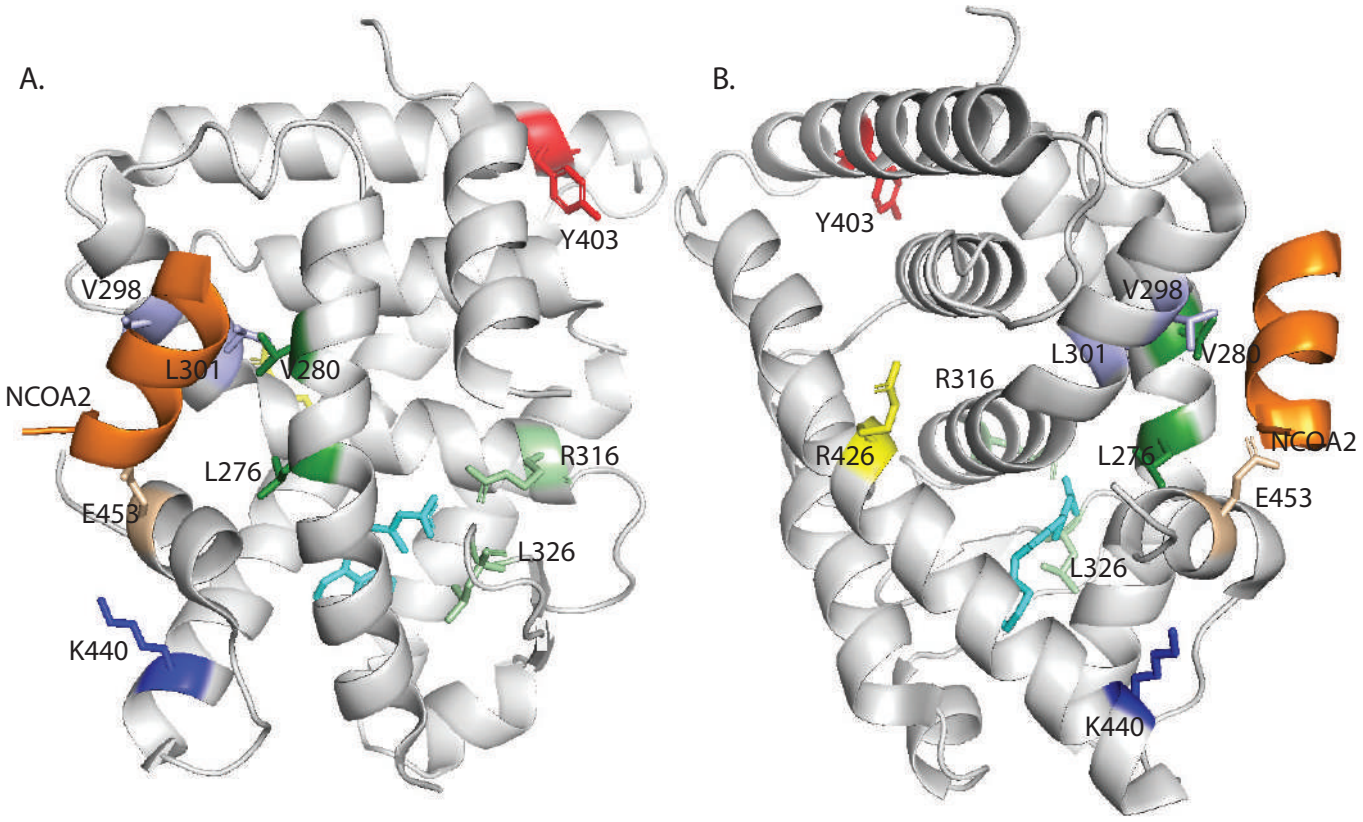


Supplemental Figure 3.

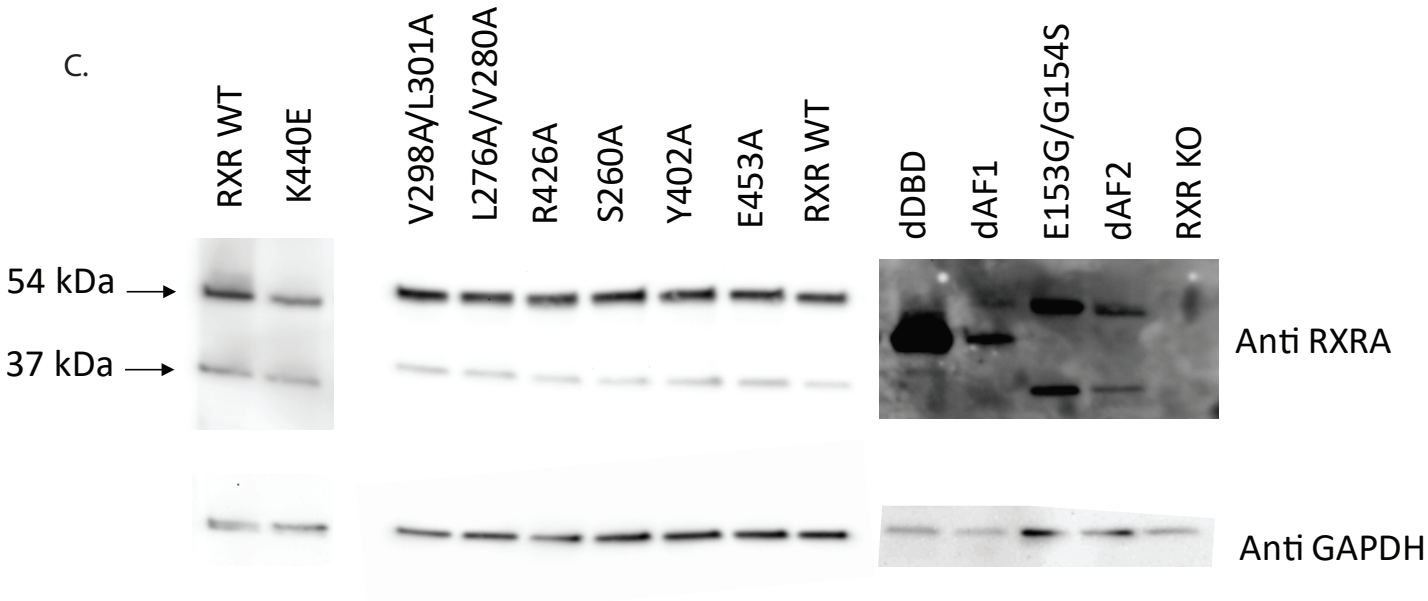


Supplemental Figure 4.

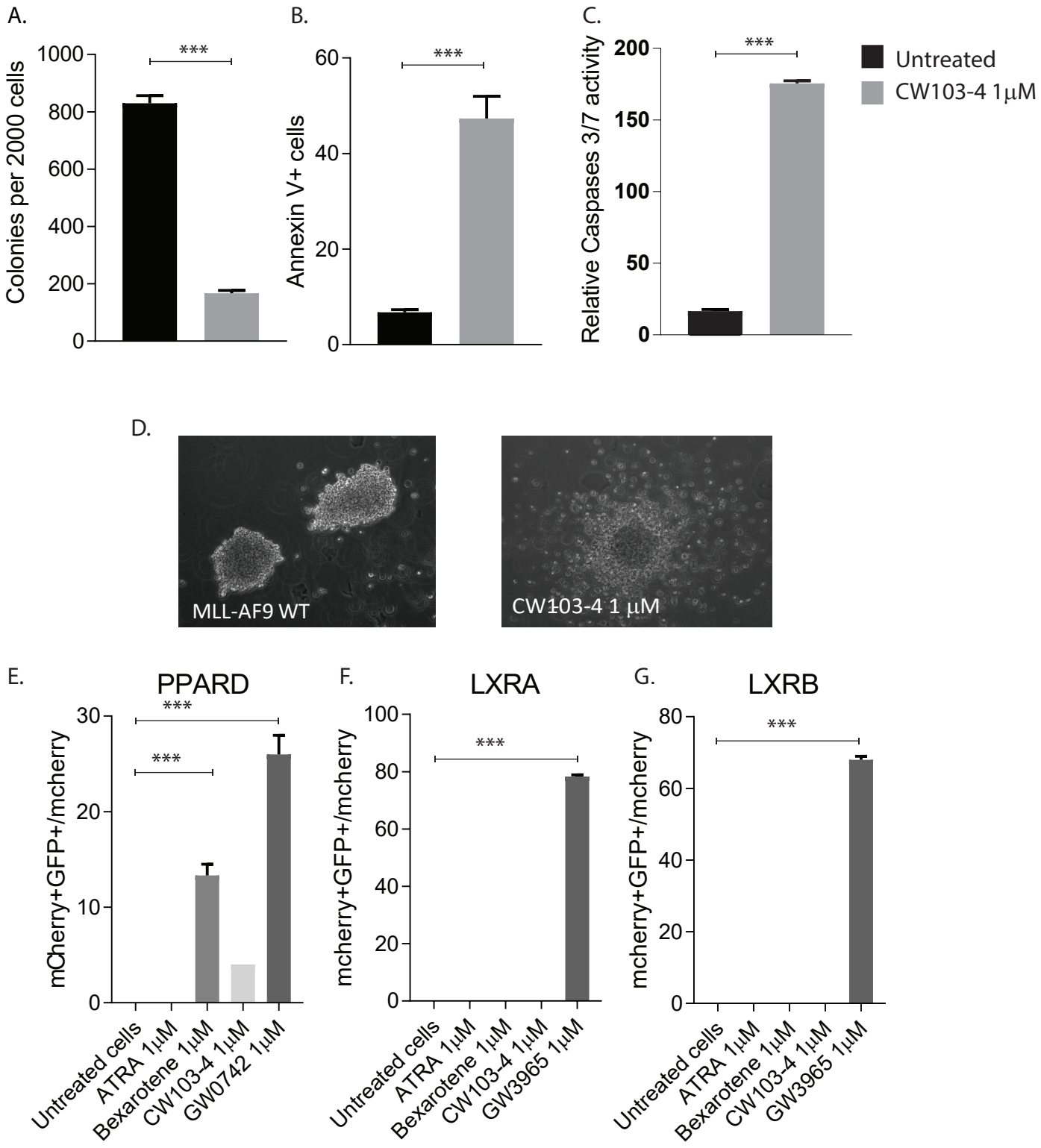
RXRA mutations highlighted within ligand binding domain (PDB: 4K4J)



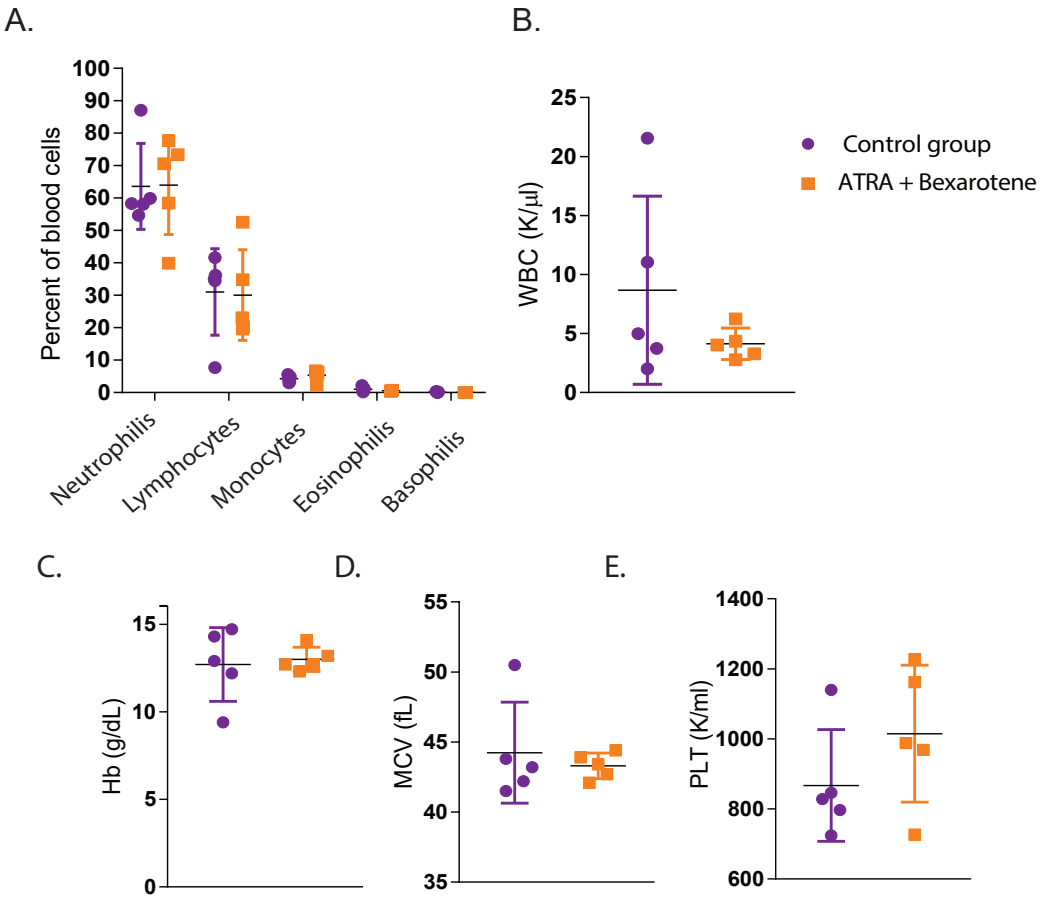
Amino acids associated with:
Co-activator binding: L276/V280 (green), V298/L301 (light blue), E453 (brown)
Co-repressor binding: K440 (blue)
Heterodimer binding: R426 (yellow), Y403 (red)
Ligand binding: R316/L326 (light green)

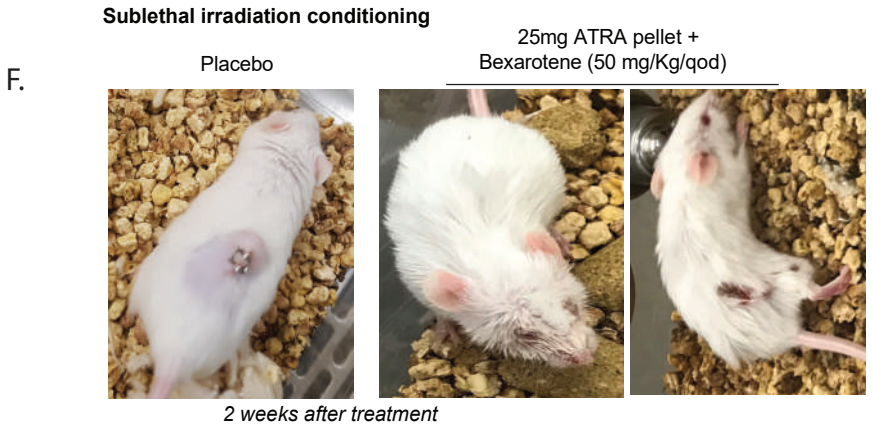


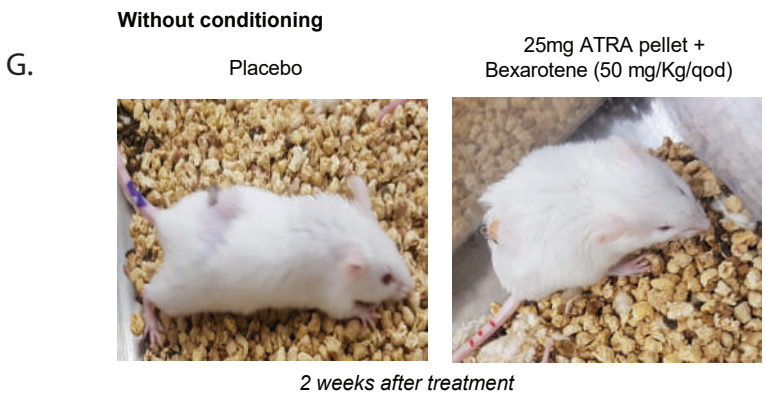
Supplemental Figure 5



Supplemental figure 6







Supplemental figure 7