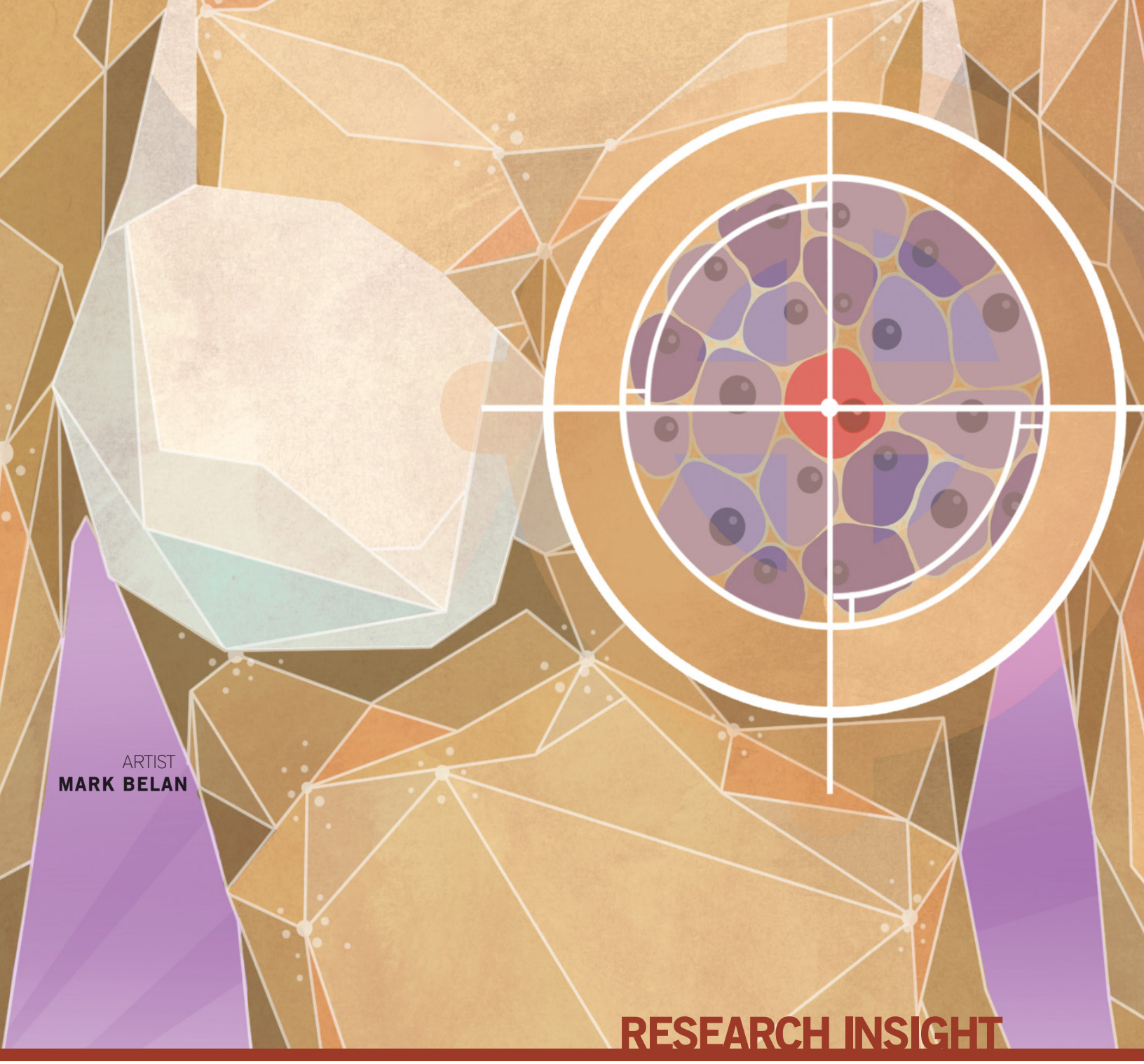

RESEARCH INSIGHT

Salinomycin: A Notch Signaling Antagonist

A NOVEL WAY OF TARGETING CANCER STEM CELLS

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ABSTRACT Dr. Hassell's research team aims to investigate the roles of therapeutically-relevant genes or gene signatures in the development of "tumour-initiating cells" or breast cancer stem cells. His research team also explores the effects of antagonistic compounds on certain regulatory receptor pathways using *in vitro* breast cancer cultures and transgenic mouse models. The following research focuses on validating the inhibitory effects of an anti-breast cancer stem cell agent, salinomycin, on downstream Notch signaling. It suggests the possibility of targeting cancer stem cells, the primary culprit in tumour initiation, chemoresistance, and metastasis, by inhibiting key regulatory pathways - such as Notch signaling - that maintain this "stem-like" population.

INTRODUCTION TO CANCER STEM CELLS

In 2012, an estimated 577,190 Americans will die from cancer, corresponding to more than 1,500 deaths per day.[1] An astonishing 15% of these individuals will be female victims of breast cancer.[1] Although major advances are being made in uncovering the mysteries and molecular dynamics of cancer biology, pharmacological treatment of cancer still relies primarily on traditional chemotherapeutic remedies.[5] As such, chemoresistance and recurrent metastases continue to contribute significantly to cancer mortality rates.[2-4] In fact, the efficacy and response to chemotherapy in a malignant tumour drops down from 60-100% in the first tumour to approximately 20% in the recurrent tumour.[6] The adaptability and heterogeneity of tumours endow them with drug-resistance, disease recurrence, and capacity for metastasis.[7,8] Hence, there is a compelling rationale to identify the mechanism by which tumours survive to seed relapse after remission and the physiological signatures of the recurrent tumour.

In the past decade, a cellular hierarchy has been theoretically established in numerous hematopoietic and solid tumours, with a rare fraction of tumour cells termed "cancer stem cells" (CSCs) or "tumour-initiating cells" (TICs) sitting at the top of this hierarchy.[9,10] The CSC hypothesis states that, in addition to the ability to resist chemotherapies and radiotherapies, CSCs share three main traits with their normal tissue-specific stem cell counterparts.[11,12,13] These traits include: the ability to differentiate into any of the heterogeneous cell types that make up the organism, organ or tumour (in the case of CSCs);[5-7,9,11,14] self-renewing capacity, or the ability to indefinitely give rise to identical daughter cells;[5-7,9,11,14] and homeostatic control, which is the ability to respond to extracellular cues and genetic constraints to balance differentiation.[11,14,15]

Cancer has typically been identified as the result of accumulated genetic mutations in a single clonal population of cells.[11,16] Specifically, the very slow cell turnover rate and self-renewing capacity of normal stem cells to maintain a stable stem cell pool increase the likelihood of these cells to accumulate mutations from one generation to the next.[9,17,18] Furthermore, the CSC hypothesis predicts that current chemotherapies are targeting the terminally differentiated, bulk tumour cells, while selecting for the treatment refractory, proliferative cells that are responsible for patient relapse, representing a paradigm shift in the conceptual approach to oncogenesis and cancer treatment (Figure 1A).[11] Eradication of CSCs is strongly thought to be a revolutionary event in permitting progression-free survival; however, long-term outcome data from clinical trials with a CSC-specific agent have yet to be compiled

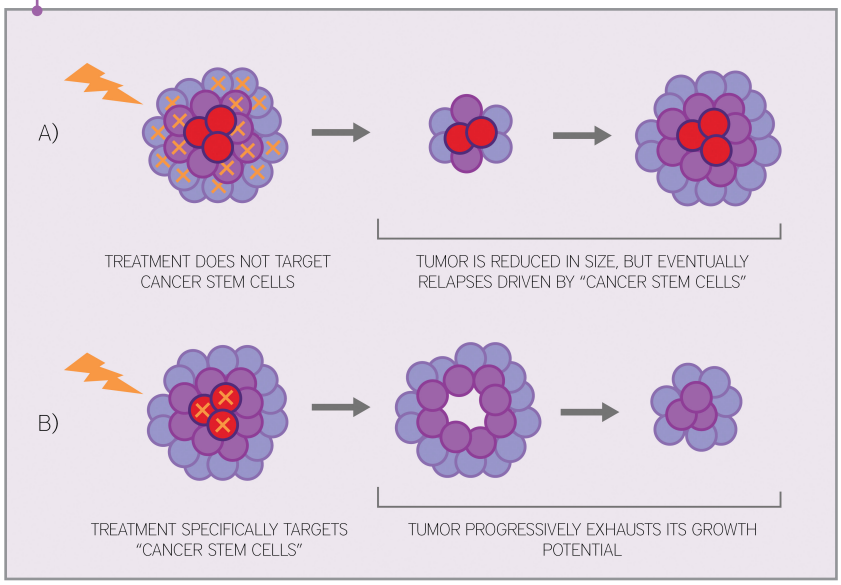

FIGURE 1: Model of how the CSC hypothesis can be incorporated in the design of anteoplastic treatments. (A) Current cancer therapies designed for broad cytotoxicity kill the majority of tumour cells within a given tissue. However, those CSCs that remain possess the potential to regenerate new heterogeneous tumours and metastases. (B) The CSC hypothesis, in contrast, proposes the utility of cancer stem cell-targeting agents that, although may not theoretically shrink the tumour immediately, can achieve eradication of self-renewal and regeneration.[11]

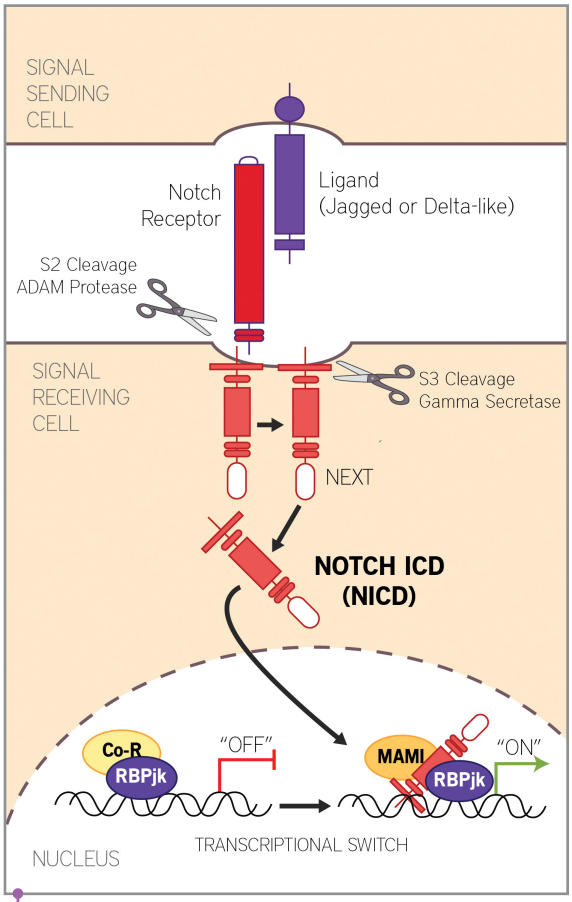

FIGURE 2: Simplified mechanism of the Notch signaling pathway. The Notch signaling pathway is activated by enzymatic cleavages that occur to the heterodimeric Notch receptor (in red). Humans possess four homologous Notch receptors, each of which consists of an extracellular domain (N^{ECD}), transmembrane domain (N^{TM}) and an intracellular domain (N^{ICD}). After being synthesized, the Notch receptors are anchored into the cell membrane, where they may bind their canonical ligands, which are also transmembrane proteins. This triggers the endocytosis of N^{ECD} and exposes N^{TM} to cleavage by an ADAM metalloprotease (S2). A Notch extracellular truncation (N^{EXT}) intermediate is produced and is further cleaved by γ-secretase (S3) to generate the active N^{ICD}. N^{ICD} is then translocated to the nucleus where it binds transcription factor CSL (or RBP-jk in mice). Upon binding CSL, which is normally in a transcriptionally repressed state, N^{ICD} replaces a co-repressor complex on CSL with a co-activator complex that includes Mastermind (MAML). This Notch transcriptional activating complex goes on to enhance transcription of Notch target genes, which code for proteins involved in self-renewal and in preventing differentiation.[43]

to confirm this hypothesis.[19] In mouse xenograft models of breast cancer, it takes thousands of injected tumour cells to generate a tumour, but only 20 to 50 putative breast cancer stem cells (BCSCs) can produce a heterogeneous tumour in keeping with the BCSC hierarchy.[6] This rare population, making up 11-35% of total cancer cells in a breast tumour, has been identified by the cell surface marker combination, $CD44^{+}/CD24^{-/low}$.[6,20] Classification of CSCs by cell-surface markers has enabled the isolation of these cells by flow cytometry for comparative studies of these cells before and after treatment.[21]

SIGNALING PATHWAYS IN BREAST CANCER STEM CELLS AS THERAPEUTIC TARGETS

Tissue differentiation is now believed to be a complex, tightly regulated process in which several developmental signaling pathways process genes responsible for a number of cell-fate decisions by integrating information from extracellular cues.[22] In mammary gland development (as well as the development of many other solid or hematopoietic tissues), many of these signaling pathways are highly implicated in stem-cell maintenance and regulation of molecular checkpoints for differentiation.[22,23,26] These include epidermal growth factors, as well as the Wnt/β-catenin, Hedgehog and Notch signaling pathways.[27,28] Interestingly, it has been found on multiple accounts that human breast tumours contain a BCSC population with similar properties to normal mammary stem cells, and these BCSCs often exhibit constitutive activation of one or more of these signaling pathways.[23] These data suggest that breast tumours may originate from pluripotent mammary stem/progenitor cells that have experienced aberrant activation in developmental signaling cascades as a result of an accumulation of genetic mutations throughout the process of self-renewal. Targeting these signaling pathways has become a topic of great interest, as their inhibition has been associated with differentiation of CSCs, loss of tumourigenic potential, and sensitization to classical chemotherapeutic regimens.[24,25]

My goal, under the supervision of Dr. John Hassell, has been to identify small-molecule compounds that inhibit Notch signaling, with the hypothesis that a specific inhibitor of the Notch signaling may result in regression and/or elimination of BCSC-mediated tumourigenesis (Figure 1B). Notch signaling has been the designated target because it is an evolutionarily-conserved pathway that functions to regulate cell-fate decisions and tissue development in all three germ layers.[6,29] Consequently, any mutation or perturbation to the canonical signaling pathway can have a vastly oncogenic effect.[30,31] This has been illustrated in breast cancer, as Notch is aberrantly activated in the malignant state when compared to normal mammary epithelium,[16,17] suggesting a mechanism for developing resistance to current cancer therapies.[32,33]

The most successful inhibitors of Notch signaling to date in both pre-clinical and clinical studies have been γ-secretase inhibitors (Figure 2).[34-36] Selective inhibition of γ-secretase prevents Notch receptor cleavage and consequently, N^{ICD}-mediated transcriptional regulation in BCSCs.[36] Preclinical studies with γ-secretase inhibitors have identified novel chemotherapeutic properties through functioning as a potent Notch inhibitor leading to tumour regression using *in vitro* and *in vivo* models of breast tumours.[39] Moreover, γ-secretase inhibitors have been shown to suppress the self-renewing capacity and anchorage-independent growth of TICs in numerous solid tumours including breast, gastrointestinal, and pancreatic cancers.[36,37] Unfortunately, multiple organ systems and molecular targets including the amyloid precursor protein implicated in Alzheimer's disease rely on the function of γ-secretase.[5] Accordingly, inhibitors of γ-secretase have revealed off-target and highly potent effects on Notch signaling with varying degrees of toxicity to patients in a dose-dependent manner.[5] Therefore, a specific, small-molecule Notch inhibitor has yet to be discovered that exhibits the same therapeutic potential as γ-secretase inhibitors without the severity of its side effects.[38]

The oncogenic effects of Notch are due to the

formation of the transcriptional activating complex, which activates transcription involved in promoting cell proliferation and blocking differentiation.[40] Our lab has focused its attention on identifying small-molecules that can disrupt the formation of this transcriptional activating complex and/or its ability to exert transcriptional control in human breast cancer cell lines. Thus, we have designed a cell-based assay that uses a set of infected breast cancer cell lines that can reliably assess the functional activity of Notch signaling downstream of γ-secretase. The goal of this research is to identify and validate a small-molecule inhibitor of the Notch pathway in multiple human breast cancer subtypes that will also target CSCs.

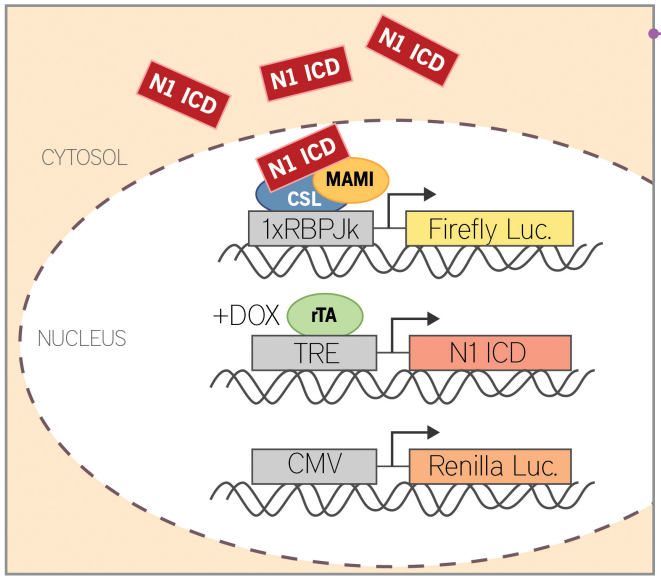

FIGURE 3: Schematic of the HCC 1954 N11R cell line and its integrated inducible system that reports on N^{ICD} activity..The HCC 1954 cell line was virally infected with three genomic constructs shown within the nucleus (dotted red line) of the cell diagram above. The resulting HCC 1954 N11R cells possess the N^{ICD} gene regulated by a Tet-On system, which is activated upon addition of doxycycline antibiotic. Over-expression of N^{ICD} permits increased transcription of the firefly luciferase reporter from the 1xRBPjk promoter sequence by forming the transcriptional activating complex with CSL and MAML. These cells also contain an internal negative control, the renilla luciferase reporter under transcriptional control of the CMV promoter, which constitutively expresses the renilla luciferase transcript.

A FUNCTIONAL CELL-BASED DOSE RESPONSE ASSAY: VALIDATION OF SALINOMYCIN-MEDIATED INHIBITION OF NOTCH SIGNALING DOWNSTREAM OF γ-SECRETASE

We have recently derived HCC 1954 N11R, a breast cancer cell line that utilizes a luciferase reporter system to report Notch signaling activity downstream of γ-secretase. This cell line has also undergone lentiviral infection to generate a collection of stable clones that contain three engineered genomic constructs. The first is a vector containing the N^{ICD} gene that can be inducibly expressed by the Tet-On promoter upon the addition of the antibiotic doxycycline. This is how the γ-secretase cleavage step is skipped, and overexpression of N^{ICD} leads to ligand-independent activation of the Notch pathway.[29] The second construct is a firefly luciferase reporter that is expressed from its promoter containing a 1x RBP-jk binding site. This is the binding sequence that the CSL Notch transcription factor binds to, and is a putative binding site amongst many Notch target gene promoters.[29] The final construct is a renilla luciferase reporter under the control of the Cytomegalovirus (CMV) promoter, a strong viral promoter that promotes constitutive expression of renilla luciferase (Figure 2). When HCC 1954 N11R cells are exposed to doxycycline, this induces over-expression of N^{ICD}, leading to high activation of N^{ICD}/CSL/Mastermind-mediated transcription of firefly luciferase. This is quantified by the measure of luminescence produced by the firefly luciferase enzyme upon addition of its substrate. When cells are treated with candidate Notch inhibitors, we expect the luminescence signal from firefly luciferase to decrease in a dose-dependent fashion, while the renilla luciferase signal remains constant because it serves as an internal negative control. Compounds that are toxic to the cells or inhibit general transcription or translation will also reduce the renilla luciferase signal, making that compound a false hit in our assay.

A previously conducted screen in the Hassell Lab, with over 1,300 natural bioactive

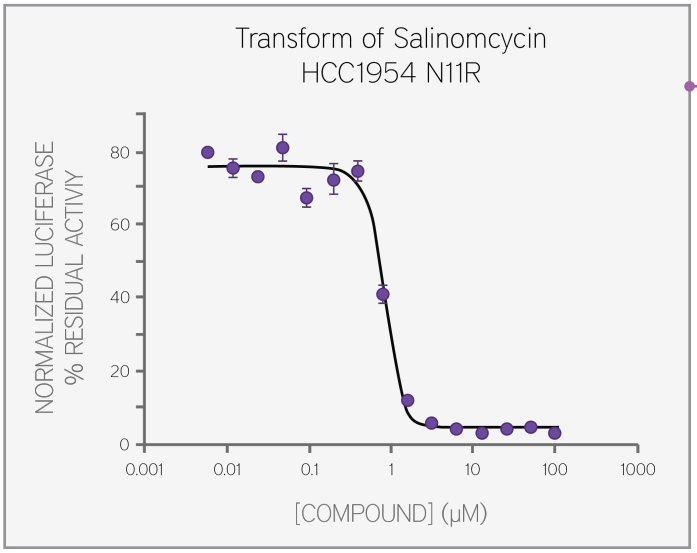

FIGURE 4: Dose-response curve of HCC 1954 N11R cells treated with salinomycin at 16 concentrations. The graph shows the percentage of firefly luciferase activity normalized to renilla luciferase activity with respect to DMSO-treated cells. Cells were treated with Salinomycin at 16 concentrations, starting at 100μM with two-fold dilutions. Firefly and renilla luciferase luminescence was read 24 hours after treatment. The decrease in normalized luciferase activity indicates a selective decline in the luminescence of firefly luciferase with respect to renilla luciferase. The IC_{50} for salinomycin in this cell line is 812nM, which is the quantity of a drug or compound required to inhibit a particular biological process by 50%.

compounds and pharmaceuticals, yielded a total of 27 unique hits. After follow-up validation experiments, salinomycin was determined to produce the most specific and reproducible inhibition of Notch signaling in our inducible system (Figure 4). The ramifications of these results are as encouraging as they are enlightening. Salinomycin has been reported to exhibit selective toxicity to BCSCs.[41] Mouse treatment with salinomycin regresses mammary tumour growth *in vivo* and induces epithelial differentiation of tumour cells.[41] In addition, salinomycin treatment results in reduced expression of characteristic BCSC genes by global gene analysis.[41]

This evidence supports the hypothesis that a pan Notch inhibitor will repress mammary tumour growth by inhibiting the proliferation of BCSCs. Furthermore, it shows that oncogenic characteristics of BCSCs, such as self-renewal and proliferation, are largely dependent on their capacity to maintain activation of Notch signaling. This sheds light on a new approach to targeting BCSCs and perhaps CSCs in other cancer types such as T-cell acute lymphoblastic leukemia, which is known to contain activating mutations in Notch.[42] It has always been difficult for investigators to isolate and validate large quantities of CSCs due to the heterogeneity of primary tumours, the minority of cells that possess the CSC phenotype in primary samples, and the ambiguity associated with the exact phenotypic definition of CSCs across cancer types.[22,24,25] However, our evidence supports the possibility of identifying inhibitors of Notch signaling (or other developmental signaling pathways) as an indirect approach to developing drugs that can target this "stem-like" population of cells. This may allow investigators to conduct high-throughput screens for this purpose.

CONCLUSION

CSCs have been at the forefront of cancer research to reveal the molecular processes that regulate tumour initiation, maintenance, chemoresistance, and metastasis.[7] Several regulatory signaling pathways are implicated in the maintenance of this CSC population, many of which are druggable targets.[14,16,22-24] Our research investigates candidate inhibitors of Notch signaling, a process shown to experience high levels of activation in BCSCs. The identification of salinomycin, an antagonist of BCSCs, as our most selective inhibitor of Notch signaling suggests the significant role of Notch in sustaining this tumourigenic population. New screening strategies may be established to indirectly identify antagonists of BCSCs by identifying compounds that inhibit the regulatory pathways that endow them with tumourigenicity.

REVIEWED BY DR. ROBIN HALLETT

Dr. Robin Hallett is a post-doctoral research fellow in the department of Biochemistry and Biomedical Sciences at McMaster University and is supervised by Dr. John Hassell (director of the Centre for Functional Genomics). Dr Hassell's group focuses on repurposing old drugs for use as anti-cancer agents and uses a variety of experimental techniques, such as high-throughput screening, gene expression profiling and connectivity mapping to achieve these ends.

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