Salinomycin: A Notch Signaling Antagonist

A NOVEL WAY OF TARGETING CANCER STEM CELLS
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. An astonishing 15% of these individuals will be female victims of breast cancer. 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. As such, chemoresistance and recurrent metastases continue to contribute significantly to cancer mortality rates. 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. The adaptability and heterogeneity of tumours endow them with drug-resistance, disease recurrence, and capacity for metastasis. 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. 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. 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); self-renewing capacity, or the ability to indefinitely give rise to identical daughter cells; and homeostatic control, which is the ability to respond to extracellular cues and genetic constraints to balance differentiation.

FIGURE 1: Model of how the CSC hypothesis can be incorporated in the design of antitumoral 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.
The Notch signaling pathway is activated by enzymatic cleavages that occur at the heterodimeric Notch receptor (in red). Humans possess four homologous Notch receptors, each of which consists of an extracellular domain (N\text{ECD}), transmembrane domain (N\text{TM}) and an intracellular domain (N\text{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\text{ECD} and exposes N\text{ICD} to cleavage by an ADAM metalloprotease (S2). A Notch extracellular truncation (N\text{ECD}) intermediate is produced and is further cleaved by γ-secretase (S3) to generate the active N\text{ICD}. N\text{ICD} is then translocated to the nucleus where it binds transcription factor CSL (or RBP\text{jk} in mice). Upon binding CSL, which is normally in a transcriptionally repressed state, N\text{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.

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. 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.

**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 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. 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. 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

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**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 1xRBP-jk 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.

**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 IC₅₀ 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 inductor 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.51 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 leukaemia, 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.22,24,43 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.