REPORT (BY THE CANDITATE)

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/ Research abroad.

1	Name and designation of ICMR- IF	**Dr. Indrakant Kumar Singh** Professor
2	Address	Department of Zoology, Deshbandhu College, University of Delhi, Kalkaji, New Delhi-110019, India
3	Frontline area of research in which training/research was carried out	Medical Oncology, Cancer Biology
4	Name & address of Professor and host institute	**Prof. Heinz-Josef Lenz** Professor of Medicine and Preventive Medicine USC/Norris Comprehensive Cancer Center USC Keck School of Medicine 1441 Eastlake Ave, Los Angeles, CA 90033, United States
5	Duration of fellowship with exact date	From March 29 to June 29, 2023 [Three (3) Months] [Sanctioned for Six (6) Months]
6	Highlights of work conducted	
	i) Technique/expertise acquired	**i) Technique/expertise acquired:** **(a) Cell culture maintenance:** I gained expertise in culturing cancer cell lines for three cancer types: colorectal cancer (HCT116, HT29, SW620), pancreatic cancer cell lines (Mia-PaCa-2, PANC-1, BxPC-3, AsPC-1), and breast cancer (MDA-MB-231, HCC1395, HCT1937), and non-malignant cell line MCF-10A. All cell lines were obtained from the American Type Culture Collection (ATCC, USA) (https://www.atcc.org/), and maintained at 37°C with 5% CO2 in humidified chamber (95%). The cell lines were maintained in complete DMEM or

		RPMI-1640, both media supplemented with antibiotics (0.5 U/mL penicillin/streptomycin) and 10% FBS (Fetal Bovine Serum), according to manufacturer's protocol. All the cell lines were passaged regularly using trypsinization and routinely tested for mycoplasma contamination. **(b) Cell viability assays:** The MTS assay was performed to measure the cell viability upon treatment with selected compounds. I learned that MTS assay is more efficient than MTT assay (that we regularly use in my Indian lab) and produces water-soluble formazan the does not require DMSO dissolution step and thus is a more efficient way to measure cell viability. Briefly, pancreatic, colorectal, and breast cancer cell lines were counted (Invitrogen Countess 3 Automated Cell Counter) and seeded into the 96-well plates at a density of 5000 cells per well and maintained overnight. Cells were then treated with different concentrations (1μM to 100μM) of the compounds for 72 h at 37°C. After the treatment period, the medium was removed, cells were washed with DPBS, and incubated with EBM (100μL) and MTS (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) reagent (20μL) for 2 h at 37°C. After, we measured the amount of MTS formazan at 490 nm by microplate reader (SpectraMax iD5, Molecular Devices). The results were expressed as the percentage of cell viability in relation to DMSO control.

		(c) Apoptosis assay: To assess apoptosis after treatment with ROR1 inhibitors, Caspase 3/7 staining was performed using the Muse Caspase 3/7 assay kit (Luminex MCH100108). The cells were seeded at a density of 250,000 cells/well in a 6-well plate. The cells were treated with either ROR1 inhibitors at the IC50 or DMSO vehicle control for 72 h. Next, the cells were trypsinized, resuspended in an assay binding buffer, and stained with Caspase 3/7 to be analyzed for apoptosis using a Muse Cell Analyzer. The USC Flow Cytometry Facility at Keck School of Medicine was used for learning how to operate the cell sorter/analyzer (Attune NxT Flow cytometer) and to select the gating strategies and analyze the results. **(d) Western blotting:** After ROR1 inhibitor treatment (10 μM) and vehicle (DMSO) control treatment, the cells were collected by scraping. The proteins were lysed using RIPA buffer (150mM NaCl, 1.0% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 1 mM PMSF (Protoc, 2017). Bradford Assay measured cell lysates' protein concentration using Pierce 660 nm reagent (Thermofisher, #22660). The Protein lysates were processed by mixing with laemmli buffer at a 1:1 ratio, followed by boiling. Equal amounts of proteins were loaded into the

		wells of an SDS-PAGE gel, followed by electrophoresis. The proteins were transferred to a nitrocellulose membrane, then blocked for 30 minutes in 5% bovine serum albumin. The membrane was incubated overnight at 4 o C in anti-ROR1 antibody (Cell signaling, #D6T8C), then washed with TBST (20 mM Tris, 150mM NaCl, 0.1% Tween 20), then incubated in horseradish-peroxidase-linked anti-Rabbit IgG (Invitrogen, #65-6120) for 1 hour at room temperature. Protein bands were imaged by chemiluminescence. GAPDH (Cell signaling, #D16H11) or β-actin (Santa Cruz Biotechnology, #sc47778) were loading controls. Western blotting is a commonly used technique; however, we generally use the wet-transfer method which is time consuming and labor-intensive. This time, I gained expertise for the semidry-transfer method which is relatively time efficient and reliable. **(e) Clinical data analysis:** Dr. Lenz's lab is actively involved in clinical trials, including 80405, FIRE-3, TRIBE, and MAVERICC, and analyzes large amounts of expression (RNA-seq and NGS) data from colorectal patients. Through this fellowship I was able to learn how large clinical data is handled and got a chance to run some survival outcome analysis for my target gene, ROR1. Kaplan-Meier plots were generated for ROR1 in 80405 expression data, and its association with targeted treatments, bevacizumab (VEGF-A inhibitor) and

		cetuximab (EGFR inhibitor) were analyzed. I also submitted the Letter of Intent (LOI, which is an extensive review on ROR1) to CARIS Life Sciences, which is a major collaborator with Dr. Lenz's lab for further clinical data analysis of ROR1 in different cancers, but the LOI results are pending.
	ii) Research results, including any papers, prepared/submitted for publication	In a preliminary analysis (Figure 1-3) to correlate the expression of ROR1 with clinical outcome in colorectal cancer, we observed that ROR1 expression had a prognostic value in CRC patients with irinotecan/5-FU/leucovorin (FOLFIRI) or oxaliplatin/5-FU/leucovorin (mFOLFOX6) with bevacizumab (BEV) or cetuximab (CET) treatment and KRAS-wildtype metastatic adenocarcinoma of the colon or rectum (mCRC) enrolled in 80405 phase III trial [12]. Patients with high ROR1 expression showed lower median progression-free survival (PFS): 8.9 months (95% CI: 7.6-10.3) compared to patients with lower ROR1 expression: 13.4 months (95% CI: 11.2-16.1) ($p = 0.0019$, log-rank test); and patients with high ROR1 expression showed lower median OS: 21.1 months (95% CI: 16.9-25.5) compared to patients with lower ROR1 expression: 37.1 months (95% CI: 34.1-41.1) ($p = 1.5e{-}05$, log-rank test). A similar trend was reported for patients treated with cetuximab, high ROR1 expression showed, lower median PFS: 8.3 months (95% CI: 6.2-10.9) vs low ROR1: 14.2 months (95% CI: 10.6-19.4) ($p = 0.0012$, log-rank test), and lower median OS:

		19.1 months (95% CI: 13.9-22.8) vs low ROR1: 40.8 months (95% CI: 34.1-56.6) (p = 1.4e−06, log-rank test). But no significant association was found between ROR1 expression and bevacizumab treatment arm of the trial. Interestingly, ROR1 expression showed significant correlation with MSS status of patients, high ROR1 expression showed, lower median PFS: 9.1 months (95% CI: 7.9-10.9) vs low ROR1: 14.8 months (95% CI: 11.9-17.7) (p = 3.9e−05, log-rank test), and lower median OS: 20.9 months (95% CI: 16.1-26.3) vs low ROR1: 38.6 months (95% CI: 34.4-42.2) (p = 3.5e−06, log-rank test). But no significant association was found between ROR1 expression and MSI high/low status of the patients in the trial. The preliminary cell viability assays for compound 4 (CID1261330), identified after the extensive virtual screening approach, were done on all the available cell lines, including colorectal, breast, and pancreatic. The lowest and best IC50 (half-maximal inhibitory dose) was obtained for the triple negative breast cancer (TNBC) cell lines, MDA-MB-231 and HCC1395 cells, at dose of approximately 2 μM. Therefore, further in vitro analysis was done on TNBC cell lines and results were compared with the non-malignant breast cell line, MCF-10A.
	iii) Proposed utilization of the experience in India	iv) The *in vitro* techniques that were learnt during this fellowship will be implemented in my research lab in India and the same expertise will be passed on to my doctoral students which will help them in their research work.

		v) The clinical data analysis was mainly performed on clinical trials involving the European population. The expertise will be used to apply the similar analysis on data obtained from the Indian population, which will help in determining the efficacy of potential ROR1 inhibitors as personalized treatment strategy for Indian colorectal patients. vi) Apart from the experimental techniques, the fellowship also provided me with a glimpse of the working culture in cancer labs in USA, and I will try to implement the weekly lab meetings, lab management skills, etc. vii) Also, this fellowship has opened many new avenues for future collaborations with people that complement my research interests, and I am looking forward to several joint international research fundings in the future.

Dr. Indrakant Kumar Singh
Professor
(Signature of ICMR-IF)

ICMR Sanction No.
Office Order No.: INDO/FRC/452/Y-63/2022-23-IHD

Supplementary File

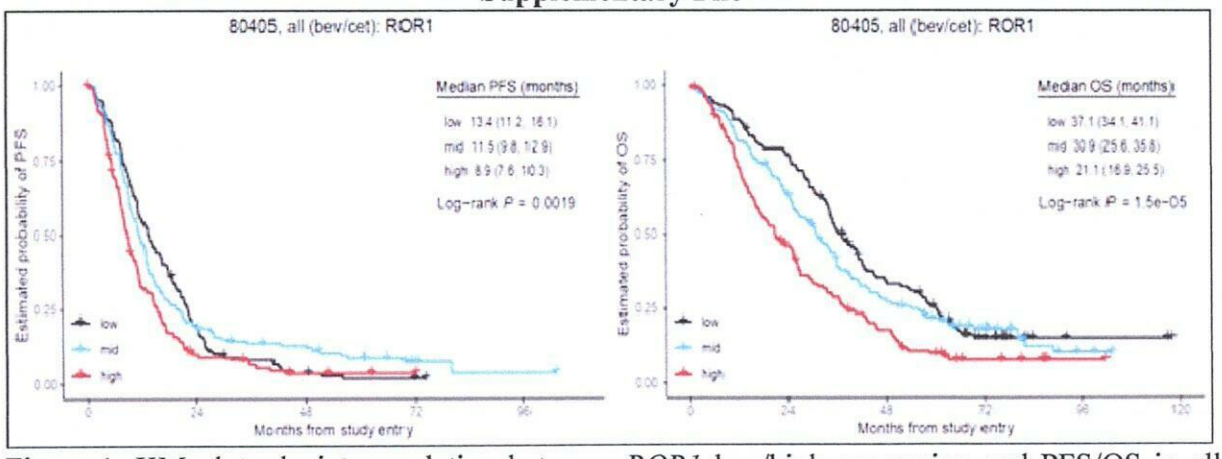


Figure 1. KM plots depict correlation between *ROR1* low/high expression and PFS/OS in all mCRC patients in 80405 trials.

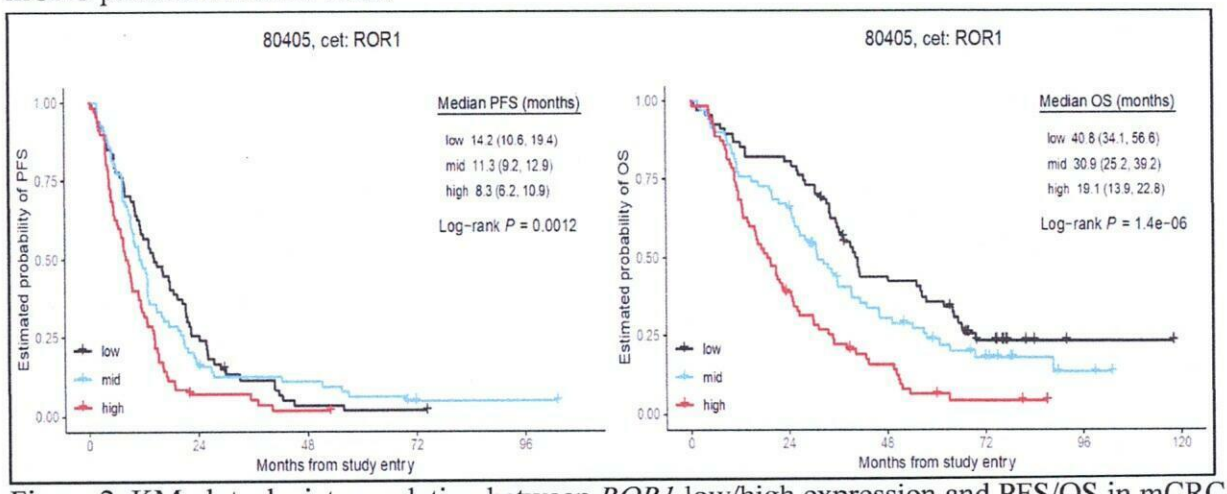


Figure 2. KM plots depict correlation between *ROR1* low/high expression and PFS/OS in mCRC patients treated with cetuximab in 80405 trials.

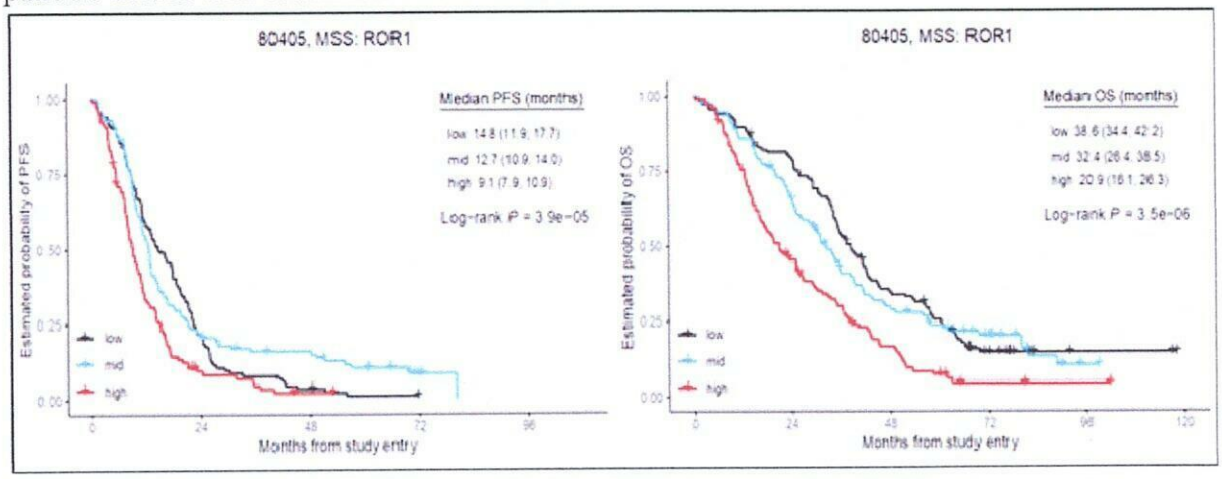
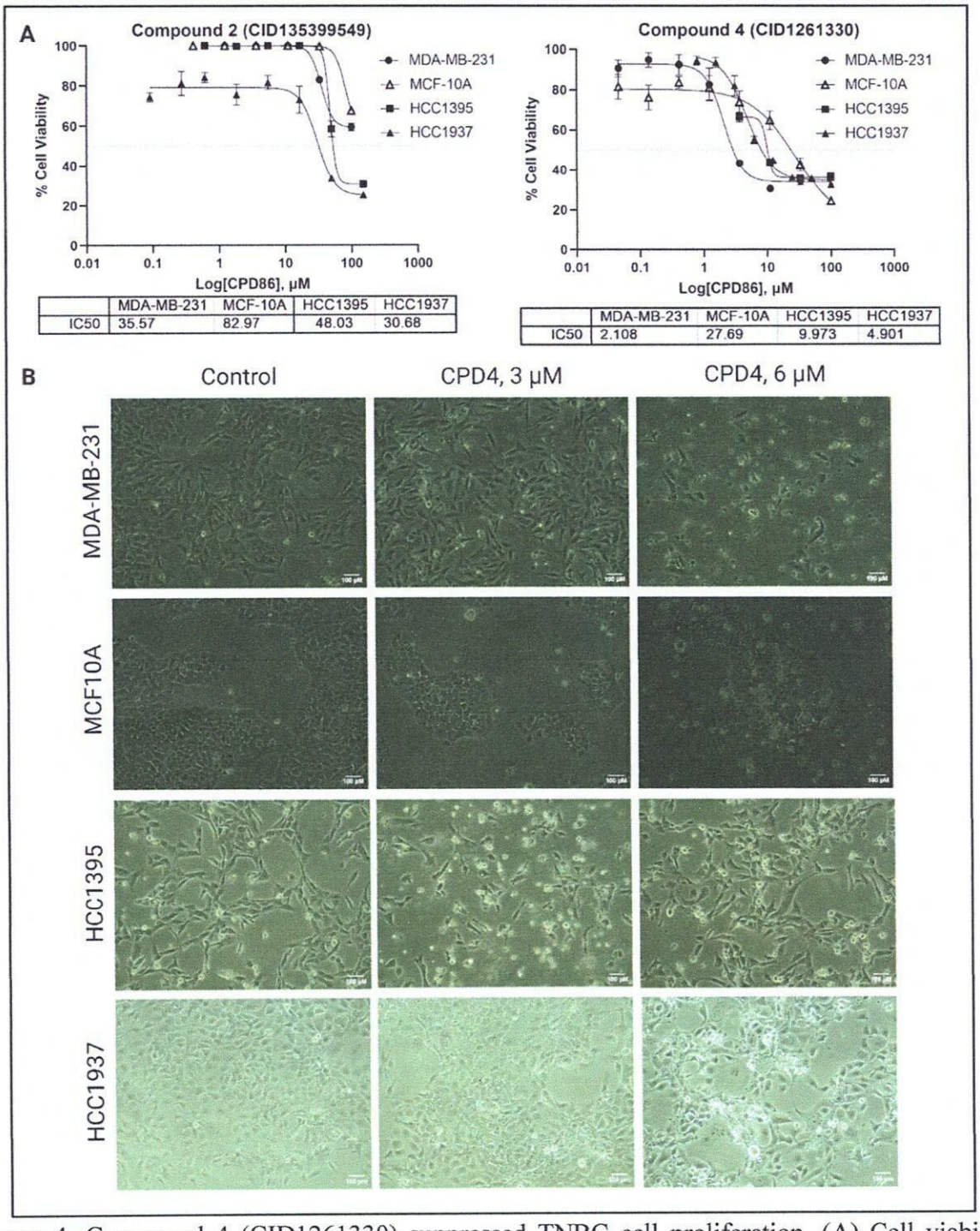


Figure 3. KM plots depict correlation between *ROR1* low/high expression and PFS/OS in mCRC patients with MSS tumor status in 80405 trials.



	MDA-MB-231	MCF-10A	HCC1395	HCC1937
IC50	35.57	82.97	48.03	30.68

	MDA-MB-231	MCF-10A	HCC1395	HCC1937
IC50	2.108	27.69	9.973	4.901

Figure 4. Compound 4 (CID1261330) suppressed TNBC cell proliferation. (A) Cell viability assays to assess the IC50 values of Compound 2 (CID135399549) (left) and Compound 4 (CID1261330) (right) of three TNBC cell lines (MDA-MB-23, HCC1395 and HCC1937) and a non-malignant MCF-10A line (n = 3 biological replicates). (B) Morphology of the three cell

lines after treatment with compound 4 (CID1261330) or vehicle control (0.3% DMSO) at 10X magnification post 72 hr treatment.

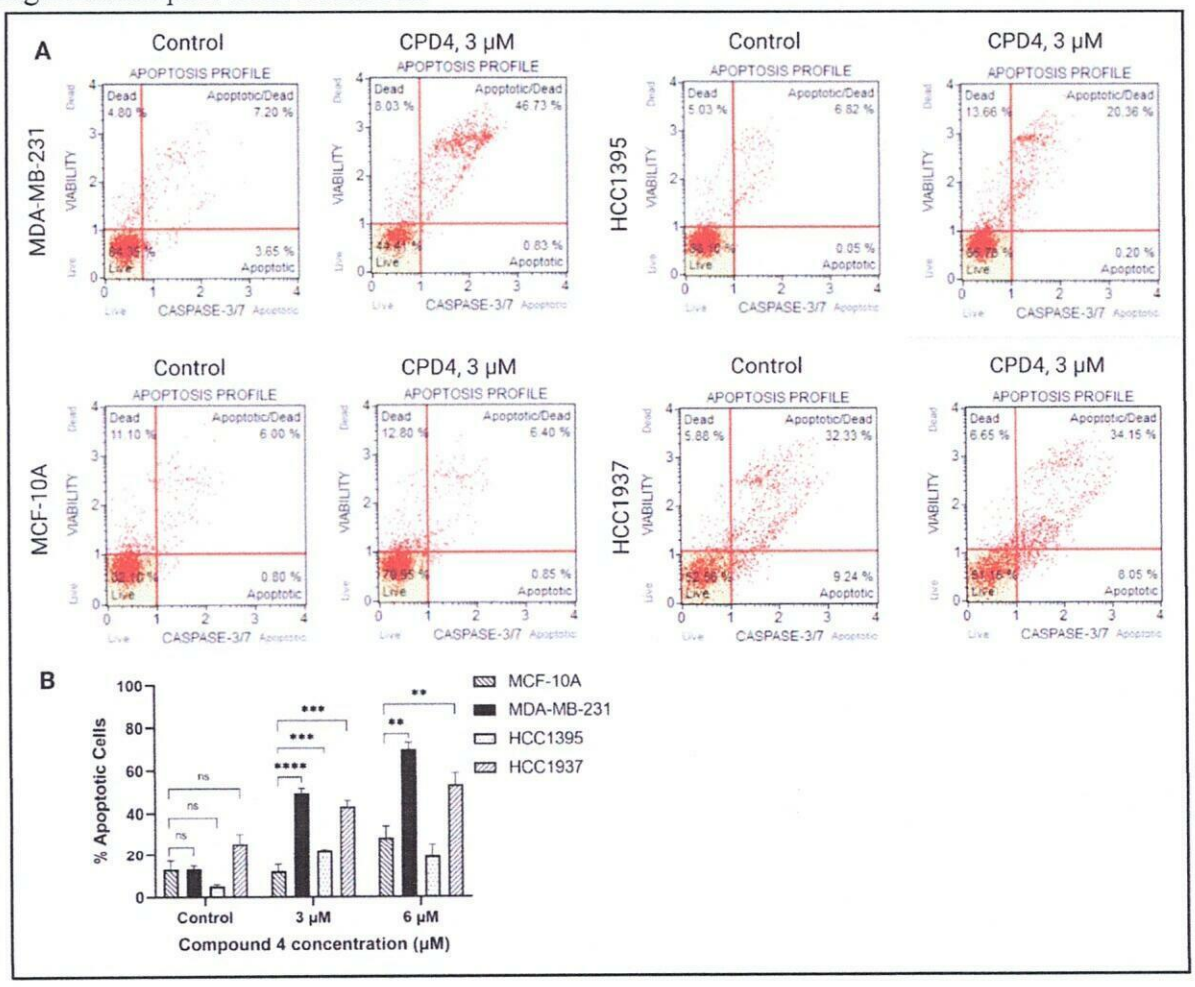


Figure 5. Compound 4 (CID1261330) at a concentration of 3 µM induced apoptosis in TNBC cells post 72 hr treatment. (A) Caspase 3/7 staining and 7-AAD assay to assess apoptosis in the four cell lines after treatment with compound 4 (CID1261330). (B) Quantification of apoptosis induced by compound 4 (CID1261330) (total of top and bottom right quadrants) in three TNBC cell lines and normal MCF-10A cells (**p < 0.01, ***p < 0.001, ****p < 0.0001, n = 1-3 biological replicates).

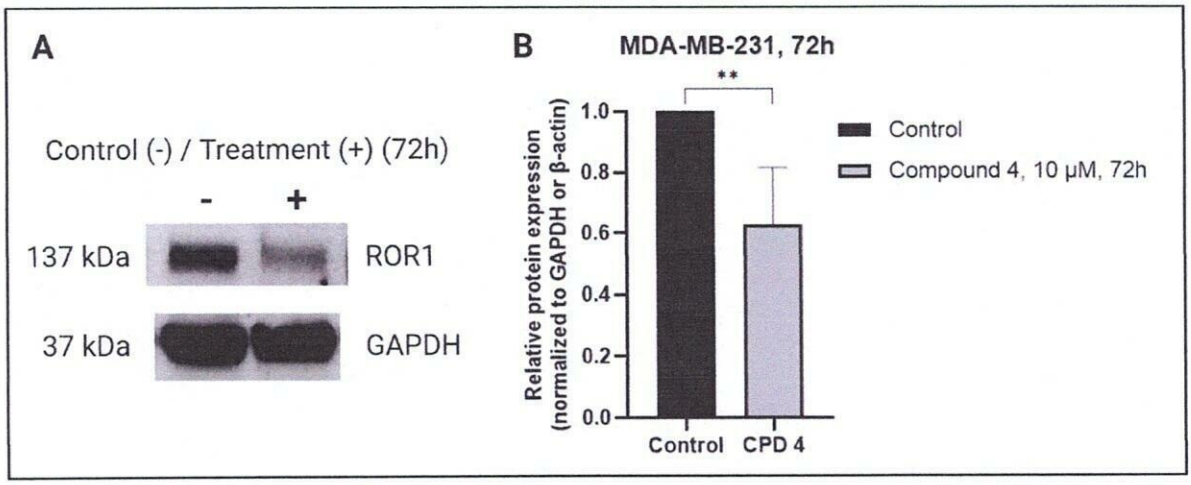


Figure 6. Compound 4 inhibits ROR1 expression levels of TNBC cells. (A) Representative western blots of ROR1 on MDA-MB-231 cells treated with vehicle and 10 μM of Compound 4 for 72 hours. (B) Quantifying panel A immunoblot bands of ROR1 normalized to GAPDH or β-actin. (**$p < 0.01$, Similar trends were observed in n=3 biological replicates.